The Involvement of Retroviruses in Human T Cell Leukaemias and Lymphomas

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Abstract

Human T lymphotropic virus type I (HTLV-I) causes adult T cell leukaemia/lymphoma (ATL), a neoplasm of $CD4^+$ T cells. The related virus HTLV-II has been isolated from cases of $CD8^+$ T cell variant hairy cell leukaemia but has not been definitively associated with neoplasia. Bovine leukaemia virus (BLV), which causes B cell leukaemia and lymphoma in cattle, belongs to the same group of retroviruses. The hypothesis tested in this study was that HTLV-I, HTLV-II or related viruses are associated with T cell leukaemias and lymphomas in the United Kingdom, particularly mycosis fungoides (MF) and Sézary syndrome (SS). A combination of cell and molecular biology techniques was used in an attempt to identify retroviruses in patients with these neoplasms.

Lymphocytes were cultivated from blood, skin and lymph nodes of patients with cutaneous lymphoid infiltrates to establish continuous T cell lines that might propagate HTLV-I, HTLV-II or related retroviruses. Techniques for the establishment of cultures included stimulation with mitogens, cytokines, conditioned medium and cocultivation. Cultured cells were examined for evidence of retroviruses by electron microscopy (EM), reverse transcriptase (RT) assay and the polymerase chain reaction (PCR). No retroviruses were isolated from 158 cultures initiated from 18 patients with cutaneous T cell lymphomas (predominantly MF and SS) and three patients with cutaneous B cell lymphomas (CBCLs). Four interleukin 2-dependent $CD8^+$ T cell lines derived from skin of three patients with MF were maintained in culture for 6 to 8 months but no viruses were detected in these cultures by EM or PCR. One Epstein-Barr virus-infected B lymphoblastoid cell line was cultured for 5 months from a skin sample of a patient with CBCL. Supernatants tested from cell cultures derived from these and 10 other patients were negative for RT activity.

PCR was used to test for LTR, *gag*, *pol*, *env* and *pX* sequences of HTLV-I or HTLV-I/II in clinical samples and cultured cells from 36 patients with cutaneous lymphoid infiltrates (predominantly MF and SS), six cases of large granular lymphocytic leukaemia and six cases of acute lymphoblastic leukaemia (ALL). Patients with ALL were tested by PCR for BLV infection since all were butchers from Cardiff with occupational exposure to cattle. There was no definitive evidence of HTLV-I, HTLV-II or BLV infection.

The HTLV-I Tax protein has transforming properties *in vitro* and is important in HTLV-I leukaemogenesis. A model for Tax oncogenesis is c-Myc, which activates pathways leading to either proliferation or apoptosis, depending on the availability of survival factors. It was hypothesised that Tax or other *pX* region proteins have anti-apoptotic properties, since several oncogenic viruses produce proteins that inhibit apoptosis. The *pX* genes *tax*, *rex*, $p21^{III}$, $p30^{II}$, $p13^{II}$ and $p12^{I}$ were cloned and expressed in Rat1 fibroblasts stably transfected with a gene encoding a chimaeric c-Myc-modified oestrogen (tamoxifen) receptor protein. The chimaeric protein translocates to the nucleus in the presence of 4-hydroxytamoxifen, inducing apoptosis when Rat1 cells are deprived of serum. *pX* gene products were tested for their ability to protect Rat1 cells from c-Myc-induced apoptosis in a low concentration of serum. It was demonstrated that Tax induces apoptosis in Rat1 cells deprived of serum but there was no inhibition of c-Myc or Tax-induced apoptosis by other *pX* region gene products.

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Abbreviations

Abbreviations are used throughout this thesis for frequently used technical terms and nomenclature. The full name is given when a term is first used in a chapter, followed by the abbreviation in brackets. Terms are redefined when used in each subsequent chapter. Definitions are sometimes repeated within a chapter to improve clarity. In some cases where a term is used only once, for example APES, an abbreviation is given if it is the usual form recognised by the scientific community. Some terms, for example JAK/STAT, are not defined in the text if this would result in loss of clarity. Standard symbols are used in most places for the names of chemicals. Système International d'Unités (SI) and derived units are used except where alternative units are commonly accepted (Aylward and Findlay 1974). Accepted letter codes for nucleotides and amino acids, including nomenclature for incompletely specified bases, are listed in Appendix 2. Reference to genes, mRNA transcripts and proteins of the HTLV/BLV group of viruses is for HTLV-I unless otherwise designated and the numbering of the HTLV-I genome is according to Seiki *et al* (1983) (Appendix 6).

%G+C	Percentage of G and C nucleotides
Ω	Ohm
$[M^+]$	Adjusted monovalent cation concentration
2ME	2 (β) mercaptoethanol
^{3}H-TTP	Tritiated thymidine triphosphate
ADF	ATL-derived factor
AIDS	Acquired immune deficiency syndrome
ALCL	Anaplastic large cell lymphoma
ALL	Acute lymphocytic leukaemia
APC	Antigen presenting cell
APES	3-aminopropyltriethoxysilane
APS	Ammonium persulphate
ATF	Activating transcription factor
ATL	Adult T cell leukaemia/lymphoma
bHLH	Basic helix loop helix
B-LCL	B lymphoblastoid cell line
BLV	Bovine leukaemia virus
bp	Base pair
BPV	Bovine papillomavirus
BSA	Bovine serum albumin
bZIP	Basic region leucine zipper
C terminus	Carboxy terminus
C	Coulomb
CA	Capsid
cAMP	Cyclic adenosine monophosphate
CBCL	Cutaneous B cell lymphoma
CBP	CREB binding protein
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CLA	Cutaneous lymphocyte-associated antigen
CLL	Chronic lymphocytic leukaemia

CM	Conditioned medium
CNS	Central nervous system
ConA	Concanavalin A
CRE	cAMP response element
CREB	cAMP response element binding protein
CREM	cAMP response element modulator
CSF	Cerebrospinal fluid
CTCL	Cutaneous T cell lymphoma
CTL	Cytotoxic T lymphocyte
Da	Dalton
dA	DNA-dependent DNA polymerase activity (poly dA template)
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DEAE	Diethylaminoethyl
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
EBNA	Epstein-Barr virus-associated nuclear antigen
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELAM	Endothelial leucocyte adhesion molecule
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EMA	Epithelial membrane antigen
Env	Envelope
ER	Oestrogen receptor
ER^{TM}	Modified oestrogen receptor (4-hydroxytamoxifen-responsive)
F	Faraday
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FLK	Foetal lamb kidney
Gag	Group antigen
GCG	Genetics Computer Group
GCSF	Granulocyte colony stimulating factor
GMCSF	Granulocyte-macrophage colony stimulating factor
HAM	HTLV-I-associated myelopathy
HBSS	Hanks' balanced saline solution
HBV	Hepatitis B virus
HCL	Hairy cell leukaemia
HCV	Hepatitis C virus
HEPA	High efficiency particulate air
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HERV	Human endogenous retrovirus
HHV	Human herpesvirus

HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen/Histocompatibility locus antigen
HMW	High molecular weight
HPV	Human papillomavirus
HRES	HTLV-related endogenous sequence
HRV	Human retrovirus
HSV	Herpes simplex virus
HT	Hydroxytamoxifen
HTLV	Human T cell lymphotropic virus
HTLV-I	Human T lymphotropic virus type I
HTLV-II	Human T lymphotropic virus type II
IκB	Inhibitor of NFκB
ICAM	Intercellular adhesion molecule
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
IL2R	Interleukin 2 receptor
IN	Integrase
IPTG	Isopropyl-1-thio-β-D-galactoside
ISH	In situ hybridisation
IVDU	Intravenous drug user
J	Joules
JAK	Just another kinase (JANUS family kinase)
KS	Kaposi's sarcoma
L	Length of the DNA:DNA duplex in bp
Large T	Large tumour antigen
LB	Luria-Bertani
LCA	Leucocyte common antigen
LDLGL	Lymphoproliferative disease of large granular lymphocytes
LFA	lymphocyte function-associated antigen
LGL	Large granular lymphocyte/lymphocytic
$liqN_2$	Liquid nitrogen
LMP	Latent membrane protein
LRF	Leukaemia Research Fund
LTR	Long terminal repeat
MA	Matrix
MAb	Monoclonal antibody
MDV	Marek's disease virus
MEKK	Mitogen-activated protein/extracellular signal-regulated kinase kinase
MF	Mycosis fungoides
MHC	Major histocompatibility complex
MOPS	3-(N-morpholino)-2-hydroxypropane sulphonic acid
mRNA	Messenger RNA
MS	Multiple sclerosis
MSC	Microbiological safety cabinet
MuLV	Murine leukaemia virus
MW	Molecular weight
N terminus	Amino terminus
$NaPP_i$	Tetrasodium pyrophosphate

NC	Non-coding
NES	Nuclear export signal
NF	Nuclear factor
NF-AT	Nuclear factor of activated T cells
NFκB	Nuclear factor κ B
NF-IL6	Nuclear factor-interleukin 6
NIB	Non-ionic detergent buffer
NK	Natural killer
NLS	Nucleolar localisation signal
NP-40	Nonidet P-40
NRK	Normal rat kidney
OD	Optical density
oligo-dT	Oligo-thymidine deoxyribonucleic acid
ORF	Open reading frame
P	Percentage base mismatch
PAB	PBS-sodium azide-bovine serum albumin
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PERT	Product-enhanced reverse transcriptase
PHA	Phytohaemagglutinin
PKC	Protein kinase C
Pol	Polymerase
poly-dA	Poly-adenosine deoxyribonucleic acid
poly-dT	Poly-thymidine deoxyribonucleic acid
poly-rA	Poly-adenosine ribonucleic acid
PR	Protease
pre-B cell	Precursor-B cell
pre-T cell	Precursor-T cell
Pro	Protease
PTHRP	Parathyroid hormone-related protein
PTK	Protein tyrosine kinase
rA	RNA-dependent DNA polymerase activity (poly rA template)
RDA	Representational difference analysis
RE	Restriction endonuclease
REF	Rat embryo fibroblast
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNase H	Ribonuclease H
RPE	R-phycoerythrin
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase-polymerase chain reaction
RxRE	Rex response element
SAF	Sézary T cell activating factor
SB	Southern blot
SCID	Severe combined immunodeficiency

SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)
SI	Système International d'Unités
SIV	Simian immunodeficiency virus
SLE	Systemic lupus erythematosus
SnRV	Snakehead fish retrovirus
SP	Signal peptide
SRE	Serum response element
SRF	Serum response factor
SS	Sézary syndrome
SSC	Sodium saline citrate
STAT	Signal transducer and activator of transcription
SU	Surface
SV40	Simian virus 40
TAE	Tris-acetic acid-EDTA
TBE	Tris-boric acid-EDTA
TCA	Trichloroacetic acid
TCGF	T cell growth factor
TCL	T cell lymphoma
TCR	T cell receptor
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	Transforming growth factor
Th	T helper
TIF-1	Tax interacting factor 1
TIL	Tumour infiltrating lymphocytes
T_m	Melting temperature
TM	Transmembrane
TNE	Tris-NaCl-EDTA
TPA	12-o-tetradecanoyl phorbol-13 acetate
TRAIL	TNF-related apoptosis-inducing ligand
TRE	Tax response element
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA
Ts	T suppressor
TSP	Tropical spastic paraparesis
U3	3' unique region
U5	5' unique region
UCMC	Umbilical cord blood mononuclear cell
UK	United Kingdom
UNG	Uracil N-glycosylase
USA	United States of America
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
WB	Western blot
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Chapter 1

Literature Review: The Role of Retroviruses in Human T Cell Leukaemias and Lymphomas

1.1 Introduction

Neoplasms are abnormal proliferations of tissue, unresponsive to normal mechanisms for control of cell growth, differentiation and death. Viruses, particularly members of the Families *Retroviridae*, *Herpesviridae*, *Papillomaviridae* and *Hepadnaviridae*, have been implicated as aetiological agents in many neoplasms, including T and B cell leukaemias and lymphomas (Zur Hausen 1991). In leukaemias neoplastic cells of haematopoietic lineage circulate in the blood. Lymphomas are solid growths of neoplastic lymphoid cells. The association of human T lymphotropic virus type I (HTLV-I) with adult T cell leukaemia/lymphoma (ATL) has led to the hypothesis that HTLV-I or related retroviruses are involved in the aetiology of other T cell neoplasms (Hall 1994, Lessin *et al* 1994).

This thesis describes studies conducted at the Leukaemia Research Fund (LRF) Virus Centre using cell and molecular biology techniques to search for HTLV-I or related retroviruses in cutaneous T cell lymphomas (CTCLs), large granular lymphocytic (LGL) leukaemia and adult acute lymphoblastic leukaemia (ALL) in the United Kingdom (UK). It also describes experiments to investigate the role of HTLV-I *pX* gene products in oncogenesis by studying their effects on apoptosis in cultured cells. This chapter reviews the biology of HTLV-I and related retroviruses, diseases associated with these viruses and the mechanisms of oncogenesis of HTLV-I. The classification and pathogenesis of CTCLs and LGL leukaemia are reviewed and evidence for the involvement of HTLV-I and related viruses in their aetiology is discussed.

1.2 Retroviruses

1.2.1 Classification of retroviruses

Viruses in the family *Retroviridae* possess reverse transcriptase (RT), which transcribes viral RNA to DNA during the retroviral replication cycle (Baltimore 1970, Temin and Mizutani 1970). Retroviruses have been categorised according to their biological behaviour into the oncornaviruses (oncogenic RNA viruses), lentiviruses (slow viruses) and spumaviruses (foamy viruses) (Teich 1985). A more recent classification is based on the structure of the retroviral genome (Van Regenmortel *et al* 2000) (Table 1.1). Replication competent simple retroviruses contain *gag* (group antigen), *pro* (protease), *pol* (polymerase) and *env* (envelope) genes from 5' to 3', flanked by unique 5' (U5) and 3' (U3) sequences (Coffin *et al* 1997).

Table 1.1: Classification of Family *Retroviridae* (Van Regenmortel *et al* 2000).

Genus	Species
Simple retroviruses	
Alpharetrovirus	Avian leucosis virus-RSA* Rous sarcoma virus (Prague C) Avian myeloblastosis virus (defective) Avian myelocytomatosis virus 29 (defective)
Betaretrovirus	Mouse mammary tumour virus* Mason-Pfizer monkey virus Jaagsiekte sheep retrovirus (ovine pulmonary adenocarcinoma virus) Squirrel monkey retrovirus
Gammaretrovirus	Murine leukaemia virus* Abelson murine leukaemia virus Friend murine leukaemia virus Moloney murine leukaemia virus Feline leukaemia virus Gibbon ape leukaemia virus Viper retrovirus Reticuloendotheliosis virus
Complex retroviruses	
Deltaretrovirus	Bovine leukaemia virus* Human T lymphotropic virus type I Human T lymphotropic virus type II Simian T lymphotropic virus type I Simian T lymphotropic virus type II Simian T lymphotropic virus type III
Epsilonretrovirus	Walleye dermal sarcoma virus* Snakehead retrovirus (tentative)
Lentivirus	Human immunodeficiency virus type 1* Human immunodeficiency virus type 2 Simian immunodeficiency virus Bovine immunodeficiency virus Equine infectious anaemia virus Feline immunodeficiency virus Caprine arthritis encephalitis virus Maedi/visna virus
Spumavirus	Chimpanzee (formerly human) foamy virus* Bovine foamy virus Feline foamy virus Simian foamy virus 1 Simian foamy virus 3

* Type species

Complex retroviruses, which include the lentiviruses (Genus *Lentivirus*), the HTLV/bovine leukaemia virus (BLV) group of viruses (Genus *Deltaretrovirus*) and the foamy viruses (Genus *Spumavirus*), have an additional region, designated *pX*, which encodes regulatory proteins. Viruses in the HTLV/BLV group include HTLV-I, HTLV-II, simian T lymphotropic virus type I (STLV-I), STLV-II and BLV.

The recognised exogenous retroviruses of humans are HTLV-I (Miyoshi *et al* 1980, Poiesz *et al* 1980a), HTLV-II (Kalyanaraman *et al* 1982b), human immunodeficiency virus type 1 (HIV-1) (Barré-Sinoussi *et al* 1983, Gallo *et al* 1984, Levy *et al* 1984, Popovic *et al* 1984) and HIV-2 (Clavel *et al* 1986). Humans can also be infected with non-human primate spumaviruses (simian foamy viruses) (Heneine *et al* 1998) and the previously designated human foamy virus is now thought to be of chimpanzee (*Pan troglodytes*) origin (Achong *et al* 1971, Herchenroder *et al* 1995, Meiering and Linial 2001). Many endogenous retroviruses and retrovirus-like elements are present in genomic DNA, including some with sequence similarity to HTLV-I (Mager and Freeman 1987, Perl *et al* 1989, Fujihara *et al* 1994).

1.2.2 Structure and organisation of retroviruses

Retrovirus virions are enveloped structures approximately 100 nm in diameter (Coffin *et al* 1997). Proteins produced from the *env* gene are expressed on the envelope in the form of trimers. The conical nucleocapsid consists of two identical molecules of single stranded ribonucleic acid (RNA), three or four proteins cleaved from a polyprotein produced by the *gag* gene, a protease produced by the *pro* gene and an RT/integrase/ribonuclease (RNase) H produced by the *pol* gene. Each viral RNA molecule is associated with a transfer RNA (tRNA) molecule of host origin.

1.2.3 Replication cycle of retroviruses

Infection by retroviruses is initiated by fusion of the retroviral envelope with the host cell membrane allowing entry of the nucleocapsid into the cytoplasm. Double-stranded complementary deoxyribonucleic acid (cDNA) is synthesised from the two single-stranded viral RNA templates by the viral RT (Coffin *et al* 1997). The associated tRNA molecules act as primers for synthesis of the minus strand by reverse transcription. The 5' and 3' long terminal repeats (LTRs) of the provirus are formed by duplication of the U5 and U3 regions of the viral genome, with an intervening repeat (R) region. The cDNA translocates to the nucleus where it is integrated as a provirus into the DNA of the host cell by the viral integrase. Regulatory and structural messenger RNA (mRNA) transcripts are produced and virions are assembled from translated structural proteins and viral genomic RNA. Complete virions are released from the cell by budding through the plasma membrane to acquire a lipid envelope containing viral proteins. Most retroviruses, including the HTLVs, establish persistent, life-long infections in their hosts and production of infectious virions is not dependent on host cell lysis.

1.3 Human T lymphotropic viruses

1.3.1 Terminology

Human T lymphotropic virus type I (HTLV-I) has also been referred to as ATL-associated virus (ATLV), human T cell leukaemia virus type I and human T cell lymphoma/leukaemia virus type I (Poiesz *et al* 1980a, 1981, Yoshida *et al* 1982, Blattner *et al* 1983). HTLV-II is also known as human T cell lymphoma/leukaemia virus type II.

1.3.2 Propagation of human T lymphotropic viruses

1.3.2.1 Discovery of HTLV-I and HTLV-II

HTLV-I was first detected in $CD4^+$ T cell lines established from lymph node cells and peripheral blood mononuclear cells (PBMCs) of a patient in the United States of America (USA) with a form of ATL initially diagnosed as mycosis fungoides (MF) (Gazdar *et al* 1980, Poiesz *et al* 1980a, b) (Sections 1.6.1.2 and 1.11.1.2). A second isolate was obtained from a $CD4^+$ T cell line derived from PBMCs of a patient also with a form of ATL but initially diagnosed as having Sézary syndrome (SS) (Poiesz *et al* 1981). Serum from patients with ATL reacted with antigens expressed by a cell line producing retrovirus-like particles established from a case of ATL in Japan (Miyoshi *et al* 1980, Hinuma *et al* 1981). Retrovirus particles, initially designated ATLV but later shown to be identical to HTLV-I, were also detected in a cell line established by cocultivation of umbilical cord blood mononuclear cells (UCMCs) with PBMCs from a patient with ATL (Miyoshi *et al* 1981a, b). Subsequently, HTLV-I has been identified in numerous other T cell lines established from patients with ATL (Hinuma *et al* 1982a, Yoshida *et al* 1982, Hoshino *et al* 1983, Markham *et al* 1983) and HTLV-I-associated myelopathy (HAM)/tropical spastic paraparesis (TSP) (Jacobson *et al* 1988) (Section 1.6.3), as well as asymptomatic people (Gotoh *et al* 1982, Sugamura *et al* 1984a). HTLV-II was first isolated from a $CD8^+$ T cell line derived from a patient with a T cell variant of hairy cell leukaemia (HCL) (Saxon *et al* 1978b, Kalyanaraman *et al* 1982b) (Section 1.7.1).

1.3.2.2 Cellular tropism of HTLV-I and HTLV-II

HTLV-I establishes productive infection of activated $CD4^+$ T cells of both T helper (Th) 1 and 2 phenotypes *in vitro* and *in vivo* (Merl *et al* 1984, Richardson *et al* 1990, Fan *et al* 1992, Macchi *et al* 1998) (Section 1.10.1.3). $CD8^+$ T cells and B cells are also susceptible to infection but have lower levels of viral transcriptional activity (Longo *et al* 1984, Mann *et al* 1984, Franchini *et al* 1985, Koyanagi *et al* 1993). Natural killer (NK) cells can be infected with HTLV-I following activation but do not produce infectious virions (Macchi *et al* 1987, Lo *et al* 1992). A wide range of other cell types is susceptible to infection with HTLV-I, including monocytes, macrophages, microglial cells, dendritic cells, fibroblasts, epithelial cells including enterocytes and mammary epithelial cells, synoviocytes, endothelial cells and neurones, but the role of these cells in maintaining viral infection *in vivo* or in transmitting HTLV-I is uncertain (Hoxie *et al* 1984, Kitajima *et al* 1991, Fan *et al* 1992, Hoffman *et al* 1992, Macatonia *et al* 1992, Zacharopoulos *et al* 1992, Ali *et al* 1993, De Revel *et al* 1993,

Koyanagi *et al* 1993, Sakai *et al* 1993, Lehky *et al* 1994, Southern and Southern 1998). The receptor for HTLV-I has not been identified but appears to be expressed by many types of cells (Gavalchin *et al* 1995). T cells from PBMCs, UCMCs or bone marrow can be infected with HTLV-I following cocultivation with lethally irradiated HTLV-I-infected cells, whereas the infectivity of cell-free virus is low (Miyoshi *et al* 1981c, Yamamoto *et al* 1982b, De Rossi *et al* 1985, Fan *et al* 1992). $CD8^+$ T cells are the main cells infected by HTLV-II, although $CD4^+$ T cells, B cells and other lymphoid cells are also susceptible to infection (Rosenblatt *et al* 1988b, Ijichi *et al* 1992, Lal *et al* 1995).

1.3.2.3 Transformation of T cells by HTLV-I

In vitro transformation is the conversion of cells with a limited life span into immortalised cells (cells that are able to grow indefinitely) with an altered phenotype and often a reduced requirement for growth factors such as those present in serum. Upon transformation in culture, normally adherent cells such as fibroblasts exhibit focus formation (piling up due to loss of contact inhibition) and colony formation in soft agar or suspension cultures (due to loss of anchorage dependence). Transformed cells may also be tumorigenic in athymic (nude) mice or other animal systems. T cells are considered to be transformed when they are able to proliferate indefinitely in the absence of exogenous interleukin (IL) 2 (Grassmann *et al* 1994).

Normal human T cells can be transformed *in vitro* following infection with HTLV-I and HTLV-II (Miyoshi *et al* 1981c, Chen *et al* 1983, Markham *et al* 1983, Popovic *et al* 1983, Merl *et al* 1984, Aboud *et al* 1987, Graziano *et al* 1987). T cells undergo polyclonal expansion 1 to 7 weeks following cocultivation of recipient PBMCs or UCMCs with HTLV-I-infected cells (Lando *et al* 1983, Kimata and Ratner 1991). Eventually there is selective outgrowth of one or a few dominant clones, usually $CD4^+$ T cells although some may be $CD8^+$, with oligoclonal integration of HTLV-I provirus (De Rossi *et al* 1985, Kimata and Ratner 1991). The efficiency of infection and transformation is increased if T cells have been activated but HTLV-I infection per se activates T cells (Merl *et al* 1984, Martin and Southern 1996, Höllsberg 1999) (Section 1.8.8). HTLV-I-infected T cell clones derived from PBMCs have variable expression of HTLV-I mRNA; T cells with transcriptionally silent proviruses do not spontaneously proliferate (Richardson *et al* 1997). HTLV-I-infected T cell lines often have defective proviruses with preferential retention of the *pX* region (Kobayashi *et al* 1984, Bhat *et al* 1993) (Section 1.3.3).

Infection of T cells with HTLV-I is associated with increased expression of IL2 and IL2 receptor (IL2R), as well as alterations in expression and function of many other cellular constituents (Section 1.8). HTLV-I-infected T cell lines may be IL2-dependent (immortalised) or IL2-independent (transformed) (Arima *et al* 1986, Katoh *et al* 1986, Arima *et al* 1987). HTLV-I-transformed T cells form colonies in soft agar in the absence of exogenous IL2 (Duc Dodon and Gazzolo 1987). In most *in vitro* models of HTLV-I infection of T cells, there is an initial phase of IL2-dependent growth following which the cells become independent of IL2, usually after 6 to 12 months (Markham *et al* 1983, Yssel *et al* 1989, Höllsberg *et al* 1992, Rohwer *et al* 1994). These results are consistent with a role for IL2 in proliferation of T cells in the early stages of HTLV-I infection and transformation, although some HTLV-I-infected T

cell lines do not produce IL2 and do not require exogenous IL2 for survival (Markham *et al* 1983, Arya *et al* 1984, Volkman *et al* 1985). Maintenance of IL2 production *in vitro* may depend on the presence of macrophages in primary cultures of PBMCs from ATL patients (Arima *et al* 1992). The roles of the HTLV-I Tax protein and IL2 in transformation of T cells by HTLV-I are discussed in Section 1.8. The use of cell culture for isolation and diagnosis of infection with HTLV-I and HTLV-II is discussed in Section 1.4.1.

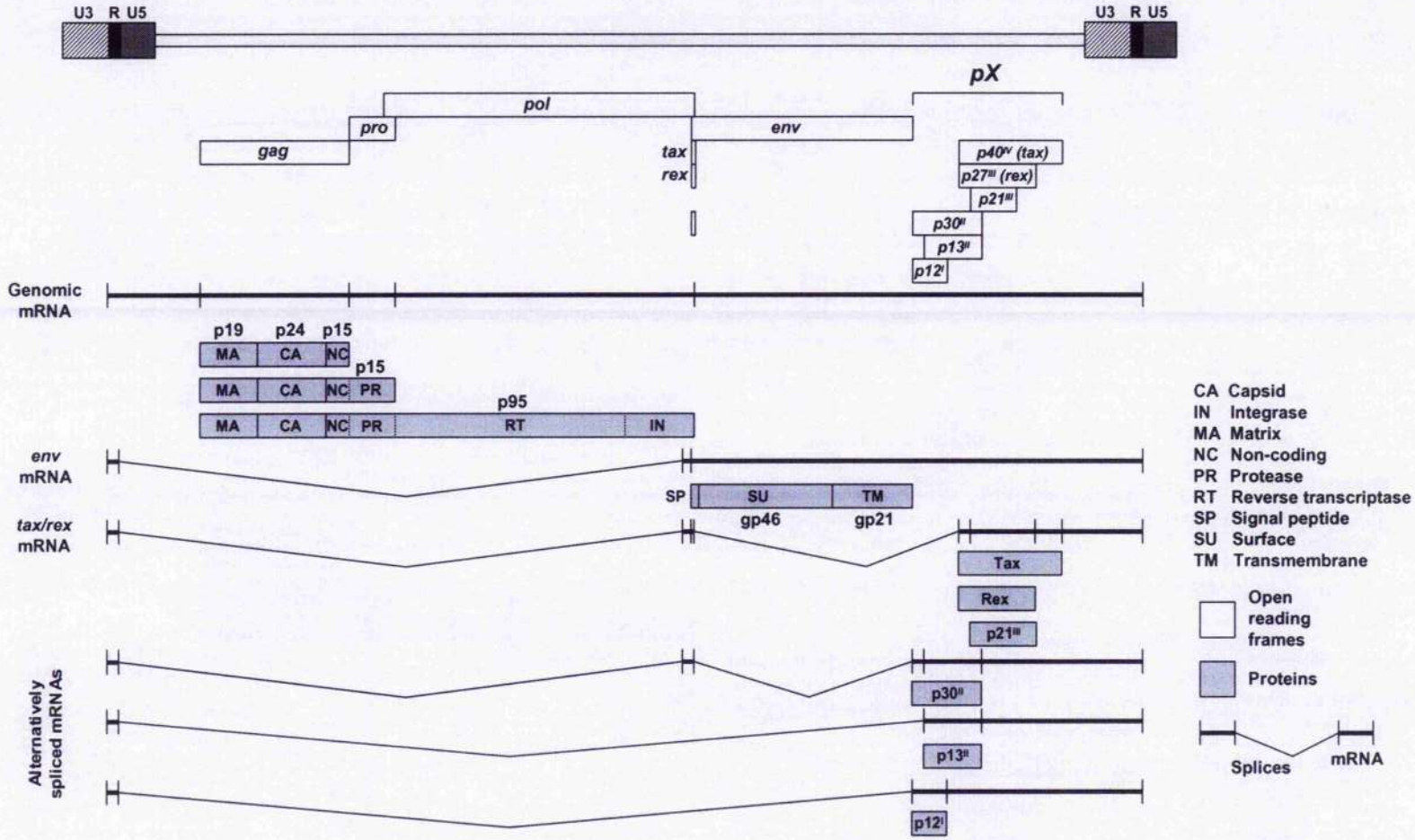
1.3.3 Genomic structure of HTLV-I

The 9,068 nucleotide HTLV-I genome is organised from 5' to 3' into U5, *gag*, *pol*, *env*, *pX* and U3 regions (Seiki *et al* 1983, Gallo *et al* 1988, Myers *et al* 1991) (Figure 1.1). The genomic structures of HTLV-II, STLV-I, STLV-II and BLV are similar (Sagata *et al* 1985, Shimotohno *et al* 1985b, Watanabe *et al* 1985). HTLV-I *gag* encodes the group antigens $p19^{Gag}$, $p24^{Gag}$ and $p15^{Gag}$, which are capsid proteins, *gag-pol* encodes a protease, $p14^{Pro}$, *pol* encodes the RT/integrase/RNase H $p95^{Pol}$ and *env* encodes the envelope glycoproteins $gp46^{Env}$ and $gp21^{Env}$. The *pX* region encodes regulatory proteins (Section 1.3.4).

1.3.4 HTLV-I pX gene expression

The proteins $p40^{IV}$ (Tax), $p27^{III}$ (Rex), $p21^{III}$, $p30^{II}$ (Tof), $p13^{II}$ and $p12^{I}$ are encoded by the four main open reading frames (ORFs I, II, III and IV) in the *pX* region of HTLV-I (Kiyokawa *et al* 1984, Lee *et al* 1984, Slamon *et al* 1984, Kiyokawa *et al* 1985b, Shimotohno *et al* 1985a, Ciminale *et al* 1992, Koralnik *et al* 1992) (Appendix 1). Tax (transactivator) and Rex (regulator of expression) are the major proteins expressed from *pX*, whereas $p21^{III}$, Tof, $p13^{II}$ and $p12^{I}$ are minor products derived by translation from alternatively spliced transcripts (Kiyokawa *et al* 1985b, Seiki *et al* 1985, Berneman *et al* 1992a, Ciminale *et al* 1992, Koralnik *et al* 1992). mRNAs expressed from the *pX* region derive their initiation codons by splicing with short transcripts from the *gag-env* region using several donor and acceptor sites (Figure 1.1). The 2 kb doubly spliced *tax/rex* mRNA that encodes Tax, Rex and $p21^{III}$ consists of a non-coding exon (exon 1) from the 5' LTR (HTLV-I 1 to 119), a small first coding exon (exon 2) overlapping the 3' end of *pol* and the 5' end of *env* (containing the AUG initiation codons and using splice acceptor sites at HTLV-I 4641, 4685 or 4701 and a single splice donor site at HTLV-I 4831) and a large second coding exon (exon 3) in the distal portion of the *pX* region with a splice acceptor site at HTLV-I 6950 (Seiki *et al* 1983, Kiyokawa *et al* 1985b, Seiki *et al* 1985, Wachsman *et al* 1985, Nagashima *et al* 1986). Tof is encoded by a doubly spliced mRNA consisting of the same exon 1 from the 5' LTR, exon 2 from HTLV-I 4641 to 4831 and exon 3 from the splice acceptor site HTLV-I 6478 in ORF II of the *pX* region (Ciminale *et al* 1992, Koralnik *et al* 1992). $p13^{II}$ is encoded by a singly spliced mRNA consisting of exon 1 spliced to exon 2 at HTLV-I 6875 in ORF II of the *pX* region (Berneman *et al* 1992b). $p12^{I}$ can be encoded by singly or doubly spliced *pX* ORF I mRNA transcripts (Koralnik *et al* 1992). The singly spliced mRNA consists of exon 1 spliced to exon 2 at HTLV-I 6383 in *pX* ORF I. The doubly spliced mRNA consists of exon 1 spliced to exon 2 (HTLV-I 4641 to 4831), with exon 3 starting from the splice acceptor site at HTLV-I 6383 in *pX* ORF I, as for the singly spliced mRNA. The initiation codons for both of the mRNA transcripts encoding $p12^{I}$ are located in *pX* ORF I.

Figure 1.1: Human T lymphotropic virus type I genome organisation and proteins (Koralnik *et al* 1992, Coffin *et al* 1997).



HTLV-II, STLV-I and BLV also make use of complex splicing for expression of *pX* products (Alexandersen *et al* 1993, Orita *et al* 1993b). Tax_2 and Rex_2 are encoded by the *pX* region of HTLV-II (Rosenblatt *et al* 1988a). Tax_{BLV} and Rex_{BLV} are produced by BLV (Rice *et al* 1987) (Section 1.9.2.1).

In HTLV-I-infected T cells Tax and $p13^{II}$ are predominantly localised in the nuclear matrix, Rex and Tof in the nucleolus, $p21^{III}$ in the cytoplasm and $p12^{I}$ in the endoplasmic reticulum and Golgi apparatus (Goh *et al* 1985, Slamon *et al* 1988, Nosaka *et al* 1989, Koralnik *et al* 1993).

A 152 amino acid *pX* product $p17^{I}$ (Rof) has been identified in cells transfected with a molecular clone of HTLV-I, but has not been detected in naturally infected cells (Ciminale *et al* 1992). RNA transcripts arising from several ORFs on the negative strand of HTLV-I have been detected in infected cells but their significance is unknown (Larocca *et al* 1989, Chou *et al* 1995). The *pX* ORFs I and II that encode Tof, $p13^{II}$ and $p12^{I}$ are not necessary for HTLV-I replication and immortalisation in cultured cells but may be necessary for efficient replication *in vivo* (Derse *et al* 1997, Collins *et al* 1998, Robek *et al* 1998, Albrecht *et al* 2000, Bartoe *et al* 2000).

1.3.5 Structure and function of HTLV-I pX gene products

1.3.5.1 Tax ($p40^{IV}$)

Tax is a 353 amino acid phosphoprotein translated from ORF IV of the doubly spliced *tax/rex* mRNA transcript (Kiyokawa *et al* 1984, Lee *et al* 1984, Slamon *et al* 1984, Kiyokawa *et al* 1985a, Seiki *et al* 1985) (Section 1.3.4) (Appendix 1). Its main functions are to activate transcription from the HTLV-I LTR and control expression of cellular genes (Sodroski *et al* 1984, Cann *et al* 1985). A zinc finger motif located in the amino (N) terminal region is responsible for dimerisation and localisation of Tax to the nucleus (Smith and Greene 1992). Separate regions in the N terminus mediate transactivation through the nuclear factor κB (NFκB) and cyclic adenosine monophosphate (cAMP) response element binding (CREB)/activating transcription factor (ATF) pathways (Wachsman *et al* 1987, Smith and Greene 1990, Semmes and Jeang 1992, Adya and Giam 1995). The carboxy (C) terminal region also interacts with transcription factors. The role of Tax in HTLV-I leukaemogenesis is discussed in Section 1.8.

1.3.5.2 Rex ($p27^{III}$)

Rex is a 189 amino acid phosphoprotein expressed from a different reading frame (ORF III) of the same doubly spliced mRNA as Tax (Nagashima *et al* 1986, Adachi *et al* 1992a) (Appendix 1). A domain at amino acids 57 to 69 allows Rex to form multimers (Weichselbraun *et al* 1992). An N terminal nucleolar localisation signal (NLS) directs Rex to the nucleolus (Siomi *et al* 1988, Nosaka *et al* 1989). The NLS also binds to the HTLV-I Rex response element (RxRE), a sequence of approximately 254 base pairs (bp) that forms a stable stem loop structure in U3 of the full-length viral mRNA transcript, inhibiting production

of the doubly spliced mRNA transcripts encoding Rex and Tax (Hidaka *et al* 1988, Ballaun *et al* 1991, Unge *et al* 1991, Gröne *et al* 1994). A leucine motif/activation domain at amino acids 82 to 93 of Rex acts as a nuclear export signal (NES) by interacting with cellular proteins that export mRNA from the nucleus, allowing Rex to shuttle between the nucleus and the cytoplasm (Bogerd *et al* 1996, Kubota *et al* 1996a, Palmeri and Malim 1996). Rex promotes transport of unspliced (*gag-pol*) and singly spliced (*env*) viral mRNA transcripts from the nucleus to the cytoplasm instead of doubly spliced (*tax/rex*) mRNA (Inoue *et al* 1987, Hidaka *et al* 1988, Nosaka *et al* 1989). It thus acts as a switch from early expression of regulatory proteins (Tax, Rex) to late expression of structural proteins (Gag, Pol, Env). Virions are not produced in the absence of Rex. The function of Rex is modulated by phosphorylation and may be influenced by expression of $p21^{III}$ (Adachi *et al* 1992a, Kubota *et al* 1996a) (Section 1.3.5.3).

1.3.5.3 $p21^{III}$

The 111 amino acid $p21^{III}$ is translated from an internal ribosomal entry site of ORF III of the doubly spliced *tax/rex* mRNA, as well as from an alternative singly spliced ORF III mRNA transcript (Kiyokawa *et al* 1985b, Nagashima *et al* 1986, Furukawa *et al* 1991, Orita *et al* 1991, Kubota *et al* 1996a) (Appendix 1). It represents a truncated form of Rex (amino acids 79 to 189) that lacks the NLS but retains the NES and may compete with Rex for cellular proteins that export mRNA (Kubota *et al* 1996a). This leads to retention of unspliced viral mRNAs in the nucleus. $p21^{III}$ could thus have a role in reducing the expression of structural proteins, maintaining latency and minimising the host immune response. Although *$p21^{III}$* mRNA has been detected by RT-PCR in PBMCs of HTLV-I-infected people, Orita *et al* (1993a) associated $p21^{III}$ expression with defective proviral genomes in HTLV-I-infected T cell lines and its function *in vivo* has not been confirmed.

1.3.5.4 Tof ($p30^{II}$)

Tof is a protein of 241 amino acids derived from a doubly spliced ORF II mRNA transcript (Ciminale *et al* 1992, Koralnik *et al* 1993) (Section 1.3.4) (Appendix 1). It has serine, arginine and threonine-rich regions with sequence similarity to activation domains of the POU family of transcription factors and interacts with CREB binding protein (CBP)/p300 (Ciminale *et al* 1992, Zhang *et al* 2001) (Section 1.8.2.3). Two arginine-rich regions act as a NLS by allowing Tof to bind to the RxRE and point towards a role for this protein in regulation of mRNA expression (D'Agostino *et al* 1997).

1.3.5.5 $p13^{II}$

$p13^{II}$ is a protein of 87 amino acids translated from an internal initiation codon in *pX* ORF II and derived from singly spliced and doubly spliced mRNAs (Berneman *et al* 1992a, Ciminale *et al* 1992, Koralnik *et al* 1992) (Section 1.3.4) (Appendix 1). $p13^{II}$ interacts with farnesyl pyrophosphate synthetase but its function is unknown (Lefèbvre et al 2002).

1.3.5.6 p12^{I}

p12^{I}, expressed from singly or doubly spliced *pX* ORF I mRNA transcripts (Section 1.3.4), is a 99 amino acid hydrophobic protein with two predicted transmembrane domains, between which is a proline-rich region (Ciminale *et al* 1992, Koralnik *et al* 1992, 1993) (Appendix 1). It forms dimers and localises in the endoplasmic reticulum and Golgi apparatus in association with calreticulin and calnexin (Koralnik *et al* 1993, Trovato *et al* 1999, Ding *et al* 2001). In these sites p12^{I} binds to major histocompatibility (MHC) class I molecules, preventing their association with β_2 microglobulin and enhancing MHC-I degradation by the proteosome complex (Johnson *et al* 2001). This may prevent surface expression of MHC-I and interfere with presentation of antigens, allowing HTLV-I to escape immune recognition. p12^{I} also binds to the 16 kDa vacuolar H^+ adenosine triphosphatase (ATPase) and the β and γ_c chains of IL2R (Franchini *et al* 1993, Koralnik *et al* 1995, Mulloy *et al* 1996). A role for p12^{I} in oncogenesis may be to stimulate proliferation of T cells by binding to molecules involved in cell signalling pathways (Section 1.8.4).

1.3.6 Control of HTLV-I gene expression

Tax and Rex are the main proteins controlling HTLV-I gene expression (Green and Chen 1990). Tax transactivates the HTLV-I promoter by interacting with cellular transcription factors that bind to Tax response elements (TREs) in the U3 region of the proviral LTR (Brady *et al* 1987, Rosen *et al* 1987, Fujisawa *et al* 1991, Zhao and Giam 1991, Seeler *et al* 1993). The TREs comprise three copies of a 21 bp imperfect repeat sequence TRE-1 and one copy of a 47 bp sequence TRE-2. Members of the CREB/ATF family, including CREB and ATF-1 homodimers, CREB-ATF-1 heterodimers, activating protein (AP) 1 (Jun-Fos) heterodimers and AP-2, bind to TRE-1 (Zhao and Giam 1992, Suzuki *et al* 1993a, Fujii *et al* 1995, Mori and Prager 1996, Barnhart *et al* 1997). Cellular proteins that bind to TRE-2 include Tax interacting factor 1 (TIF-1), Sp1, Myb and Ets-1 (Bosselut *et al* 1990, Marriott *et al* 1990, Nyborg and Dynan 1990, Bosselut *et al* 1992, Dasgupta *et al* 1992). Responsiveness to Tax requires two copies of TRE-1 or one copy each of TRE-1 and TRE-2 (Shimotohno *et al* 1986).

Each TRE-1 can be further divided into three elements (A, B and C) with different binding specificities for a variety of transcription factors (Montagne *et al* 1990, Yoshimura *et al* 1990, Tsujimoto *et al* 1991, Muchardt *et al* 1992, Morita *et al* 1993, Nyunoya *et al* 1993). Most of these factors bind to TRE-1 in the absence of Tax but their affinity for the HTLV-I LTR and thus transcriptional activation of the viral promoter is augmented by dimerisation in the presence of Tax (Matthews *et al* 1992, Zhao and Giam 1992, Armstrong *et al* 1993, Franklin *et al* 1993) (Section 1.8.2). Alternatively, Franklin and Nyborg (1995) have suggested that CREB/ATF proteins anchor Tax to the promoter site, allowing Tax to induce transcription from its own C terminal activation domain.

Repressive sequences in the U5 and R regions may also be involved in control of HTLV-I gene expression (Kashanchi *et al* 1994, Xu *et al* 1994, Okumura *et al* 1997). Other mechanisms of transcriptional control include differential methylation of the LTR, *gag-pol-env*

and *pX* regions (Clarke *et al* 1984, Kitamura *et al* 1985, Saggioro *et al* 1990, Caβens *et al* 1994), phosphorylation of Tax (Green *et al* 1992b, Saggioro *et al* 1994a), *cis*-acting inhibitory elements in the *pol-env* region (Saiga *et al* 1997) and sequestration of Tax by the NFκB2 precursor Lyt-10 (Béraud *et al* 1994) (Section 1.8.2.1).

Whereas expression of HTLV-I mRNA and protein in leukaemic cells of ATL patients is low and selective (Section 1.6.1.6), there is upregulation of expression of HTLV-I genes when PBMCs from HTLV-I-infected people, including those with ATL, are cultivated *in vitro* (Poiesz *et al* 1980a, Hinuma *et al* 1982a, Hoshino *et al* 1983, Salahuddin *et al* 1983, Clarke *et al* 1984, Setoyama *et al* 1992). Expression of HTLV-I genes is also induced following activation with mitogens or through induction of the cellular stress (heat shock protein) response (Andrews *et al* 1995, Lin *et al* 1998). The effects of Tax on expression of cellular genes and mechanisms of regulation of these genes are described in Sections 1.8.1 and 1.8.2.

As discussed in Section 1.3.5.2, Rex and possibly $p21^{III}$ are involved in post-transcriptional control of HTLV-I gene expression, increasing the expression of structural proteins by regulating mRNA processing and thus directing the production of virions (Inoue *et al* 1987, Hidaka *et al* 1988, Nosaka *et al* 1989). The roles of Tof, $p13^{II}$ and $p12^{I}$ in the control of HTLV-I gene expression are uncertain.

1.4 Detection of infection with human T lymphotropic viruses

The sensitivity and specificity of diagnostic tests for the detection of infection with HTLVs are critical in establishing associations between HTLV-I, HTLV-II or related viruses and disease entities. This section summarises the virological, serological and molecular assays commonly used for diagnosis of infection with HTLVs. Serological tests are used in the clinical diagnosis of HTLV infection.

1.4.1 Virus isolation

HTLV-I and HTLV-II are usually isolated by cultivating PBMCs from infected people and promoting the selective outgrowth of immortalised $CD4^+$ or $CD8^+$ T cell lines, respectively (Poiesz *et al* 1980a, Yoshida *et al* 1982, Rosenblatt *et al* 1986) (Section 1.3.2). Conditioned medium (CM) or purified cytokine preparations, especially those containing IL2, are used to stimulate the proliferation of $CD4^+$ T cells for isolation of HTLV-I (Chapter 2). UCMCs are used as recipient cells that become infected with HTLV-I and proliferate to form T cell lines when cocultivated with donor PBMCs from infected people (Miyoshi *et al* 1981a, b, Hjelle *et al* 1992b).

Virus particles in cultured T cells may be difficult to distinguish by electron microscopy (EM) from non-virus particles such as cell membrane blebs. Detection of RT activity in supernatants from infected cell lines is of diagnostic value, but HTLV-I or HTLV-II-specific RT activity has to be distinguished from that produced by other exogenous and endogenous retroviruses and from non-specific RT activity. Stronger evidence for the presence of a retrovirus is obtained by density gradient centrifugation and association of RT activity with a

particular density of particle. Virus-specific antigens can be demonstrated in infected cell cultures by immunofluorescence (IF) and other immunological techniques and viral genomes and transcripts can be detected by molecular methods (Section 1.4.3).

1.4.2 Serological assays

Strong humoral immune responses are mounted against HTLV-I Env ($gp21^{Env}$, $gp46^{Env}$), Gag ($p19^{Gag}$, $p24^{Gag}$) and Tax ($p40^{IV}$) antigens, whereas antibodies against Rex ($p27^{III}$)/$p21^{III}$, Tof ($p30^{III}$) and $p12^{I}$ are detected at lower titres or in only a small proportion of HTLV-I-infected people (Kamihira *et al* 1989, Dekaban *et al* 1994, Saiga *et al* 1996, Chen *et al* 1997, Dekaban *et al* 2000). Immunological assays used for screening serum samples for antibodies against HTLV-I and HTLV-II include the enzyme-linked immunosorbent assay (ELISA) and particle agglutination (PA) assay (Beilke 1992). Supplemental tests for serological confirmation of HTLV-I and HTLV-II infection include the western blot (WB) ELISA and radioimmunoprecipitation assay (RIPA) (Aboulafia *et al* 1992). Criteria for HTLV-I/II seropositivity are repeated reactivity in the ELISA and reactivity against proteins from two genes, for example HTLV-I $p19^{Env}$ and $p24^{Gag}$, by WB ELISA or RIPA. Often both supplemental tests are required for comprehensive detection of reactivity against HTLVs, as reactivity tends to be stronger against HTLV Env proteins by WB ELISA and stronger against Gag proteins by RIPA. The delay between infection and seroconversion can result in false negative results (see Section 1.5.1.1). In people who become infected with HTLV-I following blood transfusions, antibodies against $p24^{Gag}$ are detectable earlier than antibodies against $p19^{Gag}$, resulting in indeterminate seroreactivity in testing conducted during seroconversion (Manns *et al* 1991). A further problem with interpretation of serological results is that HTLV-I and HTLV-II could not be clearly distinguished using early tests (Lee *et al* 1989, Chen *et al* 1990, Wiktor *et al* 1990, Hjelle *et al* 1991a). Variations in pattern of seroreactivity and geographical clustering of seroindeterminate results have been suggested as evidence for the presence of variant HTLVs (Maruyama *et al* 1989, Nerurkar *et al* 1992, Madeleine *et al* 1993, Re *et al* 1993, Busch *et al* 2000). Anti-Tax antibodies have been reported in 4% of intravenous drug users (IVDUs) negative for HTLV-I in standard serological assays (Ehrlich *et al* 1989a) (Section 1.11.1.5). The sensitivity and specificity of the tests used and the method of sampling can affect estimates of prevalence and incidence of infection (Section 1.5.2).

1.4.3 Molecular assays

Integrated HTLV-I and HTLV-II proviral DNA can be detected in infected patients by the polymerase chain reaction (PCR), Southern blot (SB) hybridisation and *in situ* hybridisation (ISH) (Beilke 1992) (Chapter 3). Viral RNA genomes and mRNA transcripts are detected by RT-PCR, northern blot hybridisation and DNA-RNA ISH. Selection of probes and control of the stringency of hybridisation is important in optimising the sensitivity and specificity of reactions in SB and northern blot hybridisation. PCR is highly sensitive but this sensitivity also introduces the risk of false positive results due to contamination. Most studies comparing PCR with serological assays have found few virus-positive antibody-negative people (Lee *et al* 1989, Kinoshita *et al* 1993, Miyata *et al* 1995).

1.5 Epidemiology of human T lymphotropic viruses

1.5.1 Transmission

1.5.1.1 HTLV-I

HTLV-I is a cell-associated virus and transmission occurs more efficiently by transfer of intact infected T cells than by cell-free fluids (Fan *et al* 1992). Transmission occurs through sexual intercourse, blood transfusions and other transfers of cellular blood products, sharing of needles by IVDUs and ingestion of breast milk by babies (Okochi *et al* 1984, Hino *et al* 1985, Kajiyama *et al* 1986, Nakano *et al* 1986, Glaser *et al* 1988, Chen *et al* 1989, Lee *et al* 1989, Khabbaz *et al* 1990, Kaplan *et al* 1996). Sexual and lactational transmission are the main modes of infection in endemic regions, whereas in non-endemic regions intravenous drug use accounts for a substantial proportion of transmission; transplacental and perinatal transmission are less important (Blattner *et al* 1983) (Section 1.5.2.1). Since the main vehicles for infectivity during sexual intercourse are lymphocytes in semen, the rate of transmission is higher from male to female than from female to male (Kajiyama *et al* 1986). The proportion of people seroconverting after transfusion with HTLV-I infected blood constituents is 35 to 60% and occurs 20 to 90 days following transfusion (Manns *et al* 1991).

1.5.1.2 HTLV-II

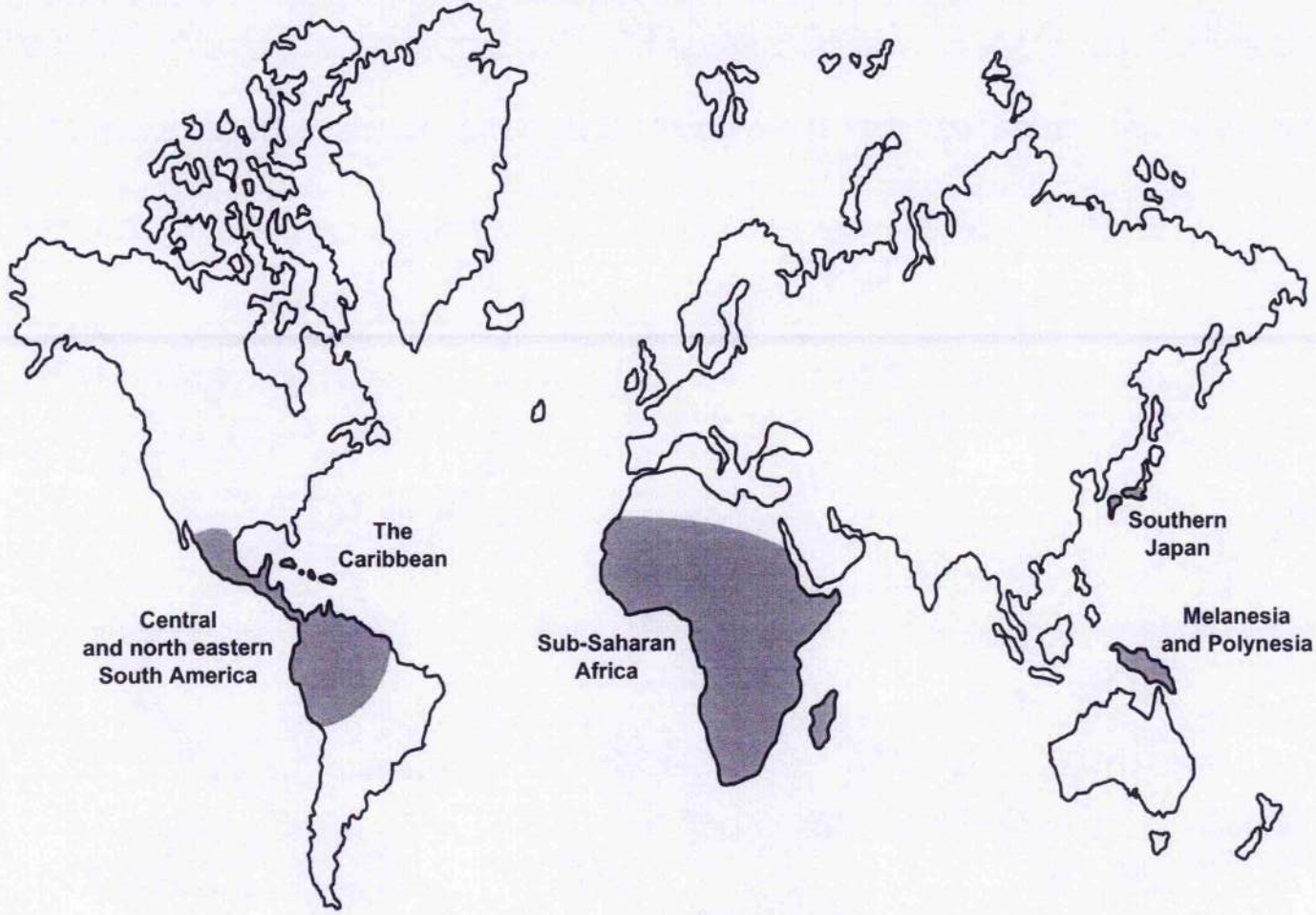
The main modes of transmission of HTLV-II appear to be through sharing of needles by IVDUs and, prior to the introduction of screening tests, through blood transfusions (Robert-Guroff *et al* 1986, Lee *et al* 1989). HTLV-II is also transmitted by sexual intercourse and through breast milk, as for HTLV-I, and these appear to be the main modes of transmission in endemic populations (Hjelle *et al* 1990).

1.5.2 Geographical distribution and high risk groups

1.5.2.1 HTLV-I

HTLV-I is distributed throughout the world, with endemic foci defined by geographical regions and ethnic groups. The virus is endemic (greater than 2% prevalence) in southern Japan, the Caribbean, central America, north eastern South America, central and southern Africa, Melanesia and Polynesia (Hinuma *et al* 1982b, Levine *et al* 1988a) (Figure 1.2). Areas in which the prevalence of HTLV-I is less than 2% (non-endemic regions) include North America, Great Britain, Europe, the Mediterranean and the Middle East. The seroprevalences of infection reported among randomly selected donors in endemic regions tested by RIPA were 12% in Japan and 3.5% in the West Indies, compared to 1.5% in non-endemic regions (Blattner *et al* 1983). The seroprevalence of HTLV-I in Japan ranges from 0 to 35%, endemic regions being the islands of Kyushu, Shikoku and the Ryukyu chain, including Okinawa.

Figure 1.2: World map showing distribution of human T lymphotropic virus type I endemic regions (shaded areas).



HTLV-I seroprevalences of up to 14% have been detected in some populations in the Caribbean (Levine *et al* 1988a). Countries with high prevalences of HTLV-I seropositivity in this region include Jamaica, Trinidad and Tobago, Martinique, Guadeloupe, Barbados and Haiti. Countries of South America with foci of high prevalences of seropositivity for HTLV-I include Colombia, Brazil, Venezuela, Surinam and Guyana. HTLV-I is prevalent (3.7 to 21.0% seropositivity by RIPA) in Africans from the Ivory Coast, Ghana, Nigeria, Zaire, Kenya, Tanzania and South Africa (Saxinger *et al* 1984, Biggar *et al* 1985, 1993). There is serological and virological evidence of infection with HTLV-I and related viruses in indigenous populations of Papua New Guinea, the south western Pacific islands and Australia (Kazura *et al* 1987, Asher *et al* 1988, May *et al* 1990, Yanagihara *et al* 1991). Foci of endemicity have also been reported in Italy (Manzari *et al* 1985).

In the UK the prevalence of HTLV-I infection is low, although certain ethnic groups derived from HTLV-I endemic regions have high prevalences of infection (Greaves *et al* 1984, Tedder *et al* 1984, Mowbray *et al* 1989, Banatvala *et al* 1990, Brennan *et* al 1990, Salker *et al* 1990, Tosswill *et al* 1990, Simms *et al* 1994, Hale *et al* 1997). In women attending antenatal clinics in England, antibodies were detected against HTLV-I by ELISA in 0.05% and against HTLV-II less than 0.01% (Ades *et al* 2000). The seroprevalence of HTLV-I in Afro-Caribbean (0.97%) and African (0.26%) women was higher than in Caucasian women (0.03%). In north London 5.2% of IVDUs had antibodies against HTLV-I/II, whereas no antibodies against HTLV-I/II were detected in IVDUs in Scotland (The HTLV European Research Network 1996, McIntyre *et al* 2001).

1.5.2.2 HTLV-II

HTLV-II is prevalent in some groups of American Indians and in IVDUs (Reeves *et al* 1990, Maloney *et al* 1992, Black *et al* 1994). Endemic foci are located predominantly in South America. The prevalence is high in American Indians in Florida, New Mexico, Arizona and Panama. Cayapo and Kraho Indians of the Amazon Basin in Brazil have HTLV-II seroprevalences of 12 to 33%, whereas other tribes in the same region have low prevalences of infection (Maloney *et al* 1992). High prevalences of HTLV-II infection (11 to 18%) were reported among IVDUs in the USA using serological and molecular assays (Robert-Guroff *et al* 1986, Ehrlich *et al* 1989b, Lee *et al* 1989, Kwok *et al* 1990). Prevalences of HTLV-II infection in IVDUs were 2.6% in the UK and 1.5% in Italy (Tedder *et al* 1984, Zella *et al* 1990).

1.5.3 Molecular epidemiology

1.5.3.1 Subtypes of human T lymphotropic viruses

HTLV-I and HTLV-II have approximately 60% nucleotide sequence identity (Seiki *et al* 1983, Shimotohno *et al* 1985b, Kwok *et al* 1988b, Lee *et al* 1993). HTLV-I has been classified into Cosmopolitan (I_A), Central African (I_B), Melanesian (I_C) and Pigmy (I_D) subtypes (Mahieux *et al* 1997). The prototype HTLV-I_A strain is the Japanese isolate HTLV-I_{ATK-1} and Cosmopolitan isolates from Japan, the Caribbean, North and South America and Africa have greater than or equal to 97% nucleotide sequence identity across the full-length genome. Melanesian

strains have 92% identity with HTLV-I_{ATK-1} (Gessain *et al* 1993). Three subtypes of HTLV-II have been identified: II_A, II_B and II_C (Switzer *et al* 1995, Biggar *et al* 1996).

1.5.3.2 Origins and global spread

STLV-I, which is closely related to HTLV-I, is present in Old World primates in Africa and Japan and it has been hypothesised that HTLV-I and STLV-I originated in Africa (Gallo *et al* 1983b, Gessain *et al* 1992b). The viruses appear to have evolved in non-human primates with multiple independent events of transmission to humans accounting for different subtypes of HTLV-I (Koralnik *et al* 1994, Liu *et al* 1996). HTLV-I may have been spread to the Americas by infected Africans in more recent times. In Japan, HTLV-I appears to have been introduced to the Joman people between 300 and 100 BC, resulting in high prevalences of infection in their descendents, the Ainu and Ryukuans (Vidal *et al* 1994). The Melanesian subtype (HTLV-I_C) appears to have evolved in relative isolation from other strains (Gessain *et al* 1993). Foci of HTLV-II_A endemicity have been identified in American Indians, consistent with introduction of HTLV-II to the Americas at least 15,000 years ago (Biggar *et al* 1996). However, it is uncertain whether STLV-II is present in New World monkeys (Chen *et al* 1994). HTLV-II_B has been identified in isolated populations of Pygmies in Africa and thus HTLV-II appears to be an ancient virus (Gessain *et al* 1995).

1.6 Diseases associated with HTLV-I

HTLV-I is aetiologically associated with ATL and HAM/TSP and has been implicated in other neoplastic or immune-mediated diseases, which are described in this section (Table 1.2). Evidence for the involvement of HTLV-I or related viruses in MF/SS, other CTCLs and LGL leukaemia is discussed in Section 1.11.

1.6.1 Adult T cell leukaemia/lymphoma

Adult T cell leukaemia/lymphoma (ATL) is an aggressive T cell neoplasm of adults caused by HTLV-I. It was first described in Japan and defined on the basis of distinctive clinicopathological features and geographical distribution (Uchiyama *et al* 1977, Hanaoka *et al* 1979, Shimoyama *et al* 1991).

1.6.1.1 Epidemiology

Cases of ATL are clustered in regions reflecting the distribution of HTLV-I infection, particularly south western Japan and the Caribbean (Blattner *et al* 1982, 1983, Hinuma *et al* 1982b, Robert-Guroff *et al* 1982) (Section 1.5.2.1). Sporadic cases occur in other parts of the world where HTLV-I infection is uncommon; many of these cases are in Japanese or Afro-Caribbean migrants from endemic regions (Greaves *et al* 1984, Wyld *et al* 1990, Pawson *et al* 1998a and b, Levine *et al* 1999). Over their life span, 1 to 5% of people infected with HTLV-I develop ATL (Kondo *et al* 1989, Murphy *et al* 1989, Tokudome *et al* 1989, Tajima *et al* 1990).

Table 1.2 Proposed association of human T lymphotropic virus type I with human diseases.

Adult T cell leukaemia/lymphoma*
HTLV-I-associated myelopathy/tropical spastic paraparesis*
HTLV-I-associated myositis
HTLV-I-associated uveitis
HTLV-I-associated arthritis
HTLV-I-associated pneumonitis
Infective dermatitis
HTLV-I-associated lymphadenitis
Autoimmune thyroiditis
Autoimmune thrombocytopaenia
Systemic lupus erythematosus
Sjögren's syndrome
Multiple sclerosis
Chronic fatigue syndrome
B cell chronic lymphocytic leukaemia
B cell hairy cell leukaemia
B cell non-Hodgkin lymphoma
Acute and chronic myeloid leukaemia
Mycosis fungoides/Sézary syndrome
Lymphoproliferative disease of large granular lymphocytes

* Aetiological role accepted

In Japan, 0.25 to 3.90 cases/100,000 people/year are diagnosed, although in some areas the rate may be substantially higher. The latent period between exposure to HTLV-I and occurrence of ATL is usually 20 years or more. Most ATL cases present from 25 years of age and the peak occurrence is at 50 to 55 years of age, although much younger cases have been reported (Murphy *et al* 1989, Tajima *et al* 1990, Pombo de Oliveira *et al* 2002). Exposure to HTLV-I early in life, particularly through breast milk, is associated with a higher risk of development of ATL (Murphy *et al* 1989).

1.6.1.2 Clinical and pathological features

ATL usually presents as an acute form with leukaemia and neoplastic T cells infiltrating the lymph nodes, skin, liver, spleen and bone marrow (Uchiyama *et al* 1977, Blayney *et al* 1983b, Jaffe *et al* 1984, Shimoyama *et al* 1991). This form is characterised clinically by enlargement of lymph nodes, nodular skin lesions, hypercalcaemia and lytic bone lesions. Among the less common variant ATL syndromes is a chronic form with erythaema but without hypercalcaemia or hepatosplenomegaly. A smouldering form with an indolent course involves the skin and is typified clinically by erythaema (Yamaguchi *et al* 1983). A lymphomatous form characterised by lymphadenopathy without leukaemia includes primary T cell lymphomas (TCLs) involving lymph nodes (Ohshima *et al* 1998b). A variety of extranodal locations, including the gastrointestinal tract, may also be sites of primary lymphomas in ATL. Infection with secondary pathogens in ATL is associated with immunosuppression (White *et al* 1995). Spontaneous remission of ATL is rare and the disease usually has an aggressive clinical course (Shimamoto *et al* 1993). In patients with leukaemia, the circulating neoplastic lymphocytes are known as "flower" cells because of their lobulated nuclei (Jaffe *et al* 1984). The neoplastic infiltrate in solid tumours consists of a mixture of small and large pleomorphic lymphocytes with lobulated nuclei, sometimes interspersed with multinucleated cells.

1.6.1.3 Immunology

The neoplastic cells in ATL are usually $CD4^+$ T cells that are $CD7^-$, $CD8^-$ and $CD25^+$ ($IL2R\alpha^+$, Tac^+) (Waldmann *et al* 1984, Kamihira *et al* 1992). In addition to CD25, markers of activation expressed by ATL cells include CD30, CD71, MHC-II (HLA-DR), Ki67 and the lymphocyte common antigen (LCA) isoform CD45RA (Shirono *et al* 1989). In functional studies, ATL cells exhibit T suppressor (Ts) activity (Tatsumi *et al* 1980), whereas the pattern of cytokine secretion is variable (Tendler *et al* 1994, Yamada *et al* 1996). $CD8^+$ and $CD4^+$ cytotoxic T lymphocytes (CTLs) against HTLV-I $gp21^{Env}$, $gp46^{Env}$, $p19^{Gag}$, $p24^{Gag}$, Tax and Rex antigens can be induced *in vitro* from PBMCs of patients with ATL, as well as asymptomatic carriers (Katahira *et al* 1995). Fresh ATL cells have low susceptibility to lysis by Tax-specific CTLs due to reduced HTLV-I expression *in vivo* (Kozuru *et al* 1989, Kannagi *et al* 1993). NK cells are able to lyse HTLV-I-infected T cells that express viral antigens and evasion of NK cell-mediated destruction may be important in survival of neoplastic T cells (Feuer *et al* 1995, Stewart *et al* 1996). Plotnicky *et al* (1994) noted that IL2-dependent HTLV-I-infected T cell lines are resistant to NK cell-mediated lysis, whereas IL2-independent cell lines are susceptible. An immunosuppressive effect of HTLV-I $gp21^{Env}$ has been demonstrated but its relevance to the pathogenesis of neoplasia is uncertain (Cianciolo *et al* 1984).

1.6.1.4 Molecular and genetic characteristics

HTLV-I proviruses are clonally integrated in neoplastic T cells in patients with ATL (Wong-Staal *et al* 1983, Seiki *et al* 1984, Yoshida *et al* 1984, Takahashi *et al* 1988) and ATL cells have clonal rearrangements of the T cell receptor (TCR) β genes (Jarrett *et al* 1986, Matsuoka *et al* 1988, Tanaka *et al* 1989, Ohshima *et al* 1990, Kanekura *et al* 1993). Although clonal numerical and structural chromosomal abnormalities are found in neoplastic T cells in a high proportion of patients, no common defects have been identified (Kamada *et al* 1992, Ohshima *et al* 1998a). The TCR α locus may be involved in ATL cases, as well as HTLV-I negative T cell leukaemias, with breakpoints at chromosome 14q11 (Sadamori *et al* 1986). As with many neoplasms, *p53* mutations are frequent in leukaemic cells from ATL patients (Nagai *et al* 1991, Sugito *et al* 1991, Cesarman *et al* 1992, Sakashita *et al* 1992, Yamato *et al* 1993) (Section 1.8.5). Deletions are present in 10 to 56% of HTLV-I proviruses in neoplastic cells but there is preferential retention of the *pX* region (Manzari *et al* 1983, Konishi *et al* 1984, Korber *et al* 1991, Ohshima *et al* 1991, Sakurai *et al* 1992a, Chou *et al* 1995, Tamiya *et al* 1996). Mutations in *pX* ORF II preventing expression of Tof and $p13^{II}$ have been identified in ATL cells *in vivo* indicating that these *pX* proteins may not be essential for leukaemogenesis (Chou *et al* 1995).

The sites of integration of HTLV-I proviruses differ between patients with ATL and appear to be non-specific (Seiki *et al* 1984, Chou *et al* 1996, Ohshima *et al* 1998a). In some cases integration of HTLV-I adjacent to cellular genes such as IL2R has been demonstrated, but insertional mutagenesis is not considered to be the principal mode of oncogenesis (Okamoto *et al* 1986, Macera *et al* 1992, Nakamura *et al* 1994, Kubota *et al* 1996b, Chi *et al* 1997) (Section 1.8). A small number of patients with ATL-like leukaemia or lymphoma have anti-HTLV-I serum antibodies but no evidence of monoclonal integration of proviral DNA, suggesting that HTLV-I could induce leukaemia indirectly or by a hit-and-run mechanism (Yoshida *et al* 1989b, Fujiwara *et al* 2001). The rare occurrence of HTLV-I-negative cases of ATL-like neoplasia also supports the hypothesis that other molecular and cytogenetic events are important in the pathogenesis of ATL (Shimoyama *et al* 1987). ATL and HAM/TSP do not appear to be caused by different strains or molecular variants of HTLV-I, as the two diseases may develop in the same person or in members of the same family (Kawai *et al* 1989, Uozumi *et al* 1991, Major *et al* 1993, Tamiya *et al* 1995). Sequence variations appear to be related to geographical origin of HTLV-I types rather than pathogenicity (Daenke *et al* 1990, Kinoshita *et al* 1991, Komurian *et al* 1991). It has also been shown that the HTLV-I LTR directs expression in a wide range of tissues independent of the source of virus (Gonzalez-Dunia *et al* 1993).

1.6.1.5 Viral aetiology

The viral aetiology of ATL was established following the isolation of HTLV-I and its association with T cell leukaemias and lymphomas in Japan, the Caribbean and the USA (Blattner *et al* 1982, Hinuma *et al* 1982b, Blattner *et al* 1983, Blayney *et al* 1983a). Patients with ATL are almost invariably infected with HTLV-I and have serum antibodies against the virus (Posner *et al* 1981, Kalyanaraman *et al* 1982a, Robert-Guroff *et al* 1982, Gallo *et al* 1983a). Infection precedes the development of ATL and HTLV-I genomes can be detected in

leukaemic cells. Thus, although ATL develops in only a small proportion of HTLV-I-infected people after a long latent period, the evidence for an aetiological association is compelling. A small number of patients with ATL are negative in standard serological assays but have molecular evidence of HTLV-I infection; in some cases this may be due to the presence of defective HTLV-I (Korber *et al* 1991, Ohshima *et al* 1991, Kubota *et al* 1995). ATL-like neoplasia has also been observed in a small number of patients without serological or molecular evidence of HTLV-I infection, but these cases appear to be exceptional (Shimoyama *et al* 1986).

1.6.1.6 Pathogenesis

HTLV-I does not possess a classical viral oncogene with homology to a cellular gene, as carried by acutely transforming simple retroviruses, and does not appear to induce neoplasia by insertional mutagenesis (Section 1.6.1.4). Instead, there is strong evidence that proteins encoded by the *pX* region are responsible for oncogenicity (Section 1.3.5). Tax and Rex are essential for replication of HTLV-I (Chen *et al* 1985) and Tax is the main HTLV-I-encoded protein implicated in leukaemogenesis (Nerenberg *et al* 1987, Tanaka *et al* 1990, Grassmann *et al* 1992) (Sections 1.8.1, 1.8.2 and 1.8.3). There is also evidence that $p12^{I}$ has transforming properties (Section 1.8.4), but the oncogenic roles of Tof, $p21^{III}$ and $p13^{II}$ are uncertain (Chou *et al* 1995, Derse *et al* 1997, Robek *et al* 1998).

Most leukaemic cells from ATL patients do not express viral antigens detectable by methods of low sensitivity such as IF or WB immunoassay but viral antigens are readily detected following *in vitro* cultivation of PBMCs from these patients due to growth of HTLV-I in non-leukaemic cells (Poiesz *et al* 1980a, Hinuma *et al* 1982a, Hoshino *et al* 1983, Salahuddin *et al* 1983). Similarly, HTLV-I mRNA cannot be detected in most leukaemic cells from ATL patients by northern blot hybridisation, whereas transcripts are readily detected by this method in cultured PBMCs (Clarke *et al* 1984, Franchini *et al* 1984) and ATL cells infiltrating skin (Setoyama *et al* 1992). Virus particles are produced in abundance in most HTLV-I-infected T cell lines, including ATL cells adapted to grow in culture (most of which are not derived from the leukaemic clone), whereas virus particles are rarely produced by ATL cells *in vivo* (Yoshida *et al* 1982, Vital *et al* 1993). A low level of expression of HTLV-I mRNA and proteins in ATL patients could be a manifestation of viral latency due to regulation by viral or cellular gene products or by the immune system of the host (Section 1.3.6). Serum antibodies against HTLV-I suppress viral antigen expression in infected T cells in culture (Tochikura *et al* 1985). HTLV-I $p24^{Gag}$ can be detected more frequently in the serum of patients with acute or advanced ATL than in HTLV-I carriers or patients with chronic ATL (Ishibashi *et al* 1987). The host immune response may suppress populations of infected cells that express viral antigens until advanced stages of disease when the immune response is weakened. The increase in mRNA and protein expression in cultivated cells may result from activation of latent HTLV-I provirus in ATL cells. However, most HTLV-I-transformed cell lines that grow in culture from PBMCs of ATL patients are not derived from leukaemic clones (Yoshida *et al* 1982). Salahuddin *et al* (1983) observed limited expression of HTLV-I antigens in UCMCs following HTLV-I infection. These results indicate that a high level of expression of viral antigens is not required for maintenance of the transformed phenotype in patients with ATL.

Using more sensitive RT-PCR, HTLV-I genomic, *env* and *pX* mRNA transcripts, including those encoding Tax, Rex, Tof, $p21^{III}$, $p13^{II}$ and $p12^{I}$, can be detected in uncultured PBMCs of most patients with ATL, as well as unaffected HTLV-I carriers, but levels are 10^{5} to 10^{6} times less than that in HTLV-I-infected T cell lines (Kinoshita *et al* 1989, Berneman *et al* 1992b, Ciminale *et al* 1992, Koralnik *et al* 1992). HTLV-I *tax/rex* mRNA transcripts were detected in 0.001% of PBMCs in five of six ATL patients by RT-PCR (Kinoshita *et al* 1989). Setoyama *et al* (1994) detected *tax/rex* mRNA by the less sensitive ISH in 1 to 90% of leukaemic cells in peripheral blood of four patients with ATL and 1 to 50% of leukaemic cells infiltrating skin lesions in 10 patients. Ohshima *et al* (1996) detected *tax/rex* mRNA by RT-PCR-ISH in the cytoplasm of 10% of PBMCs containing HTLV-I proviruses from ATL patients. Mutations in *pX* ORF II preventing expression of Tof and $p13^{II}$ have been identified in ATL cells *in vivo* (Chou *et al* 1995).

In asymptomatic people infected with HTLV-I there is polyclonal integration of provirus in $CD4^{+}$ T cells (Yamaguchi *et al* 1988). With progression to ATL the proportion of circulating lymphocytes infected with HTLV-I increases and oligoclonal or monoclonal integration of provirus becomes detectable (Yoshida *et al* 1984, Kinoshita *et al* 1985, Leclercq *et al* 1998). HTLV-I-infected $CD4^{+}$ T cell clones may persist for many years in carriers (Etoh *et al* 1997). In the preclinical stages of ATL there may be leucocytosis or the presence of abnormal lymphocytes in the peripheral blood (Kinoshita *et al* 1985, Ikeda *et al* 1993). Some patients experience phases of smouldering or chronic ATL before the onset of acute ATL (Shimoyama *et al* 1991). Upregulation of IL2 and IL2Rα (CD25) by Tax, generating an autocrine loop, is thought to be one of the mechanisms by which HTLV-I stimulates proliferation of T cells early in the course of infection (Arima *et al* 1986, Maeda *et al* 1987, Maruyama *et al* 1987, Siekevitz *et al* 1987) (Section 1.8.1.1). Many other biochemical pathways are altered in HTLV-I-infected T cells and could contribute to abnormal pre-neoplastic proliferation or maintenance of the neoplastic phenotype. Accumulation of mutations in proliferating T cells leads to the emergence of neoplastic clones (Fukuhara *et al* 1983, Sanada *et al* 1985, 1986, Mortreux *et al* 2001). The roles of Tax, $p12^{I}$, p53, c-Myc, apoptosis and Env in HTLV-I leukaemogeneis are discussed in Section 1.8.

1.6.2 HTLV-I and non-T cell neoplasia

Serological studies have associated HTLV-I with B cell, myeloid and other non-T cell neoplasia but an aetiological role is unconfirmed (Blomberg and Fäldt 1985, Asou *et al* 1986).

1.6.2.1 B cell neoplasia

A patient with B cell chronic lymphocytic leukaemia (CLL) had HTLV-I infection of T cells; leukaemic B cells were negative for HTLV-I but expressed immunoglobulins reactive with HTLV-I Gag and Env proteins (Mann *et al* 1987). HTLV-I infection of T cells may have resulted in chronic antigenic stimulation of B cells, promoting oncogenic events leading to leukaemia. HTLV *tax* sequences have also been amplified by PCR from PBMCs of other patients with B cell CLL, B cell HCL and B cell non-Hodgkin lymphoma, but there is insufficient evidence for an aetiological role in these neoplasms (Cardoso *et al* 1996).

1.6.2.2 Myeloid neoplasia

HTLV-I/II seroreactivity has been detected in patients with acute and chronic myeloid leukaemia (Kalyanaraman *et al* 1982a, Möstl *et al* 1992). HTLV-I-related antigens, RT activity and retrovirus-like particles visible by EM were detected by Xu *et al* (1996a) in leukaemic PBMCs from patients with acute myeloid leukaemia. DNA from PBMCs of an HTLV-I seropositive patient with chronic myeloid leukaemia hybridised to a probe for the HTLV-I 3' LTR, but not to probes for other regions, suggesting the presence of variant or defective HTLV-I (Wong-Staal *et al* 1983). However, there is insufficient evidence to incriminate HTLV-I in the aetiology of myeloid neoplasia.

1.6.3 HTLV-I-associated myelopathy/tropical spastic paraparesis

HTLV-I has been aetiologically linked with HTLV-I-associated myelopathy (HAM) and tropical spastic paraparesis (TSP), which are considered to be identical syndromes (Gessain *et al* 1985, Bartholomew *et al* 1986, Osame *et al* 1986, Román and Osame *et al* 1988).

1.6.3.1 Epidemiology

HAM/TSP occurs in areas endemic for HTLV-I (Levine *et al* 1988a) (Section 1.5.2.1). Sporadic cases have been reported from non-endemic areas, mainly in immigrants from HTLV-I endemic areas (Sheremata *et al* 1992). HAM/TSP develops in approximately 0.25% of people infected with HTLV-I over a lifetime of 75 years and the incidence in Japan is 3.1 cases/100,000 HTLV-I infected people/year (Kaplan *et al* 1990). Recipients of blood transfusions appear to be at higher risk of developing HAM/TSP, but infection by sexual intercourse is also a risk factor (Kramer *et al* 1995). The latent period between exposure to HTLV-I and the development of disease is shorter for HAM/TSP than for ATL (Osame *et al* 1986).

1.6.3.2 Clinical and pathological features

HAM/TSP is a chronic demyelinating disorder of the central nervous system (CNS) with a slowly progressive course characterised by weakness, spastic paraparesis or paraplegia (Gessain *et al* 1985, Osame *et al* 1986, Nakagawa *et al* 1995). Atypical neurodegenerative disorders not matching the case definition for HAM/TSP have also been associated with HTLV-I (Kazadi *et al* 1990, Hyer *et al* 1991).

1.6.3.3 Molecular and genetic characteristics

Integration of HTLV-I provirus in PBMCs of HAM/TSP patients is usually polyclonal or oligoclonal (Greenberg *et al* 1989b, Gessain *et al* 1990b, Furukawa *et al* 1992). HTLV-I infects 3 to 30% of PBMCs and 15 to 18% of circulating T cells in HAM/TSP patients; most infected cells have a single integrated provirus (Richardson *et al* 1997).

1.6.3.4 Viral aetiology

Anti-HTLV-I antibodies are present in the serum and cerebrospinal fluid (CSF) of patients with HAM/TSP, supporting the viral aetiology of this disease (Gessain *et al* 1985, Osame *et al* 1987). HTLV-I sequences can be detected in PBMCs, CSF and CNS tissue (Bangham *et al* 1988, Bhagavati *et al* 1988, Iannone *et al* 1992) and HTLV-I-infected T cell lines can be generated from PBMCs and CSF (Jacobson *et al* 1988).

1.6.3.5 Immunology and pathogenesis

HAM/TSP is thought to be an immune-mediated demyelinating disorder of the CNS with pathological changes induced by CTLs. Acute lesions in the CNS are infiltrated with activated $CD4^+$ T cells, followed by accumulation of $CD8^+$ T cells and macrophages in the chronic stages (Umehara *et al* 1993). A strong humoral immune response is mounted against HTLV-I Env, Gag and Tax antigens (Dekaban *et al* 1994). CTL responses are directed predominantly against Tax (Jacobson *et al* 1990, Parker *et al* 1994). Most HTLV-I-infected T cells are transcriptionally silent in HAM/TSP patients; expression of *tax/rex* mRNA has been demonstrated in 0.001 to 1% of PBMCs (Beilke *et al* 1991, Gessain *et al* 1991). The quantity of provirus in the peripheral blood is usually higher in HAM/TSP patients than in HTLV-I-infected people without neurological disease and, paradoxically, the level and frequency of expression of *tax* in infected PBMCs may be higher than in ATL (Yoshida *et al* 1989a, Gessain *et al* 1990b, Furukawa *et al* 1995). HAM/TSP patients may have a defective immune response to HTLV-I infection, permitting increased replication and expression of viral antigens coupled with development of autoimmunity. Certain MHC-I alleles, particularly *HLA-A*02*, are associated with reduced proviral load and reduced risk of HAM/TSP (Jeffery *et al* 1999). HTLV-I *tax* mRNA has also been detected by ISH in the CNS of HAM/TSP patients (Lehky *et al* 1995).

1.6.4 Other HTLV-I-associated immune-mediated diseases

HTLV-I has been associated with myositis, uveitis, arthritis, pneumonitis and several other apparently immune-mediated diseases. Attempts have been made to implicate HTLV-I in Sjögren's syndrome, multiple sclerosis (MS), systemic lupus erythematosus (SLE) and rheumatoid arthritis but the evidence for an association with these diseases is unconvincing.

1.6.4.1 HTLV-I-associated myositis

In Jamaica 11 of 13 patients with polymyositis had antibodies against HTLV-I (Morgan *et al* 1989). HTLV-I sequences have been detected in muscle tissue of patients with polymyositis and antigens were localised immunohistochemically to mononuclear inflammatory cells (Sherman *et al* 1995). In contrast, only one of 54 Japanese patients with polymyositis was seropositive for HTLV-I (Nishikai *et al* 1991). Virus could not be detected by immunohistochemistry or ISH in muscle fibres of a series of HTLV-I-infected patients in Japan with polymyositis (Higuchi *et al* 1992). Thus, other factors may be involved in the pathogenesis of myositis in patients with HTLV-I infection in the Caribbean.

1.6.4.2 HTLV-I-associated uveitis

Antibodies against HTLV-I were detected in a higher proportion of patients with idiopathic uveitis (35%) in Japan than patients with uveitis of defined aetiology (10%) or other ocular diseases (16%), compared to a seroprevalence of 11.5% in the local population (Mochizuki *et al* 1992). HTLV-I proviral sequences can be detected in the inflammatory exudate in the anterior chamber of patients with HTLV-I-associated uveitis.

1.6.4.3 HTLV-I-associated arthritis

HTLV-I is associated with arthritis and polyarthritis in endemic regions (Nishioka *et al* 1989, Sato *et al* 1991). Antibodies against HTLV-I are present and virus has been identified in the synovium and infiltrating T cells from joints of affected patients (Kitajima *et al* 1991, Eguchi *et al* 1992b). In contrast, no HTLV-I sequences were detected by PCR in one study of rheumatoid arthritis in a non-endemic region (Di Giovine *et al* 1994).

1.6.4.4 HTLV-I-associated pneumonitis

HTLV-I-associated pneumonitis, characterised by T lymphocytic alveolitis, has usually been observed in patients with other HTLV-I-related diseases (Sugimoto *et al* 1987, Vernant *et al* 1988, Higashiyama *et al* 1994). Therefore, HTLV-I-associated pneumonitis may not be a distinct clinicopathological entity.

1.6.4.5 Infective dermatitis

Infective dermatitis, a chronic dermatitis associated with *Staphylococcus aureus* or *Streptococcus* spp infection, has been associated with HTLV-I infection (LaGrenade *et al* 1990). This may be a consequence of immunosuppression due to HTLV-I; similarly an increase in parasitic and bacterial diseases has been epidemiologically linked to HTLV-I infection (Nakada *et al* 1987, Murphy *et al* 1997). A lymphadenopathy characterised by infiltration of lymph nodes with polyclonal $CD4^+$ T cells has also been attributed to immune dysfunction in HTLV-I-infected people (Ohshima *et al* 1992).

1.6.4.6 HTLV-I and other immune-mediated diseases

Serological and virological markers of HTLV-I infection have been identified in patients with several other immune-mediated diseases, including Sjögren's syndrome (Eguchi *et al* 1992a, Terada *et al* 1994), MS (Koprowski *et al* 1985), SLE (Olsen *et al* 1987, Lipka *et al* 1996), autoimmune thyroiditis (Kawai *et al* 1992), autoimmune thrombocytopaenia (Dixon *et al* 1989) and chronic fatigue syndrome (DeFreitas *et al* 1991, Honda *et al* 1993), but evidence for an aetiological role in these conditions remains inconclusive.

Sjögren's syndrome and MS have been of particular interest because of their possible association with endogenous retroviruses that cross-react antigenically with HTLV-I. An apparent HTLV-I $p19^{Gag}$ seroreactivity in some cases of Sjögren's syndrome may be due to a cross-reactive antigen expressed in the salivary gland from the endogenous retroviral

sequence HRES-1 (Banki *et al* 1992, Shattles *et al* 1992). Several groups have detected low copy numbers of HTLV-I *tax* sequences by PCR in labial salivary gland samples from some patients with Sjögren's syndrome (Mizokami *et al* 1998, Mariette *et al* 2000). It will be necessary to verify the methodology used in these studies and determine the pathogenetic role of the *tax* sequences before an aetiological link can be accepted.

HTLV-I sequences and antibodies have been identified in MS patients (Koprowski *et al* 1985, Greenberg *et al* 1989a, Reddy *et al* 1989) but an extensive series of follow-up studies failed to confirm the involvement of HTLV-I or related viruses in this disease (Ehrlich *et al* 1991). mRNA transcripts of MS-associated retroviral element (MSRV), which belongs to the human endogenous retrovirus (HERV) family HERV-W, can be detected in retrovirus-like particles from cultured PBMCs of patients with MS, as well as in normal human placenta (Blond *et al* 1999). HERV-H sequences are also expressed in Epstein-Barr virus (EBV)-infected B lymphoblastoid cell lines (B-LCLs) established from MS patients (Haahr *et al* 1994, Christensen *et al* 2000). Further independent studies are necessary to determine if retroviruses are involved in MS.

1.7 Diseases associated with HTLV-II

The role of HTLV-II in the aetiology of disease is uncertain. The virus has been associated with $CD8^+$ T cell lymphocytosis and leukaemia and chronic neurodegenerative disease resembling HAM/TSP but is not a cause of ATL (Kiyokawa *et al* 1991) (Table 1.3). Kaplan *et al* (1991) identified a syndrome of severe skin disease, eosinophilia, and dermatopathic lymphadenopathy in patients with HTLV-II complicating HIV-1 infection. There is also evidence that HTLV-II is involved in immune-mediated diseases; HTLV-II *pol* and *tax* sequences were amplified from PBMCs of 52% of patients with Hashimoto's thyroiditis and 12% of patients with Graves' disease compared to 2% of controls and 1% of blood donors in Japan, although all were seronegative for HTLV-II (Yokoi *et al* 1995). Independent confirmation of this study is required. Evidence for the involvement of HTLV-II in MF/SS and LGL leukaemia is discussed in Section 1.11.

1.7.1 $CD8^+$ T cell lymphocytosis and leukaemia

Early isolates of HTLV-II were obtained from patients with T cell variants of HCL (atypical $CD8^+$ T cell HCL) (Kalyanaraman *et al* 1982b, Rosenblatt *et al* 1986) and $CD8^+$ T cell lymphocytosis in association with B cell HCL (Rosenblatt *et al* 1988b). Sohn *et al* (1986) detected antibodies against HTLV-I/II in two patients with T cell variants of HCL. Subsequent molecular and seroepidemiological studies have not established an association between HTLV-II and T or B cell forms of HCL (Rosenblatt *et al* 1987, Lion *et al* 1988, Hjelle *et al* 1991a).

Table 1.3 Proposed association of human T lymphotropic virus type II with human diseases.

$CD8^+$ T cell lymphocytosis and leukaemia
Chronic neurodegenerative disease
Dermatopathic lymphadenopathy
Autoimmune thyroiditis
Mycosis fungoides
Lymphoproliferative disease of large granular lymphocytes

1.7.2 Chronic neurodegenerative disease

Infection with HTLV-II has been identified in patients with chronic neurodegenerative diseases resembling HAM/TSP (Hjelle *et al* 1992, Harrington *et al* 1993, Jacobson *et al* 1993, Murphy *et al* 1993, Sheremata *et al* 1993). The virus has been isolated from PBMCs and detected by PCR in PBMCs but not CSF of patients with neurological disorders clinically indistinguishable from HAM/TSP (Jacobson *et al* 1993, Murphy *et al* 1993). HTLV-II infection has also been demonstrated serologically and by PCR in four patients with tropical ataxic neuropathy, a neurological disorder characterised by ataxia, altered mental state, optic atrophy, deafness and peripheral neuropathy and described in Africa and the Caribbean (Sheremata *et al* 1993). However, HTLV-II does not appear to be a common cause of chronic neurodegenerative disease.

1.8 Molecular mechanisms of HTLV-I leukaemogenesis

Tax is the main HTLV-I-encoded protein implicated in the pathogenesis of ATL (Franchini 1995, Franklin and Nyborg 1995) (Section 1.6.1.6). This section discusses the mechanisms by which Tax modulates expression of cellular genes in HTLV-I-infected cells, the transforming properties of Tax, $p12^I$ and Env and the roles of p53, c-Myc and apoptosis in HTLV-I oncogenesis.

1.8.1 Effect of Tax on gene expression in HTLV-I-infected cells

The expression of a wide variety of cellular genes is altered following infection by HTLV-I and this effect is mediated predominantly by Tax (Salahuddin *et al* 1984, Miyatake *et al* 1988, Kelly *et al* 1992, Yoshida *et al* 1995) (Table 1.4). Differential regulation of cellular genes by Tax is hypothesised to be the main mechanism by which HTLV-I transforms T cells *in vitro* and initiates the oncogenic events leading to leukaemia *in vivo*. Many of the cellular genes transactivated by Tax are involved in activation, proliferation and differentiation of T cells, consistent with this protein having a direct leukaemogenic role in ATL (Wano *et al* 1988, Marriott *et al* 1991, Höllsberg 1999).

1.8.1.1 Cytokines and cytokine receptors

The high affinity IL2R is composed of α (CD25), common β (CD122, β_c) and common γ (CD132, γ_c) chains (Taniguchi *et al* 1995). The β_c and γ_c chains transduce signals resulting from binding of IL2 at the cell surface but their affinity for IL2 is increased substantially by inclusion of the α chain in the receptor complex. In activated T cells transduction of signals through the IL2R normally activates the Src family protein tyrosine kinases (PTKs) Lck and Fyn (Mills *et al* 1993). These in turn activate Ras, leading to up-regulation of the cellular oncogenes *fos* and *jun*. The IL2 and IL2Rα genes are up-regulated by Tax and this effect is more pronounced in T cells than other cell types (Krönke *et al* 1985, Greene *et al* 1986, Inoue *et al* 1986, Waldmann 1986, Maruyama *et al* 1987, Siekevitz *et al* 1987).

Table 1.4: Alteration of cellular gene expression by HTLV-I Tax.

Genes up-regulated through the NFκB pathway

Interleukins 1α, 2, 3, 4, 6, 8, 10 and 15
Interleukin 2 receptor α and interleukin 15 receptor
Tumour necrosis factors α (cachectin) and β (lymphotoxin)
Granulocyte-macrophage colony stimulating factor
Granulocyte colony stimulating factor
CD106 (vascular cell adhesion molecule 1)
Bcl-xL

Genes up-regulated through CREB/ATF/AP1 pathways

Interleukin 5
Granulocyte-macrophage colony stimulating factor
Transforming growth factor β
Parathyroid hormone-related protein
β and ε globin
Egr1 and Egr2
Fos and Fra1
Nur77
Bcl-xL

Genes up-regulated through other pathways

Interleukin 1β (NF-IL6, Spi1)
Interleukin 2 (NFκB, NF-AT)
Interleukin 4 (NFκB, NF-AT, NF-IL6)
Interleukin 5 (GATA4, AP1)
Interleukin 2 receptor α (NFκB, SRF)
Platelet-derived growth factor receptor (Sis) (Sp1, Egr1)
Parathyroid hormone-related protein (CREB/ATF, SP1, EBS)
Egr1 and Egr2 (CREB/ATF, SRE, EBS)
Fos (CREB/ATF, SRF, SIE, octanucleotide direct repeat)
Fra1 (Rb, AP1)

Genes down-regulated by Tax through CREB/ATF pathway

Cyclins A and D3
DNA polymerase α

Genes down-regulated by Tax via bHLH proteins

Lck
p53
Neurofibromin (NF1)
Bax

IL2 and IL2Rα are constitutively expressed in many T cell lines transformed by HTLV-I, although expression is not always maintained and HTLV-I-infected T cell lines may be IL2-dependent or IL2-independent (Arya *et al* 1984, Wano *et al* 1984, Arima *et al* 1987, Kelly *et al* 1992) (Section 1.3.2.3). Expression of IL2Rα is increased on the surface of PBMCs of HTLV-I-infected people in association with expression of *tax/rex* mRNA (Okayama *et al* 1997). A Tax-mediated autocrine mechanism involving upregulation of IL2Rα expression and increased secretion of IL2 may thus be involved in maintenance of proliferation of T cells infected with HTLV-I *in vivo* (Arima *et al* 1986, Maeda *et al* 1987, Maruyama *et al* 1987, Siekevitz *et al* 1987).

However, other pathways are almost certainly involved in stimulation of T cell growth by HTLV-I. Lck and Fyn are not necessary for IL2-mediated signal transduction in HTLV-I-infected cells, indicating the presence of alternative signalling pathways, for example activation of JAK/STAT through binding of $p12^I$ to γ_c (Mills *et al* 1992, Franchini *et al* 1993, Migone *et al* 1995) (Section 1.8.4). Lck is expressed in IL2-dependent but not IL2-independent HTLV-I-infected T cell lines, consistent with activation of alternative pathways in the process of transformation (Koga *et al* 1989) (Section 1.8.1.5). Human T cells expressing Tax proliferate in response to IL2 but can also be stimulated by anti-CD3 monoclonal antibodies (MAbs) through an IL2-independent pathway (Akagi and Shimotohno 1993) (Section 1.8.8). Many HTLV-I-infected T cells in short-term cultures from ATL patients do not proliferate in response to IL2, despite expressing IL2Rα and β (Uchiyama *et al* 1985, Katoh *et al* 1986, Noma *et al* 1989, Höllsberg *et al* 1992). Furthermore, the IL2-dependence of T cell lines transformed by HTLV-I is unrelated to the level of expression of Tax (Katoh *et al* 1986, Arima *et al* 1987) (Section 1.3.2.3). The γ_c component of the IL2R is shared with the receptors for IL4, IL7, IL9, IL13 and IL15 (Giri *et al* 1994, Kawahara *et al* 1994, Taniguchi *et al* 1995) and could be responsible for proliferation of ATL cells in response to exogenous IL2, IL4, IL7 and IL15 (Uchiyama *et al* 1988, Umadome *et al* 1988, Mori *et al* 1993b, Persaud *et al* 1995, Yamada *et al* 1998) (Section 2.2.1.2). The β_c component of the IL2R is shared with the IL15 receptor.

The main mechanism by which Tax induces expression of IL2 and IL2Rα is through activation of the NFκB pathway (Ballard *et al* 1988, Leung and Nabel 1988, Ruben *et al* 1988, Hoyos *et al* 1989, Crenon *et al* 1993) (Section 1.8.2.1). The IL2 gene enhancer also has a response element for CD28RC, which is activated by members of the NF of activated T cells (NF-AT) family, themselves up-regulated by Tax in HTLV-I infected T cells (Curtiss *et al* 1996, Good *et al* 1996). As well as NFκB elements, the enhancer of the IL2Rα gene has a serum response element (SRE) transactivated by serum response factor (SRF) (Cross *et al* 1987) (Section 1.8.2.3).

Levels of the interleukins IL1α and β, IL3, IL5, IL6, IL8, IL10 and IL15 are also increased in HTLV-I-infected T cells and this increase is mediated by Tax (Miyatake *et al* 1988, Sawada *et al* 1992, Muraoka *et al* 1993, Wolin *et al* 1993, Mori *et al* 1994c, Yamashita *et al* 1994, Baba *et al* 1996). IL1α and β and the IL1α receptor are expressed by HTLV-I-infected T cell lines from ATL patients and the cells respond to IL1α and β, consistent with a model of self-stimulation similar to the IL2-IL2R autocrine loop (Wano *et al* 1987, Yamashita *et al* 1987,

Shirakawa *et al* 1989, Mori *et al* 1994c). Tax up-regulates the IL1β gene promoter by interacting with the transcription factors NF-IL6 and Spi1 (Tsukada *et al* 1997).

In experimental models Tax increases expression of IL4 in T cells through NFκB, NF-AT and NF-IL6 sites in the IL4 promoter (Miyatake *et al* 1988, Li-Weber *et al* 2001). ATL cells have increased expression of the IL4 receptor and have a proliferative response to IL4 (Uchiyama *et al* 1988, Umadome *et al* 1988, Mori *et al* 1993b, 1996). However, Kodaka *et al* (1989) and Mori *et al* (1994b) were unable to demonstrate IL4 mRNA expression in leukaemic cells from ATL patients. Tax acts in synergism with GATA4 and AP1 in transactivation of the IL5 gene and HTLV-I-infected T cell lines constitutively express this cytokine (Yamagata *et al* 1997). T cells do not normally produce IL6 but production of this cytokine is induced following infection with HTLV-I (Sawada *et al* 1990, Lal and Rudolph 1991, Villiger *et al* 1991). Tax transactivates the IL6 and IL8 promoters through NFκB binding sites (Muraoka *et al* 1993, Mori *et al* 1994d, Yamashita *et al* 1994, Mori *et al* 1995a).

IL10, IL15 and the IL15 receptor are also constitutively produced by ATL cells and HTLV-I-infected T cell lines through activation of the NFκB pathway (Azimi *et al* 1998, Mori and Prager 1998, Mariner *et al* 2001). IL15 may substitute for IL2 in activation of the JAK/STAT pathway in HTLV-I-infected T cell lines (Bamford *et al* 1994, Burton *et al* 1994, Migone *et al* 1995, Azimi *et al* 1998, Yamada *et al* 1998). ATL cells and HTLV-I-infected T cell lines express IL9 mRNA but this expression is independent of Tax and ATL cells do not proliferate in response to exogenous IL9 (Matsushita *et al* 1997).

Tumour necrosis factors (TNF) α and β are similarly increased in HTLV-I-infected T cells by Tax through the NFκB pathway (Tschachler *et al* 1989, Paul *et al* 1990, Albrecht *et al* 1992, Nakajima *et al* 1993). Macrophage inflammatory protein 1α is constitutively expressed in HTLV-I-infected T cells and induced by Tax (Kelly *et al* 1992, Baba *et al* 1996). HTLV-I-infected T cell lines also produce interferon (IFN) γ (Sugamura *et al* 1983). Levels of expression of granulocyte-macrophage colony stimulating factor (GMCSF), granulocyte colony stimulating factor (GCSF), macrophage activating factor and transforming growth factor (TGF) β are increased in HTLV-I-infected T cells by Tax (Jones *et al* 1988, Niitsu *et al* 1988, Wano *et al* 1988, Nimer *et al* 1989, Kim *et al* 1990, Sawada *et al* 1992, Himes *et al* 1993). Transactivation of the GMCSF promoter by Tax is mediated through a number of transcription sites including those binding NFκB and CREB/ATF (Nimer 1991, Himes *et al* 1993). NFκB-binding sites in the GCSF promoter are responsible for Tax-mediated transactivation of this gene (Himes *et al* 1993). Expression of platelet-derived growth factor (PDGF) β and its receptor are increased in HTLV-I-infected T cells and the PDGFβ receptor (c-*sis*) promoter is up-regulated by interaction of Tax with the zinc finger transcription factors Sp1 and Egr1 (Goustin *et al* 1990, Trejo *et al* 1997). The PDGFβ receptor mediates transformation by the bovine papillomavirus type 1 (BPV-1) E5 protein and could be one of the targets of HTLV-I $p12^{I}$ (Nilson and DiMaio 1993) (Section 1.8.4). HTLV-I-infected T cell lines also have increased expression of insulin-like growth factor (IGF) receptor type I and proliferate in response to IGF-I (Lal *et al* 1993).

1.8.1.2 Cell surface molecules

As well as the cytokine and growth factor receptors listed in Section 1.8.1.1, cell surface molecules up-regulated by Tax in HTLV-I-infected T cells include CD9, CD11, CD23 (Fc_{ε} receptor II), CD21 (complement receptor 2), CD40, CD71 (transferrin receptor), CD80 (B7-1), MHC-I and GD2 ganglioside (Mann *et al* 1983, Nutman *et al* 1987, Vidal *et al* 1988, Yssel *et al* 1989, Vaile *et al* 1990, Furukawa *et al* 1993, McNearney *et al* 1993, Dezzutti *et al* 1995, Ohtsubo *et al* 1997). CD54 (intercellular adhesion molecule 1, ICAM1) is expressed on uncultured leukaemic cells from ATL patients and levels of CD54 mRNA are increased in HTLV-I-infected T cell lines derived by transformation of cocultivated PBMCs or UCMCs (Fukudome *et al* 1992, Dezzutti *et al* 1995). However, CD54 and its ligand CD11 (Section 1.10.1.5) may be down-regulated in HTLV-I-infected T cell lines derived directly from leukaemic cells of ATL patients (Fukudome *et al* 1992, Tanaka *et al* 1995). Thus, although Tax induces the CD54 promoter, its role in the regulation of CD54 expression *in vivo* is uncertain (Mori *et al* 1994a, Tanaka *et al* 1995). CD62 ligand (CD62L) is expressed on ATL cells and its promoter is transactivated by Tax, whereas this leucocyte adhesion molecule is down-regulated when normal T cells are activated (Tatewaki *et al* 1995). CD106 (vascular cell adhesion molecule 1) is expressed at high levels on HTLV-I-infected T cells and transactivation of the CD106 gene by Tax is mediated through NFκB-binding sites (Valentin *et al* 2001). Tax induces CD134 (OX40) and OX40 ligand, the latter belonging to the TNF family, in HTLV-I-infected cells (Baum *et al* 1994, Higashimura *et al* 1996). Integrins $\alpha5\beta1$ and $\alpha4\beta1$, which act as fibronectin receptors, are up-regulated on HTLV-I-infected T cells (Dhawan *et al* 1993). CD151 (SFA-1, PETA-3), which associates with integrin $\alpha5\beta1$ and contributes to fibronectin adhesion, is also expressed on HTLV-I-transformed T cells and the CD151 gene is transactivated by Tax (Hasegawa *et al* 1998).

1.8.1.3 Oncogenes, transcription factors, signalling pathways and cell cycle regulators

HTLV-I-infected T cell lines have increased concentrations of mRNAs for the AP1 transcription factors Fos, Jun, JunB, JunD and Fra1 (Fujii *et al* 1988, Nagata *et al* 1989, Hooper *et al* 1991, Tsuchiya *et al* 1993). Increased expression of Fos has been detected in PBMCs of patients with smouldering ATL (Iwahashi *et al* 1994). Tax transactivates the *fos* promoter through four regulatory elements: a CREB/ATF-binding site, an SRE, a v-*sis* conditioned medium inducible element and an octanucleotide direct repeat (Fujii *et al* 1988, Alexandre and Verrier 1991). The *fra1* promoter is transactivated by Tax through a site containing a retinoblastoma control element and two AP1-binding sites (Tsuchiya *et al* 1993). Up-regulation of Egr1 and Egr2 by Tax in HTLV-I-infected T cell lines is mediated through CRE, Ets binding site and SRE sequences in the *egr1* and *egr2* enhancers (Wright *et al* 1990, Alexandre *et al* 1991, Fujii *et al* 1991, Sakamoto *et al* 1992). *Nur77*, which encodes a steroid receptor involved in TCR-mediated apoptosis, is constitutively expressed in HTLV-I-infected T cells and is up-regulated by Tax through the CREB/ATF pathway (Kelly *et al* 1992, Liu *et al* 1999). The anti-apoptotic gene *bcl-xL* is up-regulated by Tax through NFκB and CREB/ATF pathways (Mori *et al* 2001). Tax also transactivates the proliferating cell nuclear antigen (PCNA) promoter (Ressler *et al* 1997).

The activities of a number of molecules involved in signal transduction pathways, including the PTKs Lyn and Fyn, protein kinase A and protein kinase C (PKC), are increased by Tax in HTLV-I-infected T cells (Koga *et al* 1989, Yamanashi *et al* 1989, Kadison *et al* 1990, Uchiumi *et al* 1992, Lindholm *et al* 1996, Lemasson *et al* 1997, Weil *et al* 1999). Calpain II, a cysteine protease that activates PKC, is increased by Tax in HTLV-I-infected T cells (Adachi *et al* 1992b). In contrast, the activity of Lck is decreased (Koga *et al* 1989, Lemasson *et al* 1996) (Section 1.8.1.5). The JAK/STAT pathway is constitutively activated by tyrosine phosphorylation in uncultured leukaemic cells from ATL patients, IL2-independent HTLV-I-transformed T cell lines and Tax-expressing rodent fibroblast cell lines (Franchini *et al* 1993, Migone *et al* 1995, Xu *et al* 1996b, Takemoto *et al* 1997). This pathway may mediate transduction of signals through IL2R via $p12^{I}$ (Section 1.8.4). HTLV-II is able to transform T cells independently of JAK/STAT activation (Mulloy *et al* 1998b).

Tax interacts with the cyclin-dependent kinase (CDK) inhibitor $p16^{INK4a}$, releasing its repression of CDK4 and permitting the cell cycle to continue past the G_1/S arrest points (Suzuki *et al* 1996, Low *et al* 1997, Schmitt *et al* 1998). Deletions in *p16*^{INK4a}, which is a tumour suppressor gene, have been reported in patients with ATL (Ogawa *et al* 1994, Hatta *et al* 1995). Hofmann *et al* (2001) demonstrated methylation of $p16^{INK4a}$ and other genes involved in cell cycle control in a proportion of ATL patients. Tax also associates with cyclin D (Neuveut *et al* 1998) and the mitotic checkpoint protein MAD1 (Jin *et al* 1998).

1.8.1.4 Other proteins up-regulated by Tax

The parathyroid hormone-related protein (PTHRP) gene is transactivated by Tax through the CREB/ATF pathway and interactions with the transcription factors Ets1 and Sp1 (Ikeda *et al* 1993, Dittmer *et al* 1997). Elevated production of PTHRP by HTLV-I-infected cells may contribute to increased resorption of bone and retention of calcium by the kidneys in ATL patients (Fukumoto *et al* 1989, Watanabe *et al* 1990, Ejima *et al* 1993). ATL-derived factor (ADF)/thioredoxin-like protein is produced by HTLV-I-infected T cell lines but not uninfected T cell lines and the thioredoxin gene is transactivated by Tax (Wakasugi *et al* 1990, Masutani *et al* 1996). ADF induces IL2R and synergises with IL1α and β and IL2 in stimulating the growth of T cells, possibly through its effect on redox status (Wakasugi *et al* 1990). HTLV-I-infected T cell lines, as well as activated T cells, produce oncostatin M, a factor that stimulates growth of fibroblasts, but inhibits growth of melanoma cells (Nair *et al* 1992). The lymphocyte chemoattactant stromal cell-derived factor 1 and its receptor CXCR4 are preferentially expressed in HTLV-I-infected T cells and in T cells expressing Tax (Arai *et al* 1998b). Levels of expression of the cytoskeletal proteins vimentin and cytokeratin 7 are increased by Tax in HTLV-I-infected cells through the NFκB pathway (Lilienbaum *et al* 1990). Tax also transactivates several globin genes (Fox *et al* 1989).

1.8.1.5 Proteins down-regulated by Tax

Tax down-regulates expression of Lck, the tumour suppressor proteins p53 and neurofibromin (NF1) and the apoptosis accelerator Bax (Uittenbogaard *et al* 1995, Feigenbaum *et al* 1996, Brauweiler *et al* 1997, Lemasson *et al* 1997). Repression of these genes, mediated by the basic helix-loop-helix (bHLH) family of transcription factors

(Uittenbogaard *et al* 1995), may promote survival and proliferation of HTLV-I-infected T cells, contributing to oncogenesis. Although Lck and JAK/STAT are activated in some T cell neoplasms (Yu *et al* 1997) and IL2-dependent HTLV-I-infected T cell lines (Section 1.8.1.1), Lck is a regulator of apoptosis and therefore the ability of HTLV-I-infected cells to downregulate and bypass Lck may promote survival (Belka et al 2003). Repression of β polymerase by Tax may also contribute to oncogenicity by inhibiting repair of DNA, allowing mutations to accumulate (Jeang *et al* 1990). An increased frequency of random mutations has been observed in HTLV-I-infected and Tax-transfected cells (Majone *et al* 1993, Saggioro *et al* 1994b, Miyake *et al* 1999). Tax represses cyclin A, cyclin D3 and DNA polymerase α by acting through CREB/ATF binding sites in the enhancers of these genes (Kibler and Jeang 2001). Expression of the TCR-CD3 complex is down-regulated by Tax in HTLV-I-infected T cell lines and leukaemic cells from patients with ATL (De Waal Malefyt *et al* 1990, Matsuda *et al* 1994).

1.8.1.6 Clinical relevance of alterations in cellular gene expression

It is difficult to ascribe specific clinicopathological correlates to the wide variety of cellular changes associated with HTLV-I infection in patients with ATL (Section 1.6.1.2). Over-expression of receptor activator of NFκB (RANK) ligand (Nosaka *et al* 2002) and increased production of IL1α, TNFβ and PTHRP by HTLV-I-infected T cells may induce hypercalcaemia and lytic bone lesions (Motokura *et al* 1989, Ishibashi *et al* 1991, Mori *et al* 1994c). Upregulation of IL3, IL5 and GMCSF could contribute to granulocytosis and eosinophilia (Prin *et al* 1988). However, the clinical significance of many of the alterations in gene expression caused by Tax in HTLV-I-infected T cells is unknown. Since the level of expression of viral gene products in ATL cells *in vivo* may be low (Section 1.6.1.6), the effects of HTLV-I and Tax on cellular function *in vitro* cannot necessarily be extrapolated to ATL patients. Furthermore, it has been shown that clonally related ATL cells derived from the same patient may have different patterns of cytokine expression (Noma *et al* 1989).

1.8.2 Mechanisms of regulation of cellular genes by HTLV-I Tax

Dysregulation of cellular functions by Tax is mediated through a variety of cellular transcription factors that attach to specific DNA sequences and activate or suppress cascades of genes. Tax does not bind directly to DNA but interacts with proteins in the NFκB and CREB/ATF families, increasing transcriptional activity by enhancing their dimerisation or by facilitating their translocation to the nucleus following dissociation or degradation of cytoplasmic inhibitors (Wagner and Green 1993, Kanno *et al* 1994, Baranger *et al* 1995, Franklin and Nyborg 1995, Yoshida *et al* 1995). Tax also activates transcription through pathways other than NFκB and CREB/ATF, such as SRF. The NFκB, CREB/ATF and SRF pathways appear to be important in transformation of cells by Tax (Smith and Greene 1991, Yamaoka *et al* 1996, Matsumoto *et al* 1997, Rosin *et al* 1998, Robek and Ratner 1999) (Section 1.8.3).

1.8.2.1 NFκB

Members of the NFκB family are transcription factors that bind to consensus DNA sequences in the enhancers of many cellular genes. NFκB proteins include NFκB1 (p50) derived from the precursor p105, NFκB2 (p52) derived from the precursor Lyt-10 (p100), Rel (p86), RelA (p65) and RelB (p50B) (Baeuerle and Henkel 1994). The NFκB pathway is involved in proliferation and differentiation of lymphocytes and is activated in T cells following stimulation through the TCR and by cytokines, mitogens and viral infection. HTLV-I-infected T cells constitutively express NFκB proteins and Tax specifically increases the functional activity of NFκB1, NFκB2, Rel and RelA in T cells (Lindholm *et al* 1990, Arima *et al* 1991, Li *et al* 1993, Lanoix *et al* 1994, Sun *et al* 1994). Elevated expression of *NFκB1/p105* and *relA* mRNA has been demonstrated in leukaemic cells from ATL patients (Inoue *et al* 1998). Cellular genes with NFκB-binding sites that are up-regulated by Tax include IL1α, IL2, IL2Rα, IL6, IL15, IL15R, TNFα and β, GMCSF and GCSF (Section 1.8.1).

There are at least three mechanisms by which Tax activates the NFκB pathway. The most important of these is by promoting release of NFκB dimers from inhibitors (IκBs) in the cytoplasm, allowing NFκB proteins to translocate to the nucleus and transcribe cellular genes (Karin 1999). IκBs include IκBα, β, ε, γ (identical to the C terminal 607 amino acids of p105) and Bcl-3; Lyt-10 and p105 also have IκB-like activity. Tax binds to MEKK1, resulting in activation of IκB kinases (Chu *et al* 1998, Geleziunas *et al* 1998, Yin *et al* 1998). IκB kinases phosphorylate IκBs, facilitating their degradation by the proteosome complex (Sun *et al* 1994, Kanno *et al* 1995, Lacoste *et al* 1995, Maggirwar *et al* 1995, Good and Sun 1996). Released from IκBs in the cytoplasm, NFκB proteins translocate to the nucleus, where they activate cellular genes. Tax also binds to ankyrin repeat motifs in IκBs and this interaction further contributes to translocation of NFκB proteins to the nucleus by facilitating competitive dissociation of NFκB-IκB complexes (Watanabe *et al* 1993, Hirai *et al* 1994, Kanno *et al* 1994, Muñoz *et al* 1994, Suzuki *et al* 1995, Petropoulos *et al* 1996, Petropoulos and Hiscott 1998).

Furthermore, Tax mediates transactivation of NFκB-binding sites by interacting with ankyrin repeat motifs in the Rel homology domains of NFκB proteins, promoting the formation of NFκB dimers, which bind to DNA (Lacoste *et al* 1991, Armstrong *et al* 1993, Suzuki *et al* 1994, Hiscott *et al* 1995, Petropoulos *et al* 1996). NFκB-binding activity induced by Tax is thus initially due to translocation of NFκB dimers (especially NFκB1-RelA) from the cytoplasm to the nucleus and is later supplemented by complexes containing newly synthesised proteins as a result of transcriptional activation of NFκB promoters by Tax in a positive feedback loop (Li *et al* 1993, Kanno *et al* 1994, Sun *et al* 1994). Conversely, sequestration of Tax in the cytoplasm by association with Lyt-10 (and to a lesser extent p105) could form part of a negative feedback loop leading to viral latency following activation of NFκB in HTLV-I-infected T cells (Béraud *et al* 1994, Pepin *et al* 1994) (Section 1.3.6).

1.8.2.2 CREB/ATF

Proteins in the CREB/ATF and AP1 families are basic region leucine zipper (bZIP) proteins that bind to CREB response elements (CREs) in the enhancers of many genes (Lemaigre *et al* 1993, Karin *et al* 1997). CREB, ATF1 and ATF2 up-regulate expression, whereas CRE modulator (CREM) down-regulates expression. The mechanism by which Tax activates transcription through CREB/ATF and AP1 factors is by interaction of Tax dimers with the basic regions of these proteins, promoting dimerisation through their leucine zippers, which enhances their ability to bind to DNA (Zhao and Giam 1992, Wagner and Green 1993, Baranger *et al* 1995, Perini *et al* 1995, Kwok *et al* 1996). Cellular genes responsive to transactivation by Tax through CREB/ATF-binding sites include *egr1*, *egr2*, *fos*, GMCSF, PTHRP, *Nur77*, cyclin A and DNA polymerase α (Section 1.8.1).

1.8.2.3 Other mechanisms of activation by Tax

Tax interacts with SRF and enhances its binding to CArG elements in the SREs of *egr1*, *egr2*, *fos* and IL2Rα (Fujii *et al* 1992, Armstrong *et al* 1993, Suzuki *et al* 1993b) (Section 1.8.1). Other mediators of transcriptional activation by Tax include CBP/p300 (Kwok *et al* 1996, Bex *et al* 1998, Van Orden *et al* 1999), HEB1 (Béraud *et al* 1991), TATA box-binding protein (TBP) (Caron *et al* 1993), TFIIA (Clemens *et al* 1996) and NF-YB (Pise-Masison *et al* 1997). Tax mutants that selectively activate the CREB/ATF pathway localise with CBP, whereas Tax mutants that selectively activate the NFκB pathway localise with p300 (Bex *et al* 1998). Tof also binds to CBP/p300 (Zhang *et al* 2001) (Section 1.3.5.4). Repressive effects of Tax on cellular genes are mediated through CREB/ATF and bHLH pathways (Section 1.8.1.5). The enhancers of many genes have more than one site responsive to transactivation by Tax.

1.8.3 Transforming properties of Tax

Tax is able to transform established rodent fibroblast cell lines, immortalise primary rat embryo fibroblasts (REFs) and immortalise human T cells (Grassmann *et al* 1989, Pozzatti *et al* 1990, Tanaka *et al* 1990, Smith and Greene 1991, Grassmann *et al* 1992). Tax cooperates with Ras in the transformation of primary REFs, but is unable to transform them when expressed alone (Pozzatti *et al* 1990). Miyazaki *et al* (1996) demonstrated cooperation of Tax with c-Myc and Lck in the transformation of a B cell line to IL3-independence. Participation of Lck and Ras in Tax-mediated transformation in this model is consistent with involvement of the IL2R signalling pathway (Section 1.8.1.1). Primary human T cells immortalised with *Herpesvirus saimiri* or retroviral vectors expressing Tax remained dependent on IL2 and had increased expression of IL2Rα (Grassmann *et al* 1989, Akagi *et al* 1995). Mutational inactivation or deletion of *tax* from transformed cells resulted in reversion of the transformed phenotype (Sakurai *et al* 1992b, Yamaoka *et al* 1992). An established rodent fibroblast cell line (Rat2 cells) transformed by Tax lost its transformed phenotype when fused with non-transformed human fibroblasts, even though expression of functional Tax was maintained in the hybrid cells, indicating that transformation by Tax is dependent on additional oncogenic events, particularly inactivation of tumour suppressor

function (Inoue *et al* 1994). Expression of Tax is maintained in T cell lines transformed by HTLV-I even though such cell lines may not express structural genes (Sodroski *et al* 1985). Tax_2 is essential for transformation of human T cells by HTLV-II (Ross *et al* 1996, Endo *et al* 2002).

As discussed in Section 1.8.2, the NFκB, CREB/ATF and SRF pathways are involved in Tax-mediated transformation. Tax mutants that stimulate the CREB/ATF pathway but not the NFκB pathway are able to transform Rat2 cells (Smith and Greene 1991, Rosin *et al* 1998). In contrast, Yamaoka *et al* (1996) demonstrated that activation of NFκB was necessary for Tax-induced transformation of another established rodent fibroblast cell line (Rat1 cells). An IL2-dependent murine T cell line (CTLL-2) lost its dependence on IL2 for growth when transfected with a plasmid expressing Tax and this effect was mediated through the NFκB pathway rather than the CREB/ATF pathway (Iwanaga *et al* 1999). The same cell line required expression of Tax that transactivated both pathways in order to form colonies in soft agar in the absence of IL2 (Iwanaga *et al* 1999). Colony formation by *tax*-transfected Rat1 cells in soft agar was dependent on the NFκB pathway, whereas focus formation by primary REFs in cooperation with Ras was dependent on the SRF pathway (Matsumoto *et al* 1994, 1997). Immortalisation of primary human T cells (indefinite growth in the presence of IL2) was dependent on activation of the NFκB pathway by Tax but did not require an active CREB/ATF pathway (Robek and Ratner 1999). Similarly, activation of the NFκB pathway in *tax*-transfected human PBMCs conferred growth responsiveness to IL2, whereas clonal expansion of $CD4^+$ T cells from this population was associated with activation of the CREB/ATF and SRF pathways (Akagi *et al* 1997a). In HTLV-I-infected T cells the IL2-dependent phase of growth is associated with activation of the NFκB pathway, whereas loss of dependency on IL2 is associated with activation of the CREB/ATF, SRF and JAK/STAT pathways (Migone *et al* 1995) (Section 1.3.2.3). These results suggest that different signalling pathways are involved in distinct facets of the transformation process mediated by Tax.

1.8.4 Role of $p12^I$ in HTLV-I oncogenesis

$p12^I$ has oncogenic properties and may play a role in HTLV-I leukaemogenesis. Although unable to induce transformed foci when transfected alone, $p12^I$ enhances transformation of murine C127 fibroblasts when co-transfected with BPV-1 E5, a 44 amino acid oncoprotein implicated in the development of papillomas induced by BPV-1 in cattle (Schlegel *et al* 1986, Franchini *et al* 1993). Transformation of C127 cells by E5 is mediated by the PDGFβ receptor (Section 1.8.1.1); E5 transforms NIH 3T3 cells by interacting with the epidermal growth factor (EGF) receptor (Martin *et al* 1989, Nilson and DiMaio 1993). $p12^I$ and E5 have structural similarities, both being hydrophobic proteins that localise in the endoplasmic reticulum and Golgi apparatus (Koralnik *et al* 1993) (Section 1.3.5.6). The second of two putative transmembrane regions of $p12^I$ (amino acids 39 to 62) has 59% amino acid identity with the single transmembrane region of E5 (amino acids 6 to 28), a common amino acid motif LFLL and a conserved Q residue (Franchini *et al* 1993) (Appendices 1 and 2). $p12^I$ and E5 both associate with 16 kDa vacuolar H^+ ATPase, a proton pump responsible for acidification of vesicles (Goldstein *et al* 1991) (Section 1.3.5.6). However, the sites of

interaction of $p12^I$ and E5 with 16 kDa vacuolar H^+ ATPase are different and the binding domain in $p12^I$ is not the same as the domain that mediates its ability to potentiate transformation (Koralnik *et al* 1995). BPV-1 E5 enhances the binding of 16 kDa vacuolar H^+ ATPase to the PDGFβ receptor and this may be the mechanism by which the PDGFβ receptor is constitutively activated in transformed C127 cells (Goldstein *et al* 1992). It is unknown whether $p12^I$ has a similar mechanism of action.

$p12^I$ binds to the cytoplasmic portions of the β_c and γ_c chains of the IL2R (Franchini *et al* 1993, Mulloy *et al* 1996) (Section 1.3.5.6). A proline-rich region between the two transmembrane domains of $p12^I$ is involved in binding to IL2Rβ and also mediates interaction with 16 kDa vacuolar H^+ ATPase (Mulloy *et al* 1996). $p12^I$ binds to an acidic region in the cytoplasmic domain of $IL2R\beta_c$, which is the site of interaction with Lck and involved in recruitment of JAK1 to $IL2R\beta_c$ and JAK3 to $IL2R\gamma_c$ following IL2 stimulation, resulting in activation of STAT5 (Migone *et al* 1995, Nicot *et al* 2001). $p12^I$ may thus bypass the requirement of proliferating T cells for IL2. A Tax-$p12^I$-based model for HTLV-I-induced transformation of T cells is for Tax to activate the IL2-IL2R autocrine loop early in the course of infection, with $p12^I$ maintaining proliferation or preventing apoptosis later in infection through ligand-independent stimulation of the IL2R signalling pathway.

1.8.5 Role of p53 in HTLV-I oncogenesis

p53 is a tumour suppressor protein that induces cell cycle arrest and apoptosis in response to DNA damage (Liebermann *et al* 1995) (Section 1.8.7). Mutations resulting in inactivation of *p53* are found in many neoplasms and have been identified in cases of ATL (Hollstein *et al* 1991, Newcomb 1995) (Section 1.6.1.4). Leukaemic T cells in nine (42%) of 21 cases of ATL had significant mutations in *p53* (Nagai *et al* 1991, Sugito *et al* 1991, Cesarman *et al* 1992, Sakashita *et al* 1992, Yamato *et al* 1993). Nucleotide sequence changes identified were predicted to lead to amino acid substitution (seven cases), deletion (one) or premature termination (one) in the encoded protein. There was a corresponding loss of the opposite *p53* allele (loss of heterozygosity) in four cases. Mutations in *p53* were more frequent in acute than chronic ATL (Nagai *et al* 1991, Sugito *et al* 1991, Sakashita *et al* 1992, Nishimura *et al* 1995).

p53 and other tumour suppressor proteins are frequent targets of viral oncogenes (Hoppe-Seyler and Butz 1995). p53 is increased in HTLV-I-infected T cell lines but the protein is inactivated by phosphorylation and this effect is mediated by Tax (Koeffler *et al* 1986, Reid *et al* 1993, Pise-Masison *et al* 2000). T cell lines immortalised by Tax also have increased expression of p53 (Akagi *et al* 1997b). The increase in p53 is due to stabilisation of the protein (increased half life) rather than an increase in the amount of mRNA, since Tax down-regulates *p53* through binding of bHLH transcription factors to an E box element in the *p53* enhancer (Lübbert *et al* 1989, Reid *et al* 1993, Uittenbogaard *et al* 1994) (Section 1.8.1.5). Tax does not bind to p53 but induces phosphorylation of this protein through the NFκB pathway (Pise-Masison *et al* 2000). Phosphorylation blocks the interaction of transcription factors such as TFIID and MDM2 with the N terminal activation domain of p53. As a consequence, p53-mediated responses to cell damage are impaired in HTLV-I-infected cell

lines (Cereseto *et al* 1996, Pise-Masison *et al* 1998). Mulloy *et al* (1998a) found that the CREB/ATF domain of Tax was also important in inhibition of p53 transcriptional activity. An increase in the level of inactive p53 is correlated with loss of IL2 dependence of HTLV-I-infected T cell lines (Yamato *et al* 1993, Gartenhaus and Wang 1995, Cereseto *et al* 1996). Levels of p53 were also increased in leukaemic T cells in eight of 14 cases of ATL (Nagai *et al* 1991, Sugito *et al* 1991, Cesarman *et al* 1992). p53 thus appears to be an important target of HTLV-I Tax in infected cells.

1.8.6 Role of c-Myc in HTLV-I oncogenesis

The Myc family of proteins, c-Myc, N-Myc and L-Myc, are nuclear phosphoproteins with bZIP and bHLH domains that act as transcription factors with an important role in cell proliferation (Marcu *et al* 1992, Lüscher and Larsson 1999). c-Myc dimerises with Max and is involved in transformation, cell cycle progression and apoptosis (Heikkila *et al* 1987, Dang *et al* 1989, Kato *et al* 1990, Evan *et al* 1992, Amati *et al* 1993). It cooperates with Ras in transformation (Land *et al* 1983, Strasser *et al* 1990). Activation of c-Myc appears to prime cells for either proliferation or death by apoptosis, depending on whether conditions are permissive for survival (Evan *et al* 1992) (Section 1.8.7). Survival signals that allow c-Myc to induce proliferation in different contexts include cytokines present in serum such as IL2, IL3, IGF-I and PDGFβ and expression of apoptosis inhibitors such as Bcl-2 (Fanidi *et al* 1992, Harrington *et al* 1994a). c-*myc* is activated in a wide variety of neoplasms, including human, murine, feline and avian leukaemias and sarcomas (Cory *et al* 1984).

HTLV-I appears to have a variety of disparate effects on c-Myc expression and function. No mutations in c-*myc* were detected in leukaemic T cells from 10 ATL patients (Cesarman *et al* 1992) and c-*myc* mRNA could be detected by ISH in 25% of leukaemic cells (Moriuchi *et al* 1988). The level of c-*myc* mRNA was increased in an HTLV-I-infected T cell line examined by Koizumi *et al* (1989). Duyao *et al* (1992) demonstrated transactivation of c-*myc* by Tax through NFκB-response elements, whereas in a study by Fujii *et al* (1988) Tax failed to activate transcription from the c-*myc* promoter. Similarly, there was no upregulation of c-Myc in a T cell line transfected with *tax* (Nagata *et al* 1989). Inhibition of c-*myc* expression in HTLV-I-infected T cell lines was correlated with inhibition of growth (Koizumi *et al* 1989, Fujita and Shiku 1993). Tax inhibits c-Myc function, including its transforming properties in Rat1 cells, through a post-translational mechanism involving the CREB/ATF pathway (Semmes *et al* 1996). These differences probably reflect the complex pattern of regulation of expression and the diverse functions of c-Myc in cells. Experiments investigating the effects of HTLV-I *pX* genes on apoptosis induced by c-Myc in rodent fibroblasts under conditions of serum deprivation are described in Chapter 4.

1.8.7 Role of apoptosis in HTLV-I oncogenesis

1.8.7.1 Apoptosis and oncogenesis

Apoptosis is a mechanism of programmed cell death in response to pathological or physiological stimuli (Kerr *et al* 1972). In the process of oncogenesis, cells have a

requirement to switch on genes that promote growth, for example the oncogene c-*myc* (Section 1.8.6). However, c-*myc*-induced proliferation is automatically coupled to activation of an apoptosis pathway, allowing cells to respond to potentially oncogenic events by committing suicide (Evan *et al* 1992, Evan and Littlewood 1993). Therefore, additional events that prevent apoptosis in response to aberrant cell proliferation are required for oncogenesis, for example by activating the anti-apoptotic gene *bcl-2* or inactivating the tumour suppressor gene *p53* (Bissonnette *et al* 1992, Fanidi *et al* 1992, Harrington *et al* 1994b, Pan *et al* 1997). Tax up-regulates the anti-apoptotic protein Bcl-xL and down-regulates the tumour suppressor proteins p53 and NF1 and the pro-apoptotic protein Bax (Uittenbogaard *et al* 1995, Feigenbaum *et al* 1996, Brauweiler *et al* 1997, Mori *et al* 2001) (Sections 1.8.1.3 and 1.8.1.5). Apoptosis induced by expression of c-Myc is mediated by p53 (Hermeking and Eick 1994) (Section 1.8.6).

1.8.7.2 Survival signals

Suppression of apoptosis is important in maintaining survival of normal cells under physiological conditions. Cells that survive in the presence of growth factors, such as IL2 or IGF-I, may undergo apoptosis when these growth factors are withdrawn, for example by cultivation in low concentrations of serum (Evan *et al* 1992, Harrington *et al* 1994a, Kulkarni and McCulloch 1994, Preston *et al* 1994). IL2-dependent T cells undergo apoptosis when IL2 is withdrawn (Duke and Cohen 1986). IL2, IL4 and IL7 prevent apoptosis of quiescent T cells in response to γ-irradiation (Boise *et al* 1995). Similarly, apoptosis is the mechanism of death in IL3- and IL6-dependent haematopoietic cells deprived of these cytokines (Colotta *et al* 1992, Gottlieb *et al* 1994). A potential mechanism of HTLV-I leukaemogenesis would be to prevent apoptosis in T cells. However, PBMCs from patients with ATL undergo apoptosis when cultivated in serum-free medium, similar to PBMCs from normal people (Debatin *et al* 1993). This effect is only partially inhibited by IL2, indicating that other growth factors in serum are required to prevent apoptosis of ATL cells and normal PBMCs (Tsuda *et al* 1993). The growth of IL2-dependent HTLV-I-infected T cell lines is arrested in early G_1 (G_0) after withdrawal of IL2, due to inhibition of the cyclin E-CDK2 complex by $p27^{Kip1}$, whereas cell cycle progression is maintained in IL2-independent T cell lines upon serum starvation (Cereseto *et al* 1999).

1.8.7.3 CD95-mediated apoptosis

The CD95 (Fas, Apo-1)-CD95 ligand (CD95L) system is one of the pathways mediating activation-induced death in T cells (Owen-Schaub *et al* 1992, Klas *et al* 1993, Wang *et al* 1994, Alderson *et al* 1995, Brunner *et al* 1995, Dhein *et al* 1995, Ju *et al* 1995). Binding of CD95L to CD95 by cell-cell contact activates an apoptosis cascade through interleukin 1β-converting enzyme (ICE)-like proteases (Enari *et al* 1995, Los *et al* 1995). CD95 is expressed by leukaemic cells in most cases of ATL and by HTLV-I-infected T cell lines derived from ATL patients, but the level of expression is variable (Debatin *et al* 1990, 1993, Kotani *et al* 1994, Sugahara *et al* 1997, Tamiya *et al* 1998). CD95 was expressed on leukaemic cells of 46 (98%) of 47 ATL cases examined by Tamiya *et al* (1998). HTLV-I-infected T cell lines, short-term cultured PBMCs and fresh neoplastic cells from most ATL patients, all of which expressed CD95, were susceptible to apoptosis induced by anti-CD95

MAbs (Debatin *et al* 1990, 1993, Kotani *et al* 1994). In two CD95$^-$ ATL cases, leukaemic cells had mutations in both alleles of the CD95 gene and were resistant to apoptosis induced by anti-CD95 MAbs (Tamiya *et al* 1998, Maeda *et al* 1999). Resistance to CD95-mediated apoptosis in other HTLV-I-infected T cell lines (especially long-term cell lines established by cocultivation and not derived from leukaemic clones) was associated with expression of Tax and/or Fas-associated phosphatase 1 (FAP1), which acts as a negative regulator of the CD95 apoptosis pathway (Copeland *et al* 1994, Sato *et al* 1995, Arai *et al* 1998a). Expression of Tax reduced the susceptibility of uninfected CD4$^+$ T cell lines to CD95-mediated apoptosis, provided the cells were cultivated in 5 to 10% serum (Copeland *et al* 1994, Chlichlia *et al* 1995, Chen *et al* 1997, Chlichlia *et al* 1997, Arai *et al* 1998a). The effect of duration of cultivation on expression of Tax by T cells could account for differences in sensitivity to CD95-mediated apoptosis. Tax up-regulates CD95L on T cells through NFκB and CREB/ATF pathways and these cells have an increased rate of apoptosis through the CD95-CD95L autocrine cell suicide pathway (Chlichlia *et al* 1995, Chen *et al* 1997, Chlichlia *et al* 1997). CD95-CD95L interactions are involved in physiological regulation of T cell responses, but it is uncertain whether this system plays a significant role in ATL (Alderson *et al* 1995, Dhein *et al* 1995).

1.8.7.4 Tax and apoptosis

The response of cells expressing Tax to withdrawal of growth factors is similar to that of cells expressing c-Myc. Established (immortalised) rodent fibroblast cell lines undergo growth arrest in G_0 but survive when serum is withdrawn. Expression of c-Myc in the presence of serum leads to uncontrolled proliferation (transformation), whereas expression of c-Myc under conditions of serum deprivation leads to a reduction in the rate of population increase because continued proliferation is balanced by an increased rate of apoptosis (Evan *et al* 1992) (Sections 1.8.6 and 1.8.7.1). Rat1 cells transformed by Tax, as well as REFs and 3Y1 rat fibroblasts expressing Tax, undergo apoptosis when deprived of serum (Sakurai *et al* 1992b). This effect is blocked by Bcl-2 (Yamada *et al* 1994). The proportion of Rat1 cells expressing Tax that remain in S phase is maintained upon serum deprivation, indicating that Tax promotes cell cycle progression in the absence of external growth factors (Yamada *et al* 1994). Fujita and Shiku (1995) also observed that Rat1 cells transformed by *tax* underwent apoptosis following deprivation of serum, although the degree of apoptosis was less than that observed in Rat1 cells transformed by c-*myc* or *fos*. In a manner similar to c-Myc, Tax may thus activate pathways leading to either proliferation or apoptosis, depending on the availability of survival signals (Section 1.8.7.2). Appropriate survival signals would include growth factors such as those present in serum or anti-apoptotic factors such as Bcl-2. Conversely, since Tax may up-regulate c-*myc* (via NFκB) there is a possibility that c-Myc could mediate the apoptotic effect of Tax (Koizumi *et al* 1989, Fujita and Shiku 1993) (Section 1.4.6). Fos also induces apoptosis under conditions of serum deprivation and up-regulation of *fos* (via CREB/ATF or SRF) could be another indirect mechanism by which Tax activates this pathway (Alexandre and Verrier 1991, Colotta *et al* 1992, Smeyne *et al* 1993, Preston *et al* 1996). p21$^{Waf1/Cip1}$, which induces apoptosis independent of p53 activity, is also up-regulated by Tax via NFκB in HTLV-I-infected and Tax-transformed T cell lines (Akagi *et al* 1996, Cereseto *et al* 1996, Gartenhaus *et al* 1996, Parker *et al* 1996, Low *et al* 1997).

The roles of the CREB/ATF and NFκB pathways in Tax-mediated apoptosis are uncertain. Iwanaga *et al* (1999) provided evidence that the CREB/ATF pathway mediated Tax-induced apoptosis, whereas Tax inhibited apoptosis through the NFκB pathway. Tax reduced the rate of apoptosis of the IL2-dependent murine T cell line CTLL-2 in the absence of IL2 and this effect was maintained by Tax mutants defective for activation of the CREB/ATF pathway, whereas Tax mutants with an active CREB/ATF pathway, but defective NFκB pathway, accelerated the rate of apoptosis (Iwanaga *et al* 1999). In contrast, Rivera-Walsh *et al* (2001) showed that Tax-induced apoptosis was mediated by TNF-related apoptosis-inducing ligand (TRAIL) acting through the NFκB pathway and that Tax induces expression of TRAIL mRNA. An understanding of the pathways involved in Tax-induced apoptosis would allow the importance of this mechanism in HTLV-I leukaemogenesis to be better defined.

1.8.8 HTLV-I envelope

Proliferation of T cells is stimulated following infection with HTLV-I and also by contact with inactivated virions (Gazzolo and Duc Dodon 1987, Martin and Southern 1996, Höllsberg 1999). Cytokine-receptor loops such as IL2-IL2R appear to be important components in maintaining HTLV-I-induced T cell proliferation (Section 1.8.1.1) but initial activation of HTLV-I-infected T cells may be induced by viral $gp46^{Env}$ and through cell-cell contact involving CD2-lymphocyte function-associated antigen (LFA) 3, CD54-LFA1 and CD3-TCR interactions (Wucherpfennig *et al* 1992, Akagi and Shimotohno 1993, Kimata *et al* 1993, Cassé *et al* 1994, Kimata *et al* 1994a). Activation of T cells increases their permissiveness for HTLV-I infection (Section 1.3.2.2). The C terminus of HTLV-I $p21^{Env}$ has sequence similarity to the EGF receptor but there is no evidence that it plays a role in virus-induced proliferation of T cells (Lal 1991).

1.9 Animal models of HTLV-I leukaemogenesis

Animal models used in attempts to understand the pathogenesis of ATL, HAM/TSP and other HTLV-I-associated diseases include *tax*-transgenic mice, natural infections of cattle with BLV, natural infections of primates with STLV-I and experimental infections of mice, rats, rabbits and primates with HTLV-I.

1.9.1 HTLV-I tax-transgenic mice

Mice transgenic for *tax* have developed LGL leukaemia, fibroproliferative lesions, epithelial neoplasms, immune-mediated diseases and skeletal abnormalities (Hinrichs *et al* 1987, Nerenberg *et al* 1987, Furuta *et al* 1989, Green *et al* 1989, Iwakura *et al* 1991, Ruddle *et al* 1993, Grossman *et al* 1995). However, T cell neoplasms similar to ATL (Section 1.6.1) have not been demonstrated and in some *tax*-transgenic models there is no increase in the frequency of neoplasia (Furuta *et al* 1989). The observation of LGL leukaemia in *tax*-transgenic mice is most significant, since HTLV-I has been associated with this lymphoproliferative disorder in humans (Sections 1.10.2 and 1.11.2). In this model, mice transgenic for *tax* under the control of the lymphocyte-specific granzyme B enhancer, targeting expression to T, NK and lymphokine-activated killer (LAK) cells, developed LGL

leukaemia and NK cell tumours of the extremities and lymph nodes (Grossman *et al* 1995). Transgenic mice expressing *tax* in a wide variety of tissues from the HTLV-I LTR, as well as mice with *tax* expression targeted to T cells by Thy1.2 or CD3-ε, frequently develop fibroproliferative lesions at sites of trauma, mainly on the extremities (Hinrichs *et al* 1987, Nerenberg *et al* 1987, Coscoy *et al* 1998, Hall *et al* 1998). The lesions are associated with peripheral nerves but can be distinguished from neurofibromatosis. Increased frequencies of other mesenchymal tumours (fibrosarcomas and lipomas), adrenal adenomas, adenocarcinomas of the mammary glands, salivary glands and lungs and fibroproliferative lesions of the iris and ciliary body have also been observed in HTLV-I LTR-*tax*-transgenic mice (Green *et al* 1992a, Iwakura *et al* 1994). Rats transgenic for the HTLV-I *pX* region developed mammary adenocarcinomas (Yamada *et al* 1995). Proliferating cells from LGL lymphomas, fibroproliferative lesions and epithelial neoplasms in transgenic mice have high levels of expression of Tax, supporting a role for this protein in oncogenesis. However, only LGL leukaemia has been associated with HTLV-I infection in humans.

Mice and rats transgenic for *tax* are also predisposed to development of immune-mediated diseases, similar to conditions associated with HTLV-I infection in humans (Section 1.6.4). Arthritis developed in mice transgenic for the *pX* region of HTLV-I under control of the HTLV-I LTR or the metallothionein promoter (Iwakura *et al* 1991, Yamamoto *et al* 1993, Kira *et al* 1997, Saggioro *et al* 1997). Autoantibodies and CTL responses against Tax and Env in joints were demonstrated (Iwakura *et al* 1995, Fujisawa *et al* 1998). A condition resembling Sjögren's syndrome, with proliferation of ductal epithelial cells in salivary, lachrymal and Harderian glands in association with infiltration of lymphocytes and plasmacytes, has also been observed in HTLV-I LTR-*tax*-transgenic mice (Green *et al* 1989, Bieberich *et al* 1993) (Section 1.6.4.6). Lymph nodes adjacent to affected salivary glands were hyperplastic and occasionally had metastases of salivary ductal epithelium (Green *et al* 1989). Rats transgenic for the HTLV-I LTR-*env-pX* region developed arthritis, polyarteritis, polymyositis, myocarditis, sialoadenitis and thymic atrophy, consistent with an immune-mediated multisystem disease process (Yamazaki *et al* 1995). Thymic atrophy and immunosuppression in *tax*-transgenic mice is associated with growth retardation and increased mortality from bacterial and parasitic diseases (Hinrichs *et al* 1987, Nerenberg *et al* 1987, Furuta *et al* 1989, Iwakura *et al* 1991, Bieberich *et al* 1993, Hall *et al* 1998). Skeletal abnormalities in HTLV-I LTR-*tax*-transgenic mice were characterised by remodelling of bone and myelofibrosis due to increased activity of osteoclasts and osteoblasts (Ruddle *et al* 1993, Saggioro *et al* 1997).

$CD3^+$ $CD4^+$ $CD8^-$ TCLs of the abdominal lymph nodes and CNS neoplasms resembling neuroblastomas developed rapidly in 100% of mice co-transgenic for HTLV-I LTR-*tax* and *Ig*-c-*myc* (Benvenisty *et al* 1992). However, mice transgenic for c-*myc* alone under the influence of the HTLV-I LTR also developed $CD4^+$ $CD8^+$ T cell or B cell lymphomas in the mesenteric lymph nodes. Since in these experiments c-*myc* expression was driven by the HTLV-I LTR, the activity of which is up-regulated in the presence of Tax, it was not possible to determine whether Tax cooperated with c-Myc independently of its action on the HTLV-I LTR.

1.9.2 Natural animal models of HTLV-I infection

1.9.2.1 Bovine leukaemia virus

BLV is an exogenous retrovirus that infects B cells and causes B cell lymphoma and leukaemia (enzootic bovine leucosis, EBL) in naturally infected cattle throughout the world (Miller *et al* 1969, Ferrer *et al* 1974, Callahan *et al* 1976, Kettmann *et al* 1976, Paul *et al* 1977, Burny *et al* 1987). The virus is transmitted by transfer of infected cells in blood and milk (Ferrer *et al* 1981). In infected cattle polyclonal proliferation of B cells (persistent lymphocytosis) is induced in the early stages of infection, followed by oligoclonal or monoclonal expansion of neoplastic B cells (Kettmann *et al* 1980). B cell neoplasia develops in sheep infected with BLV experimentally (Kenyon *et al* 1981). Transformed B cell lines containing integrated BLV provirus can be established from infected cattle. BLV also infects and transforms fibroblasts (Onuma *et al* 1981, Rhim *et al* 1983). Seroepidemiological and molecular studies have failed to confirm BLV infection in humans or implicate BLV in human neoplasia (Burridge 1981, Donham *et al* 1987, Bender *et al* 1988, Maruyama *et al* 1989).

BLV is structurally similar to HTLV-I and contains genes in the *pX* region that express Tax_{BLV} and Rex_{BLV}, as well as accessory proteins R^{III} and G^{IV} (Sagata *et al* 1985, Rosen *et al* 1986, Willems *et al* 1987, Jensen *et al* 1991, Haas *et al* 1992, Alexandersen *et al* 1993, Willems *et al* 1994). Kettmann *et al* (1982) found that defective BLV proviruses were frequent in EBL, with preferential retention of the *pX* region, similar to HTLV-I in ATL. Other studies have reported conservation of complete BLV provirus with infrequent occurrence of deletions (Tajima *et al* 1998). Tax_{BLV} immortalises B cells and cooperates with Ras in transformation of primary REFs, although it may not be necessary for maintenance of the transformed phenotype (Willems *et al* 1990, 1992, Twizere *et al* 2000). G^{IV} also cooperates with Ras in transforming primary REFs and inducing tumours in athymic mice (Kerkhofs *et al* 1998).

1.9.2.2 Simian T lymphotropic viruses

STLV-I has been serologically associated with lymphomas in a variety of species of non-human primates (Homma *et al* 1984). ATL-like leukaemia with monoclonal integration of STLV-I has been described in African green monkeys (*Cercopithecus aethiops*) (Tsujimoto *et al* 1987). In captive colonies of baboons (*Papio hamadryas*) there is a high prevalence of STLV-I infection and most animals with TCLs are seropositive for STLV-I (Moné *et al* 1992, Voevodin *et al* 1996). STLV-II does not appear to be associated with T cell neoplasia in monkeys.

1.9.3 Experimental animal models of HTLV-I infection

Animals susceptible to experimental infection with HTLV-I include rabbits, rats, mice and primates (Miyoshi *et al* 1985, Suga *et al* 1991, Kazanji *et al* 1997b, Fang *et al* 1998). HTLV-I infects and transforms rabbit and non-human primate T cells, as well as a variety of different cell types from these and other species (Miyoshi *et al* 1982, 1983). Genetic background appears to be an important determinant of susceptibility to infection and disease (Kazanji *et al* 1997a). Among several strains of rats infected experimentally with HTLV-I, only those

derived from the WKAH strain develop ATL-like or HAM/TSP-like disease (Ishiguro *et al* 1992, Oka *et al* 1992, Kushida *et al* 1994). Mesenchymal tumours and polyarthritis have also been observed in experimentally infected rats (Kira *et al* 1997) (Sections 1.6.4.3 and 1.9.1). Rabbits infected with HTLV-I develop ATL-like T cell leukaemia and CTCL (Seto *et al* 1988, Simpson *et al* 1996). A rhesus macaque (*Macaca mulatta*) developed polyarthritis, uveitis and polymyositis following infection with HTLV-I (Beilke *et al* 1996) (Section 1.6.4). However, HTLV-I infection of non-human primates is not a useful model for ATL, since neoplasia is likely to be an infrequent and delayed consequence of experimental exposure (Kazanji 2000). $CD4^+$ TCLs of human origin can be established in severe combined immunodeficiency (SCID) mice by inoculation of PBMCs from ATL patients (Feuer *et al* 1993, Kondo *et al* 1993).

1.10 T cell and natural killer cell neoplasms

The International Lymphoma Study Group Revised European-American Lymphoma (REAL) classification is used in this thesis for terminology relating to T cell and natural killer (NK) cell neoplasms (Harris *et al* 1994), with reference to Willemze *et al* (1997) for specific classification of cutaneous T cell lymphomas (CTCLs) (Table 1.5). HTLV-I and related retroviruses have been associated with ATL (Sections 1.6.1 and 1.11), CTCLs (particularly MF/SS, cutaneous $CD30^+$ ALCL and angiocentric TCL) and LGL leukaemia. CTCLs are neoplasms of T cells that primarily involve the skin. The epidemiology, clinicopathological features and pathogenesis of these neoplasms will be described in this section. The biology of acute lymphoblastic leukaemia (ALL), small plaque parapsoriasis, Langerhans cell histiocytosis (LCH) and cutaneous B cell lymphoma (CBCL) will also be described. These entities are included in the differential diagnosis of ATL and CTCL and were among cases from which samples were received for laboratory examinations described in Chapters 2 and 3.

1.10.1 Mycosis fungoides/Sézary syndrome

Alibert (1806) introduced the term mycosis fungoides (MF) to describe a mushroom-shaped tumour of the skin that developed following a rash. MF is now established as a distinct clinicopathological entity. Sézary and Bouvrain (1938) described the syndrome of erythroderma, leukaemia and lymphadenopathy that has become known as Sézary syndrome (SS) and is now considered to be the leukaemic form of MF (Edelson 1980).

1.10.1.1 Epidemiology

MF/SS occurs sporadically and there is limited evidence for case clustering or areas of endemicity. The incidence of reported cases of MF/SS per 100,000 population per year is 0.13 to 0.14 in Europe and Australia and 0.29 in the USA (Weinstock and Horm 1988); 0.9 cases per 100,000 per year were reported in a more intensive, localised study in Minnesota, USA (Chuang *et al* 1990). About 15% of cases of MF/SS are classified as SS. The median age of onset of skin lesions is 51 years; only a small proportion of cases appear during childhood or in young adults (Zackheim *et al* 1997). The male:female adjusted ratio is 2:1 to 3:1 (Weinstock and Horm 1988, Chuang *et al* 1990). A trend to increasing incidence of

reported cases has been observed (Weinstock and Reynes 1999). MF and other lymphoid neoplasms have occasionally been observed in relatives of patients with MF/SS (Greene *et al* 1982). Cohen *et al* (1980) identified an increased risk for MF in people employed in the manufacturing or construction industries, especially in petrochemical, textile, metallurgical and mechanical occupations, whereas other studies have not confirmed these associations (Tuyp *et al* 1987, Whittemore *et al* 1989).

1.10.1.2 Clinical and pathological features

MF is a slowly growing neoplasm, usually affecting adults, characterised by infiltration of the skin with neoplastic T cells exhibiting marked epidermotropism. In the early stages of MF neoplastic T cells infiltrate the epidermis with a band pattern (lichenoid reaction, vacuolar interface dermatitis), sometimes forming intraepidermal clusters (Pautrier's microabscesses) (Shapiro and Pinto 1994). Patches, plaques and nodules (tumours) are formed as the disease progresses and there is involvement of lymph nodes and viscera in the advanced stages (Vonderheid *et al* 1994). SS is a leukaemia characterised by erythroderma, lymphadenopathy and leucocytosis, with neoplastic T cells circulating in the peripheral blood (Matutes *et al* 1990). Neoplastic lymphocytes infiltrate the skin in SS but may have less marked epidermotropism than in MF (Trotter *et al* 1997, Kamarashev *et al* 1998). Clinical erythroderma without a histologically distinct neoplastic infiltrate often precedes MF (Bakels *et al* 1991). A rare "tumeur d'emblée" form of MF presents as tumours with no apparent premycotic or plaque stages and has a rapidly progressive course (Blasik *et al* 1982). Other variants of MF include spongiotic/vesicular, bullous, follicular/mucinous, pustular, granulomatous and verrucous forms, as well as cases with altered pigmentation of the skin (LeBoit 1991).

The neoplastic cells infiltrating the skin in MF, circulating in the peripheral blood in SS and infiltrating lymph nodes in advanced forms of the disease are T cells that are similar morphologically and immunologically and are termed MF/Sézary cells (Zucker-Franklin *et al* 1974). These are round cells with highly convoluted (cerebriform) nuclear contours (Lutzner *et al* 1971). Transformation of neoplastic T cells to a large cell morphology characteristic of anaplastic large cell lymphoma (ALCL) occurs in some late stage cases of MF and SS (Salhany *et al* 1988, Wood *et al* 1993) (Section 1.10.3).

1.10.1.3 Immunology

MF/Sézary cells usually have a committed memory helper-inducer phenotype: $CD2^+$, $CD3^+$, $CD4^+$, $CD5^+$, $CD8^-$, $CD7^-$, $CD25^-$, $CD30^-$ and $CD49^+$, with expression of TCR $\alpha\beta$ or $\gamma\delta$ surface antigens and the LCA null isoform (CD45RO) (Waldmann *et al* 1984, Nasu *et al* 1985, Michie *et al* 1989, Wood *et al* 1990a, Boehncke *et al* 1993, Bagot *et al* 1996, Barzilai *et al* 1996, Willemze *et al* 1997). Cases of MF with $CD8^+$ phenotype have been identified (Agnarsson *et al* 1990). T cell markers may be lost with progression of MF/SS. In functional studies, PBMCs from some MF/SS patients exhibit Th activity, whereas in other patients Ts activity is predominant or there is a lack of effector activity (Berger *et al* 1979, Miedema *et al* 1984, Golstein *et al* 1986).

Table 1.5: Classification of T cell and natural killer cell neoplasms (Harris *et al* 1994).

I.	Precursor T cell neoplasm
	1. Precursor T-lymphoblastic lymphoma/leukaemia
II.	Peripheral T cell and natural killer cell neoplasms
	1. T cell chronic lymphocytic leukaemia/prolymphocytic leukaemia
	2. Large granular lymphocytic leukaemia
	Large granular lymphocytic leukaemia, T cell type
	Large granular lymphocytic leukaemia, NK cell type
	3. Mycosis fungoides/Sézary syndrome
	4. Peripheral T cell lymphomas, unspecified
	Medium-sized cell peripheral T cell lymphoma*
	Mixed medium-sized and large cell peripheral T cell lymphoma *
	Large cell peripheral T cell lymphoma *
	Lymphoepithelioid cell (Lennert's lymphoma)*
	Hepatosplenic $\gamma\delta$ T cell lymphoma*
	Subcutaneous panniculitic T cell lymphoma*
	5. Angioimmunoblastic T cell lymphoma
	6. Angiocentric T cell lymphoma
	7. Intestinal T cell lymphoma (+/- enteropathy associated)
	8. Adult T cell lymphoma/leukaemia, HTLV-I$^+$
	9. Anaplastic large cell lymphoma, CD30$^+$
	Anaplastic large cell lymphoma, T cell type
	Anaplastic large cell lymphoma, Null cell type
	10. Anaplastic large cell lymphoma, Hodgkin's-like*

* Provisional entities

It has been suggested that the cytokine profile of neoplastic T cells is predominantly Th1 in MF and Th2 in SS (Saed *et al* 1994, Lee *et al* 1999), but other studies support a Th2 profile in both MF and SS (Vowels *et al* 1992, Tendler *et al* 1994, Vowels *et al* 1994, Dummer *et al* 1996). Naïve (Th0) $CD4^+$ T cells are induced by IL12 to differentiate into Th1 cells, which produce IFNγ, or by IL4 to differentiate into Th2 cells, which produce IL4, IL5 and IL13 (Abbas *et al* 1996). Since these cells have different roles in the regulation of immune responses, hypotheses as to the pathogenesis of MF/SS are restricted by uncertainty about the functional categorisation of the neoplastic cells.

Immunological abnormalities observed in patients with advanced MF/SS include decreased responsiveness of T cells to antigens and mitogens, decreased NK cell activity, decreased LAK cell activity, eosinophilia and increased serum IgE and IgA (Wood *et al* 1990b). In SS there is a marked reduction in the number of non-neoplastic T cells in the peripheral circulation (Heald *et al* 1994) and an associated immune deficiency may be responsible for secondary infections in MF/SS patients (Axelrod *et al* 1992). An MHC-I-restricted cell-mediated immune response against neoplastic T cells has been observed in MF/SS patients (Berger *et al* 1996). An infiltrate of activated cytotoxic $CD8^+$ and $CD4^+$ T cells accompanies the neoplastic $CD4^+$ infiltrate in the early stages of MF/SS but decreases with advancement of disease, as does the proportion of activated $CD8^+$ T cells in the peripheral blood (Wood *et al* 1994a, Asadullah *et al* 1997). Some authors have reported restricted use of TCR Vβ epitopes by neoplastic T cell clones in MF, suggesting that exposure to a common antigen, such as a bacterial superantigen or persistent viral antigen, could contribute to chronic T cell activation and proliferation in MF (Potoczna *et al* 1996), but others have shown that a variety of Vβ genes are used (Longley *et al* 1995, Bigler *et al* 1996).

1.10.1.4 Molecular and genetic characteristics

The neoplastic character of MF/SS has been confirmed by the detection of clonal TCR β or γ gene rearrangements in skin, PBMCs and lymph nodes (Weiss *et al* 1985b, Whittaker *et al* 1991). Clonality in MF/SS has also been demonstrated by cytogenetic studies but no consistent karyotypic abnormalities have been identified (Edelson *et al* 1979, Nowell *et al* 1982, Johnson *et al* 1985). Mutations, rearrangements or deletions involving *lyt-10*, *p53* or *tal1* have been observed in some cases of MF/SS but their pathogenetic role is uncertain (Thakur *et al* 1994, Neri *et al* 1995). Mutations in *p53* develop during neoplastic progression, being found in one third of patients with tumour stage MF but not in patients with plaque stage MF (McGregor *et al* 1999).

1.10.1.5 Pathogenesis

In models of the development of MF/SS a multistage progression of oncogenic events has been hypothesised (Lessin *et al* 1994). Acute to chronic dermatitis with recruitment of a polyclonal T cell infiltrate often precedes the development of MF/SS (Sigurdsson *et al* 1997). The next stage is cutaneous lymphoid hyperplasia, with formation of preneoplastic patches in the skin containing polyclonal lymphocytes exhibiting nuclear atypia (Lange Wantzin *et al* 1988). Primary neoplasia in the form of plaques is initially localised to the skin, then tumours

develop in association with erythroderma and there is spread to lymph nodes, viscera and peripheral blood (Bunn *et al* 1980, Toback and Edelson 1985). Transformation of MF to ALCL is marked by development of aggressive disease (Wood *et al* 1993). MF/Sézary cells circulate between cutaneous and extracutaneous sites and systemic dissemination of neoplastic T cell clones can be demonstrated early in the development of the disease (Miller *et al* 1980, Veelken *et al* 1995, Muche *et al* 1997). MF/Sézary cells proliferate most rapidly in lymph nodes, with a lower rate of proliferation in the skin and limited proliferation in the peripheral blood (Shackney and Schuette 1983, Tu *et al* 1994). As disease progresses the rate of proliferation increases, while the proportion of cells undergoing apoptosis decreases (Kikuchi and Nishikawa 1997). Expression of c-Myc, Ras, Jun, Fos, PCNA and p53 increases with advancing stages of MF (Kanavaros *et al* 1994, Tu *et al* 1994, Beylot-Barry *et al* 1995, Lauritzen *et al* 1995, Qin *et al* 1999).

Selective expression of adhesion molecules is responsible for the tropism of subsets of T cells for the skin, lymph nodes and other tissues and may explain the distribution of cellular infiltrates at different stages of MF/SS (Miller *et al* 1980, Borowitz *et al* 1993, Campbell and Butcher 2002). CD54, induced on keratinocytes and dermal endothelial cells by T cell-derived IFNγ and TNFα, binds to CD11a/CD18 (LFA1) expressed by T cells, including those in MF (Vejlsgaard *et al* 1989, Imayama *et al* 1992, Uccini *et al* 1993). This interaction appears to be necessary but not sufficient for epidermotropic behaviour. Loss of CD54 expression by keratinocytes due to reduced IFNγ expression by neoplastic T cells may permit systemic dissemination in SS (Nickoloff *et al* 1989). CD62E (E-selectin, endothelial leucocyte adhesion molecule 1, ELAM1), which is expressed by dermal endothelial cells, particularly in inflamed skin, is one of the ligands for cutaneous lymphocyte-associated antigen (CLA), a carbohydrate epitope induced on CD62P (P-selectin) glycoprotein ligand 1 (PSGL1) in T cells, including those infiltrating the skin in MF (Fuhlbrigge *et al* 1997, Tietz *et al* 1998). CLA, which acts as a skin homing receptor, is expressed on CTCLs but is absent from nodal TCLs (Picker *et al* 1990, Noorduyn *et al* 1992). Similarly, integrins α3β1, α5β1 and $\alpha^{E}\beta7$, which act as extracellular matrix receptors, are expressed by epidermotropic T cells in MF but are absent from circulating MF/Sézary cells in SS (Savoia *et al* 1992, Simonitsch *et al* 1994). Most cases of MF lack expression of CD62L (L-selectin, peripheral lymph node homing receptor), whereas expression of CD62L has been associated with the involvement of lymph nodes by CTCL (Wood *et al* 1990a, Borowitz *et al* 1993). MHC-II, the ligand for CD4, is expressed by keratinocytes and other cells in lesional skin in MF/SS, but is not correlated with the presence of epidermal lymphocytes (Wood *et al* 1994b). IFNγ- and TNFα-inducible proteins (IPs) IP9, IP10 and monokine induced by IFNγ (MIG), which are chemotactic for $CD4^{+}$ T cells, are expressed by epidermal keratinocytes in MF/SS lesions and may also contribute to the epidermotropism of MF/Sézary cells (Daliani *et al* 1998, Tensen *et al* 1998).

Abnormal production of cytokines by MF/Sézary cells, keratinocytes or antigen presenting cells (APCs) may contribute to the proliferation and distribution of neoplastic T cells in MF/SS. Increased expression of IL1, IL5, IL6, GMCSF, IFNγ, TNFα, TGFα and EGF have been detected in MF (Kupper *et al* 1988, Lawlor *et al* 1990, Hansen *et al* 1991, Vowels *et al* 1992, Tendler *et al* 1994, Dummer *et al* 1996, Asadullah *et al* 1998, Daliani *et al* 1998,

Courville *et al* 1999). IL4, IL10 and IL15 expression may increase with progression of MF (Vowels *et al* 1994, Asadullah *et al* 1996a, 2000). The response of MF/Sézary cells to cytokines is discussed in Section 2. Little is known about the expression of receptors for these cytokines. Neoplastic T cells in MF express both CD28 and its ligand CD80, providing the opportunity for self-costimulation (Nickoloff *et al* 1994). Rook *et al* (1993) hypothesised that IFNγ production by tumour infiltrating lymphocytes (TILs) responding to the neoplastic infiltrate exceeds IL4 production by MF/Sézary cells in the early stages of MF, decreasing the rate of expansion of the population of neoplastic cells and promoting epidermotropism through induction of CD54 on keratinocytes. Eventually the population of neoplastic cells reaches a size at which IL4 production counteracts the production and biological activity of IFNγ, leading to reduced expression of CD54 by keratinocytes, loss of epidermotropism with systemic dissemination of neoplastic cells and reduced anti-neoplasm immunity.

Rowden and Lewis (1976) proposed that persistent antigenic stimulation was responsible for the abnormal T cell proliferation in MF/SS. APCs, including Langerhans cells and interdigitating cells, are present among the neoplastic T cell infiltrate (Tjernlund 1982). Langerhans cells have been shown to contribute to epidermotropism of T cells (Shiohara *et al* 1988). It has been hypothesised that retroviral infection of Langerhans cells leads to chemotaxis or retention of T cells in the epidermis (MacKie 1981, Slater *et al* 1985, Lessin *et al* 1994). Chronic stimulation of this population would eventually lead to the selective outgrowth of a neoplastic T cell clone. Alterations in the morphology of Langerhans cells and increased interactions between Langerhans cells and MF/Sézary cells have been described in MF (Rowden *et al* 1979, Füllbrandt *et al* 1983). In contrast, Bani *et al* (1990) observed that MF/Sézary cells were frequently associated with interdigitating cells in the epidermis, but rarely made contact with Langerhans cells. However, most evidence incriminating retroviruses in CTCLs is focussed on infection of neoplastic T cells by HTLV-I or related viruses and a direct involvement of such viruses in oncogenesis (Dosaka *et al* 1991, Hall *et al* 1991) (Section 1.11). In addition, the density of Langerhans cells is increased in the dermis and epidermis in plaque stage MF, but may decrease with progression to tumour stage lesions, indicating that Langerhans cells could play a role in suppression of CTCLs rather than facilitating progression (Igisu *et al* 1983, Meissner *et al* 1993).

1.10.2 Large granular lymphocytic leukaemia

Large granular lymphocytic (LGL) leukaemia is defined as a clonal increase in the number of circulating large granular lymphocytes (LGLs) and may be of T cell or NK cell phenotype (Loughran *et al* 1985, Chan *et al* 1986, Loughran 1993). A chronic proliferation of LGLs that cannot be proven to be clonal is referred to as lymphoproliferative disease of LGLs (LDLGLs) (Pandolfi *et al* 1990, Scott *et al* 1993, Semenzato *et al* 1997). Synonyms for LGL leukaemia include chronic T cell lymphocytosis with neutropaenia, T cell CLL and Tγ lymphocytosis/lymphoproliferative disorder (Brouet *et al* 1975, Aisenberg *et al* 1981, Reynolds and Foon 1984). Hypotheses regarding the aetiology of $CD3^+$ and $CD3^-$ LGL leukaemia have included infection by retroviruses, activation by antigens, stimulation by cytokines and accumulation of mutations (Loughran 1993). Evidence for the involvement of viruses in LGL leukaemia is discussed in Section 1.11.

1.10.2.1 Large granular lymphocytic leukaemia, T cell type

T cell LGL leukaemia is a clonal disorder of $CD3^+$ cells with LGL morphology and is associated with chronic neutropaenia, anaemia, splenomegaly, recurrent bacterial infections and rheumatoid arthritis or other autoimmune diseases (Loughran *et al* 1985, Loughran 1993, Scott *et al* 1993). Neoplastic lymphocytes have abundant, pale blue cytoplasm, azurophilic granules, an eccentric nucleus with moderately condensed chromatin and an inconspicuous nucleolus. The immunophenotype is usually $CD2^+$, $CD3^+$, $CD4^-$, $CD7^-$, $CD8^+$, $CD16^+$, $CD25^-$ and $CD56^-$, although $CD4^-$ $CD8^-$ and $CD4^+$ $CD8^{dim+/-}$ phenotypes have been observed (Pandolfi *et al* 1991, Richards *et al* 1992). Most cases are TCR $\alpha\beta^+$ but TCR $\gamma\delta^+$ expression has been reported (Foroni *et al* 1988, Loughran *et al* 1988a). Clonality has been demonstrated by cytogenetic studies (Loughran *et al* 1985, McKenna *et al* 1985) and detection of clonal rearrangements of the TCR β or γ genes (Pelicci *et al* 1987, Pandolfi *et al* 1991, Scott *et al* 1993).

1.10.2.2 Large granular lymphocytic leukaemia, natural killer cell type

The clinical features of NK cell LGL leukaemia, a clonal disorder of $CD3^-$ $CD56^+$ cells, are hepatomegaly, splenomegaly, anaemia and thrombocytopaenia, along with fever, night sweats and/or weight loss (B symptoms) (Sheridan *et al* 1988, Sivakumaran *et al* 1996). The immunophenotype is usually $CD2^+$, $CD3^-$, $CD4^-$, $CD8^-$, $CD16^+$, $CD56^+$ and $CD57^-$. NK activity has been demonstrated in leukaemic cells (Loughran *et al* 1987). There is no expression or rearrangement of the TCR or immunoglobulin genes (McKenna *et al* 1985, Loughran *et al* 1988a, Biondi *et al* 1989). Clonality has been confirmed in some cases by demonstration of clonal cytogenetic abnormalities or clonal X-linked inactivation of the phosphoglycerate kinase gene (Sheridan *et al* 1988, Taniwaki *et al* 1990, Nash *et al* 1993, Kelly *et al* 1994). Cases of $CD3^-$ $CD56^+$ LDLGLs in which clonality cannot be demonstrated usually have a less aggressive clinical course.

1.10.3 $CD30^+$ anaplastic large cell lymphoma

$CD30^+$ anaplastic large cell lymphomas (ALCLs) are aggressive solitary or multiple tumours that in the primary systemic form have a bimodal age distribution and involve lymph nodes, skin, bone or soft tissues (Agnarsson and Kadin 1988, Kaudewitz *et al* 1989, Paulli *et al* 1995). Primary cutaneous $CD30^+$ ALCLs, although indolent, limited to the skin and occasionally spontaneously regressing, paradoxically are often difficult to treat (Beljaards *et al* 1993, Paulli *et al* 1995, Willemze *et al* 1997). Secondary $CD30^+$ ALCLs appear to be derived following transformation of MF, other TCLs or Hodgkin's disease (Salhany *et al* 1988, Wood *et al* 1993) (Section 1.10.1.2). In a patient with MF and cutaneous ALCL, $CD30^+$ T cells in spontaneously regressing tumours were derived from the same clone as MF/Sézary cells in patch stage MF lesions (Woodrow *et al* 1996). $CD30^+$ ALCLs are composed of large pleomorphic lymphoid cells with abundant cytoplasm and horseshoe-shaped or wreath-like nuclei with prominent nucleoli, along with multinucleated Reed-Sternberg-like cells. Inflammatory leucocytes may infiltrate the involved tissue. CD30 is a marker of activation and $CD30^+$ ALCLs variably express other activation markers such as

CD25, MHC-II and CDw70 (Kaudewitz *et al* 1989). The origin of the neoplastic cell is uncertain, since $CD30^+$ ALCLs have been classified as having T cell, B cell or immature phenotype. Most cases of primary cutaneous $CD30^+$ ALCL appear to be of T cell origin; 95% or more are $CD4^+$ and 5% or less are $CD8^+$, with variable expression of pan T cell antigens (CD2, CD3, CD5) (Kaudewitz *et al* 1989, Beljaards *et al* 1993, Willemze *et al* 1997). Primary systemic $CD30^+$ ALCLs often express epithelial membrane antigen (EMA) and are negative for CLA, whereas most cases of primary cutaneous $CD30^+$ ALCL are CLA^+ and EMA^- (De Bruin *et* al 1993). Clonal rearrangements of TCR genes are found in 50 to 60% of primary systemic $CD30^+$ ALCLs; most cases of primary cutaneous $CD30^+$ ALCL also have clonal TCR gene rearrangements (Herbst *et al* 1989, Banerjee *et al* 1991). Many T cell and null cell primary systemic $CD30^+$ ALCLs have the chromosomal translocation t(2;5)(p23;q35), which conjoins the nucleophosmin (*NPM*) and anaplastic lymphoma kinase (*ALK*) genes (Morris *et al* 1994). This translocation does not appear to be present in most primary cutaneous $CD30^+$ ALCLs (De Coteau *et al* 1996, Li *et al* 1997). The role of EBV in the aetiology of $CD30^+$ ALCL is described in Section 1.11.3.1 and evidence for the involvement of HTLV-I in this entity is described in Section 1.11.1.7.

1.10.4 Angiocentric T cell lymphoma

Angiocentric TCLs are extranodal T cell neoplasms associated with blood vessels that frequently involve the nasopharyngeal region, lung and skin (Lipford *et al* 1988, Kanavaros *et al* 1993, Kato *et al* 1999). The nasopharyngeal/sinonasal form is also known as lethal midline granuloma or polymorphic reticulosis (Gaulard *et al* 1988, Strickler *et al* 1994). Blood vessels are surrounded and invaded by pleomorphic neoplastic lymphocytes, sometimes with a predominance of large cells, and frequently there is an associated inflammatory infiltrate, along with ischaemic necrosis. The immunophenotype is $CD2^+$, with variable expression of CD3, CD4, CD5, CD7 and CD8, indicating a T cell origin; however the neoplasms can also be $CD3^-$ $CD56^+$, consistent with a NK cell phenotype. TCR β gene rearrangements have been detected in angiocentric TCLs of T cell type (Gaulard *et al* 1988). There are usually no clonal rearrangements or expression of TCR or immunoglobulin genes in the NK cell type, but clonality has been confirmed by karyotypic studies (Emile *et al* 1996, Tien *et al* 1997). EBV has been aetiologically associated with the nasopharyngeal/sinonasal form of angiocentric TCL (Section 1.11.3.1) and some groups have associated limited numbers of cases of angiocentric TCL with HTLV-I infection (Section 1.11.1.7). The neoplastic infiltrate in cutaneous ATL may have an angiocentric distribution with necrotising vasculitis (Haynes *et al* 1983, Manabe *et al* 1988).

Lymphomatoid granulomatosis is also an angiocentric immunoproliferative condition that may involve the lungs or skin and in most cases appears to be a form of angiocentric TCL (James *et al* 1981, Kessler *et al* 1981, Gaulard *et al* 1988, Whittaker *et al* 1988). However, some pulmonary cases express B cell antigens, have clonal immunoglobulin gene rearrangements and contain EBV genomes, so represent proliferations of EBV-infected B cells (Nicholson *et al* 1996).

1.10.5 Acute lymphoblastic leukaemia

Acute lymphoblastic leukaemias (ALLs) are aggressive clonal proliferations of T or B cell lineage lymphoid cells with heterogeneous morphological, immunophenotypic and cytogenetic characteristics (Harris *et al* 1994, Copelan and McGuire 1995, Kersey 1997). Neoplastic lymphoid cells circulate in the blood and infiltrate tissues. ALL is over-represented as a neoplasm of childhood, with a peak in incidence at 2 to 5 years of age. The incidence in children under 15 years of age in the USA is approximately 30 cases/million/year (Young *et al* 1986). The incidence in adults is one third of this figure but exhibits a second peak at 50 years of age followed by an increasing incidence with age. In paediatric and adult ALL, approximately 85% of cases are of precursor-B (pre-B) or B cell type and 15% of T cell type (Copelan and McGuire 1995, Kersey 1997, Khalidi *et al* 1999). B cell ALLs express surface CD19 and include B-precursor (early pre-B) ALL (expressing cytoplasmic CD22 and nuclear terminal deoxynucleotidyl transferase), common ALL (expressing surface CD10), pre-B ALL (expressing cytoplasmic immunoglobulin heavy chains) and B-ALL (expressing surface immunoglobulin) (Janossy *et al* 1989, Copelan and McGuire 1995) (Table 1.6). T cell ALLs have cytoplasmic expression of CD3 and include precursor-T (pre-T) ALL ($CD7^+$) and T-ALL ($CD7^+$, $CD2^+$). Null ALL predominates in infants (less than 1 year of age), common ALL is predominant in children 1 to 7 years of age and T cell ALL is proportionally high in adolescents and young adults (McKinney *et al* 1993).

Clonality in ALL has been demonstrated by cytogenetic analysis, X-linked inactivation of glucose-6-phosphate dehydrogenase and analysis of immunoglobulin and TCR gene rearrangements (Williams *et al* 1985, Dow *et al* 1985, Khalidi *et al* 1999). The earlier stage B and T cell ALLs have partial or incomplete rearrangements of immunoglobulin receptor and TCR genes, respectively. Up to 5% of children and approximately 30% of adults with ALL have a Philadelphia chromosome (shortened chromosome 22) due to a t(9;22)(q34;q11) translocation that results in production of abnormal proteins, $p190^{Bcr\text{-}Abl}$ or $p210^{Bcr\text{-}Abl}$, with increased PTK activity (Chan *et al* 1987, Kurzrock *et al* 1987, Faderl *et al* 1998). Most Philadelphia positive cases have the phenotype of B cell ALL, but appear to be derived from a pluripotential stem cell; mixed lineage leukaemia may occur (Secker-Walker *et al* 1991, Copelan and McGuire 1995, Schenk *et al* 1998). A wide range of other numerical and structural chromosomal abnormalities have been observed (Faderl *et al* 1998, Kersey 1997). Translocations frequently generate oncogenic fusion proteins or result in deregulated expression of transcription factors. These include immunoglobulin H, κ or λ-c-*myc* and *TEL-AML1* in B cell ALL and TCR β-*TAL1* or TCR α-c-*myc* in T cell ALL (Croce and Nowell 1985, Nakamura *et al* 1993, Kersey 1997, Wiemels and Greaves 1999). The translocation t(4;11)(q21;q23) conjoining *MLL* and *AF-4* occurs in approximately 60% of infants with ALL, as well as in 2% of older children and 3 to 6% of adults (Pui *et al* 1991, Biondi *et al* 2000). Some cases of childhood ALL are initiated *in utero* (Wiemels *et al* 1999). Patients with ataxia telangiectasia have an increased risk of developing T cell ALL (Toledano and Lange 1980).

Geographical and temporal clustering of childhood ALL cases in the UK and elsewhere has been suggested as evidence for an infectious cause (Kinlen 1988, Cartwright *et al* 1990, Kinlen *et al* 1990, Alexander 1992, Alexander 1993, Gilman *et al* 1999, Birch *et al* 2000).

Table 1.6: Immunophenotypes of acute lymphoblastic leukaemias
(Copelan and McGuire 1995).

Leukaemia type	Surface markers	Cytoplasmic markers	Nuclear markers
B cell acute lymphoblastic leukaemia			
Early Pre-B ALL	CD19	CD22	TdT
Common ALL	CD19, CD10	CD22	TdT
Pre-B ALL	CD19	Ig	TdT
B-ALL	CD19, CD22, Ig	-	-
T cell acute lymphoblastic leukaemia			
Pre-T	CD7	CD3	TdT
T-ALL	CD1, CD2, CD3, CD4, CD7, CD8		TdT

ALL	Acute lymphoblastic leukaemia
CD	Cluster of differentiation
Ig	Immunoglobulin
TdT	Terminal deoxynucleotidyl transferase
Pre	Precursor

Studies using PCR with specific, consensus or degenerate primers have so far failed to implicate herpesviruses, polyomaviruses or parvovirus B19 as the cause of common ALL in children (Luka *et al* 1991, Gentile *et al* 1999, Heegaard *et al* 1999, MacKenzie *et al* 1999, Smith *et al* 1999, MacKenzie *et al* 2001). ATL can be distinguished from T cell ALL on the basis of clinicopathological features, particularly the presence of hypercalcaemia and lytic bone lesions, and the presence of HTLV-I infection.

1.10.6 Small plaque parapsoriasis

Small plaque parapsoriasis is a chronic dermatosis characterized by patches on the trunk and extremities that often resolve spontaneously (Harris *et al* 1994, Haeffner *et al* 1995). Histopathological examination reveals a mild superficial perivascular lymphocytic infiltrate composed of $CD4^+$ T cells admixed with $CD8^+$ T cells, Langerhans cells/indeterminate cells and macrophages, associated with mild spongiosis and parakeratosis. Both dominant clonal and polyclonal T cell populations have been demonstrated (Haeffner *et al* 1995).

1.10.7 Langerhans cell histiocytosis

Langerhans cell histiocytosis (LCH, histiocytosis X) is characterised by cutaneous infiltrations of Langerhans cells that contain Birbeck granules, express S-100 and CD1a and may have aberrant expression of CD2, CD3, CD54 and CD58 (LFA-3), consistent with an activated state (Ben-Ezra and Koo 1993, Hage *et al* 1993, De Graaf *et al* 1994, Emile *et al* 1994). As well as cells with histiocytic morphology, lesions contain giant cells, macrophages, neutrophils, eosinophils, lymphocytes, plasma cells and occasional mast cells. Using PCR and ISH, 56 cases of LCH in the USA were negative for HTLV-I, HTLV-II, HIV-1, EBV, herpes simplex virus (HSV) type 1, HHV-6, cytomegalovirus, adenovirus and parvovirus (McClain *et al* 1994). Infiltrating histiocytic cells lack TCR gene rearrangements but clonality has been determined by X-linked inactivation studies of the androgen receptor gene and with X-linked polymorphic DNA probes, consistent with LCH being a neoplastic proliferation (Willman *et al* 1994, Yu *et al* 1994, Yu and Chu 1995).

1.10.8 Cutaneous B cell lymphoma

Cutaneous B cell lymphomas (CBCLs) are described here because they represent one of the differential diagnoses of CTCL, being characterised by infiltrations of neoplastic B cells in the skin (Harris *et al* 1994, Rijaarsdam and Willemze 1994). They frequently have a follicular pattern with formation of nodules but neoplastic B cells may be distributed diffusely through the dermis and occasionally exhibit epidermotropism. Immunoglobulin gene rearrangements can be detected in most cases (Child *et al* 2001). A small proportion of cases have rearrangements in *bcl-1*, *bcl-2*, *bcl-6* and *lyt-10* genes (Neri *et al* 1995).

Herpesviruses have been excluded as likely aetiological agents in primary CBCL in immunocompetent patients (Dupin *et al* 1997, Nagore *et al* 2000), although CBCLs in acquired immune deficiency syndrome (AIDS) patients may be associated with EBV (Beylot-Barry *et al* 1999). An association between Lyme disease and CBCL has been hypothesised (Cerroni *et al* 1997); *Borrelia burgdorferi* sequences were detected by PCR in DNA from

seven (35%) of 20 lesions in Scotland (Goodlad *et al* 2000). However, further studies are required to confirm this relationship. Evidence for the involvement of HTLV-I in B cell lymphomas is discussed in Section 1.6.2.1.

1.11 Viral aetiology of cutaneous T cell lymphomas and large granular lymphocytic leukaemia

The possibility that HTLV-I or related retroviruses are involved in the aetiology and pathogenesis of CTCLs and LGL leukaemia is the main theme of this thesis. This section discusses the evidence for retroviral involvement in the aetiology of MF/SS, cutaneous $CD30^+$ ALCL, angiocentric TCL and LGL leukaemia.

1.11.1 Human T lymphotropic viruses and cutaneous T cell lymphomas

1.11.1.1 Epidemiology

The sporadic occurrence and other epidemiological features of MF/SS do not immediately support an infectious aetiology (Section 1.10.1.1). ATL and HTLV-I have distinct distributions by geographical area and population groups, whereas cases of MF/SS do not exhibit clustering and are mostly reported from Europe and the USA, which are not HTLV-I endemic regions (Weinstock and Horm 1988). Hjelle *et al* (1991b) found no difference in the incidences of MF between three ethnic groups in the USA with differences in the prevalence of HTLV-II infection. There is little evidence that MF/SS is transmissible (Stratton 1948, Grossman and Duvic 1993).

1.11.1.2 Clinicopathological features

The similarity of clinicopathological features of MF/SS and some forms of ATL is the main basis for attempts to search for HTLV-I-related viruses in CTCLs. As discussed in Section 1.6.1.5, HTLV-I is the cause of ATL. MF/SS and ATL are both $CD4^+$ T cell neoplasms that can involve the skin, lymph nodes, viscera and peripheral blood (Nagatani *et al* 1990). Cutaneous involvement occurs in 43 to 72% of patients with ATL and usually takes the form of rapid onset, generalised papules with erythaema (Chan *et al* 1985, Gibbs *et al* 1987, Maeda and Takahashi 1989, Johno *et al* 1992). Smouldering ATL is a distinct cutaneous form that usually develops slowly, with systemic involvement and leukaemia being a late event, similar to most cases of progressive MF (Yamaguchi *et al* 1983, Jaffe *et al* 1984, Chan *et al* 1985, Arai *et al* 1991, Shimoyama *et al* 1991). The acute and lymphomatous forms of ATL are aggressive diseases; likewise the rare "tumeur d'emblee" form of MF has no premycotic phase and has a rapidly progressive course (Blasik *et al* 1982). The hypercalcaemia and lytic bone marrow lesions characteristic of ATL are not observed in MF/SS (Nagatani *et al* 1990).

Whereas MF/SS is epidermotropic, the cutaneous infiltrate in acute ATL primarily involves the dermis and subcutaneous tissues, often consists of highly pleiomorphic cells with few reactive cells and is sometimes associated with vascular lesions (Hasui *et al* 1987, Maeda and Takahashi 1989, Nagatani *et al* 1990). However, the pattern of lymphocyte infiltration in

ATL and MF can be very similar and Pautrier's microabscesses can be observed in both conditions (Nagatani *et al* 1990, Johno *et al* 1992). Neoplastic T cells in ATL have moderately indented (lobulated) nuclei, whereas MF/Sézary cells have highly indented (cerebriform) nuclei (Hasui *et al* 1987, Maeda and Takahashi 1989, Nagatani *et al* 1990). The neoplastic cells in both conditions are usually $CD4^+$ $CD7^-$ T cells (Hanaoka 1984, Nasu *et al* 1985, Kamihira *et al* 1992), although Nagatani *et al* (1990) observed higher expression of CD7 by ATL cells than MF/Sézary cells. In unaffected people approximately 9% of normal T cells have a $CD7^-$ phenotype (Reinhold *et al* 1993). MF/Sézary cells rarely express CD25 (or other activation markers) whereas CD25, CD30, CD71, Ki67 and MHC-II are usually expressed by ATL cells (Waldmann *et al* 1984, Maeda and Takahashi 1989, Oishi *et al* 1994). Transformation of MF to ALCL is also associated with acquisition of CD25, CD30, CD71, Ki67 and MHC-II expression (Cerroni *et al* 1992). ATL and MF/SS share expression of cell adhesion molecules, some of which are up-regulated by Tax in HTLV-I-infected T cells (Sections 1.6.1.3, 1.8.1.2 and 1.10.1.5). ATL cells exhibit Ts activity (Tendler *et al* 1994, Yamada *et al* 1996), whereas MF/Sézary cells exhibit Th or Ts activity, as discussed in Section 1.10.1.3 (Berger *et al* 1979, Miedema *et al* 1984, Golstein *et al* 1986). Immortalised and transformed T cell lines infected with HTLV-I can be readily established from ATL cells *in vitro*, usually following the addition of IL2 or CM, whereas MF/Sézary cells, with few exceptions, do not proliferate readily under these conditions (Waldmann *et al* 1984, Abrams *et al* 1991b, Zucker-Franklin *et al* 1991) (Chapter 2).

It has been possible to distinguish most cases of MF/SS from ATL using a combination of clinicopathological criteria and the presence or absence of HTLV-I infection (Yamaguchi *et al* 1984, Levine *et al* 1994a). Numerous cases initially classified as MF/SS were reclassified as forms of ATL following the discovery of HTLV-I (Poiesz *et al* 1980a, 1981, Blattner *et al* 1982, Blayney *et al* 1983b, D'Incan *et al* 1995). Most cases of HTLV-I-associated CTCLs in HTLV-I endemic regions such as Japan probably represent cutaneous forms of ATL (Amagasaki *et al* 1984, Nakamura *et al* 1993). HTLV-I-infected patients with MF/SS-like diseases in non-endemic regions have subsequently developed ATL-like leukaemia, supporting an aetiopathological classification as ATL (Bunker *et al* 1990, Whittaker *et al* 1993, Fujihara *et al* 1997). Infiltrating neoplastic T cells in the cutaneous form of ATL contain monoclonally integrated HTLV-I provirus and thus may be distinguished from HTLV-I negative MF/SS (Takahashi *et al* 1988, Yamada *et al* 1989b, Dosaka *et al* 1991, Arai *et al* 1994, Fouchard *et al* 1998, Setoyama *et al* 1998). In some cases of smouldering ATL, clonal integration of HTLV-I and rearrangement of the TCR genes may only be found in T cells infiltrating the skin, with no evidence of a neoplastic clone in the peripheral blood (Dosaka *et al* 1991, Hamada *et al* 1992). Rare cases of ATL-like leukaemia do not have evidence of HTLV-I infection (Shimoyama *et al* 1986). Most peripheral T cell and NK cell neoplasms in non-endemic regions are not HTLV-I-associated (Henni *et al* 1990). However, HTLV-I and HTLV-II have still been implicated in a number of cases that conform to the case definition of classical MF/SS (Kaplanski *et al* 1986, Zucker-Franklin *et al* 1991, 1992, Bazarbachi *et al* 1994, Manca *et al* 1994).

In several early studies retrovirus-like particles were observed by EM and RT activity was detected in skin lesions and blood of patients with MF/SS, but it is uncertain if these are representative of true retroviruses (Van der Loo 1979, Füllbrandt *et al* 1983, Slater *et al*

1985). Positive immunostaining for HTLV-I $p19^{Gag}$ has been detected in involved skin and lymph nodes of patients with CTCL but the specificity of this reactivity is uncertain (Turbitt and MacKie 1985).

1.11.1.3 Serology

There is conflicting serological evidence that HTLV-I, HTLV-II or related viruses are involved in MF/SS in non-endemic regions. Although some studies have reported ELISA seroreactivities as less than 1% (Posner *et al* 1981, Gallo *et al* 1983a), others have reported reactivities of 11% (Lange Wantzin *et al* 1986) and 24% (Srivastava *et al* 1990). Reactivities against HTLV-I by WB ELISA have been reported as 15% (Srivastava *et al* 1990) and 86% (Ranki *et al* 1990). Interpretation of these results is confounded by the unknown specificity of the serological assays used, including their ability to discriminate HTLV-I and HTLV-II (Section 1.4.2). One group reported antibodies against HTLV-I Tax in 10% of MF/SS patients who were seronegative for HTLV-I/II structural proteins (Gag and Env) in standard serological assays (Pancake *et al* 1995) (Section 1.11.1.5).

1.11.1.4 Cell culture

Retrovirus-like particles have been detected by EM and RT activity demonstrated in T cell lines propagated from patients with MF/SS (Kaltoft *et al* 1984, 1987, Saal *et al* 1989, Zucker-Franklin *et al* 1991, Bazarbachi *et al* 1994). Reactivity with antiserum against HTLV-I $p19^{Gag}$ was detected in T cell cultures established from involved skin or lymph nodes of three patients with MF using CM and depletion of $CD8^+$ TILs (Kaltoft *et al* 1984). Extracellular C-type retrovirus-like particles could be induced by withdrawal of serum from a T cell line, Se-Ax, derived from a patient with SS, although no budding particles were observed and little or no RT activity was demonstrable in cell culture supernatants (Kaltoft *et al* 1987). There was no evidence of HTLV-I by SB hybridisation (Kaltoft *et al* 1988). C-type retrovirus-like particles were also observed by EM in cultured PBMCs from a French patient with SS and RT activity was detected in culture supernatants (Saal *et al* 1989). The patient did not have detectable serum antibodies against HTLV-I and DNA from PBMCs was negative for HTLV-I by SB hybridisation.

Retrovirus-like particles were detected by EM in 18 cell lines established from PBMCs of 17 patients with MF and one patient with SS using GMCSF and IL2 as growth stimulants (Zucker-Franklin *et al* 1991). Molecular evidence suggested that incomplete or variant HTLV-I was integrated in four cell lines and HTLV-II was integrated in one (Zucker-Franklin *et al* 1992) (Section 1.11.1.5). However, the viral nature of the observed particles was not confirmed, they were not purified and characterised, their infectivity was not demonstrated and RT activity was not reported. HTLV-I *tax* sequences were also detected by PCR in 20 (80%) of 25 T cell lines established from PBMCs of SS patients in the USA, whereas all were negative for HTLV-I *gag* (Abrams *et al* 1991b, Ghosh *et al* 1994) (Section 1.11.1.5).

Retrovirus particles were detected by EM in PBMCs from a SS patient after four months in culture and weak RT activity was detected in culture supernatants, but no HTLV-I-related antigens could be detected (Bazarbachi *et al* 1994). PBMCs from 19 patients with MF/SS

were grown in culture for approximately three months using phytohaemagglutinin (PHA) and IL2 (Capésius *et al* 1991). No retrovirus particles or budding forms were detected in cultured cells by EM and only transient RT activity was detected in culture supernatants, mainly from degenerating cultures. No specific HTLV-I products were amplified by PCR from PBMCs using primers and probes for HTLV-I *gag* and *pol*.

In comparison with the use of molecular techniques on uncultured clinical samples (skin, PBMCs and lymph nodes), there have been fewer indications of the presence of retroviruses in cultured T cells from patients with MF/SS (Section 1.11.1.5). Only two groups have demonstrated the presence of HTLV-I or HTLV-II sequences in cultured T cells from patients with MF/SS (Zucker-Franklin *et al* 1992, Ghosh *et al* 1994).

1.11.1.5 Molecular techniques

HTLV-I or HTLV-II sequences have been identified in DNA from PBMCs, skin and lymph nodes of patients with MF/SS from non-endemic regions and low prevalence populations using PCR, SB hybridisation and ISH (Hall *et al* 1991, Chan *et al* 1993). The proportion of MF/SS cases reported as positive for HTLV-I on the basis of PCR results has been 0% (Capésius *et al* 1991, Lisby *et al* 1992, Bazarbachi *et al* 1993), 1 to 2% (D'Incan *et al* 1992, Lapis *et al* 1992), 7.5% (Whittaker and Luzatto 1993), 34% (Manca *et al* 1994), 45% (Chan *et al* 1993) and 92% (Pancake *et al* 1995). It is difficult to reconcile these widely differing percentages; selected results are detailed here to illustrate the differences among groups working in this field.

Hall *et al* (1991) detected HTLV-I proviral DNA by PCR in cutaneous lesions of five HTLV-I-seronegative Swedish patients with MF using primers for the LTR, *gag*, *pol*, *env*, and *pX* regions. Sequences from all regions were amplified from both PBMCs and cutaneous lesions in one patient with leukaemic stage MF, whereas incomplete provirus was identified in cutaneous lesions in the other four patients with non-leukaemic MF; one had *gag*, *pol*, *env* and *pX*, one had LTR and *gag*, one had *gag* and *pX* and one had *pX* only.

Zucker-Franklin *et al* (1991, 1992) provided evidence for the presence of HTLV-I-related viruses in cultured PBMCs from HTLV-I/II seronegative patients with MF in the USA. PCR and SB hybridisation results suggested that incomplete or variant HTLV-I proviruses were integrated in cultured cells of four of 20 patients with MF (HTLV-I *pol* or *env* sequences amplified by PCR) and HTLV-II was integrated in cultured cells of one case (HTLV-II *pol* sequences amplified) (Section 1.11.1.4). The same group detected HTLV-I *tax* sequences by PCR and RT-PCR in PBMCs from 46 (92%) of 50 MF patients (Pancake *et al* 1995). These patients were seronegative for HTLV-I/II Gag and Env in standard serological assays but five (10%) had antibodies against HTLV-I Tax (Section 1.11.1.3). Two AIDS patients with MF also had HTLV-I *tax* and *pol* sequences amplifiable by PCR from PBMCs and skin (Zucker-Franklin *et al* 1994).

HTLV-I *tax* sequences were amplified by PCR from PBMCs of 18 (72%) of 25 SS patients and three (30%) of 10 skin lesions of MF patients in the USA, whereas no HTLV-I *gag* or HTLV-II *gag* or *tax* sequences were detected (Ghosh *et al* 1994).

HTLV-I *gag* and *pX* PCR products of identical sequence to a reference strain of HTLV-I were amplified from PBMCs of three of four HTLV-I ELISA positive patients with MF/SS in the USA (Srivastava *et al* 1992). Manca *et al* (1994) detected HTLV-I *pol* and *tax* sequences by PCR in 10 (34%) of 29 patients with MF from Italy, confirming these results in a double blind trial after a 6 month interval. HTLV-I *pol*, *env* and *tax* sequences were detected by PCR in one (2%) of 51 HTLV-I/II seronegative patients with CTCL from France (D'Incan *et al* 1992) and HTLV-I *tax* sequences were amplified by PCR from one (5%) of 20 patients with MF in the USA (Lapis *et al* 1992).

The wide range of reported frequencies of detection of HTLV-I-related sequences by PCR in patients with MF/SS suggests that there are differences in methods between groups and possible methodological problems such as PCR contamination. There might also be differences in case definitions and selection of cases for inclusion in different studies. If a retrovirus is involved in CTCL, it might be distantly related to HTLV-I or HTLV-II, there might be deletions or mutations, it might be present in the skin in quantities below the detection limit of PCR or it might have been eliminated from the skin before clinical lesions develop (hit-and-run hypothesis) (Lisby *et al* 1992, Lessin *et al* 1994).

1.11.1.6 Defective viruses

Incomplete or variant HTLV-I-related viruses have been identified in patients with MF/SS on the basis of PCR, variant restriction endonuclease (RE) patterns on SB hybridisation and sequencing (Hall *et al* 1991, Zucker-Franklin *et al* 1991, Srivastava *et al* 1992, Kiss *et al* 1993, Whittaker and Luzzato 1993, Bazarbachi *et al* 1994, Ghosh *et al* 1994, Pancake *et al* 1995). The basis for declaration of incomplete or variant HTLV in samples analysed by PCR has been the amplification of HTLV-I or HTLV-II sequences from some regions of the genome, with other regions of the genome being negative (Section 1.11.1.5). The *pX* region appears to be retained preferentially and *tax* sequences have been detected more frequently than sequences of other genes (Hall *et* al 1991, Zucker-Franklin *et al* 1991, Srivastava *et al* 1992, Ghosh *et al* 1994, Pancake *et al* 1995).

Monoclonally integrated HTLV-I-related proviral sequences were detected by SB hybridisation in DNA from PBMCs of an HTLV-I seronegative patient with SS from France (Kaplanski *et al* 1986). After digestion of DNA with *Eco*RI, which does not cleave within the provirus, RE-digested fragments were smaller (7.5 kb) than expected (9.0 kb) using a full-length HTLV-I probe, indicating integration of incomplete or variant HTLV-I. Seven Hungarian patients with MF had evidence of infection with HTLV-I-related viruses by SB hybridisation and serology (Kiss *et al* 1993). RE digestion suggested differences in the *gag* region of the integrated provirus compared with a reference strain of HTLV-I. A full-length HTLV-I probe hybridised to SBs of DNA from PBMCs of an HTLV-I seronegative French patient with SS had a RE pattern distinct from that of HTLV-I (Bazarbachi *et al* 1994). HTLV-I *pol* and *env* sequences could not be detected by PCR.

Sequences with homology to HTLV-I were identified in DNA from PBMCs and cutaneous lesions of three of 40 HTLV-I-seronegative West Indian patients with MF in the UK by SB

hybridisation using a full-length HTLV-I probe under conditions of high stringency (Whittaker and Luzzato 1993). After *Eco*RI digestion the HTLV-I probe hybridised to an 8 kb DNA fragment in one patient, indicating the presence of incomplete provirus. A 119 bp product amplified from the DNA of this patient by PCR using primers specific for the *pol* region of HTLV-I differed in sequence from the prototype HTLV-I in only one base. HTLV-I could not be detected by PCR in the other two patients using the same HTLV-I *pol*-specific primers. After *Hind*III digestion of DNA from both of these patients, a 3 kb fragment was observed in addition to the expected 2 kb fragment when hybridised to the full-length HTLV-I probe. Whittaker and Luzzato (1993) suggested that these three patients had monoclonally integrated defective HTLV-I provirus.

Monoclonally integrated HTLV-I with a 5.5 kb deletion involving the entire *pol* gene and large portions of *gag* and *env* was demonstrated by hybridisation, RE analysis, cloning and sequencing in an EBV-infected B-LCL established from PBMCs of an HTLV-I-seronegative American patient with SS, as well as in uncultured PBMCs (Hall *et al* 1991). Since the defective virus could not be demonstrated in neoplastic T cells, its pathogenetic role in the patient is uncertain.

Monoclonally integrated HTLV-I-related sequences were identified by SB hybridisation in DNA from a skin lesion of an Italian patient with CTCL (Manzari *et al* 1984). Although a single fragment of approximately 16 kb was detected by SB hybridisation after *Eco*RI digestion, *Bam*HI digestion did not reveal an expected internal 1 kb fragment, suggesting that the provirus differed from a reference strain of HTLV-I by the absence of this restriction site. A putative HTLV-I-related virus, designated HTLV-V, was subsequently identified in eight of 150 patients with MF/SS and one patient with acute T cell leukaemia from Italy whose sera had weak reactivity for HTLV-I by ELISA (Manzari et al 1987). A 5 kb probe used in this study was cloned from a continuous B cell line established from the patient with acute T cell leukaemia and may not represent a relevant viral sequence, since no sequence data have been published. Therefore, the authenticity of HTLV-V is questionable.

Evidence from a number of groups thus indicates that defective genomes of HTLV-I and possibly related viruses are sometimes present in patients with CTCL (Hall *et al* 1991, Whittaker and Luzzato 1993, Pancake *et al* 1995). Preferential retention of the *pX* region in these defective HTLV-I proviruses is evidence that *pX* genes might have a role in the pathogenesis of MF/SS (Hall *et al* 1991, Pancake *et al* 1995). As discussed in Section 1.6.1.4, defective HTLV-I proviruses have been detected in a small proportion of patients with ATL (Konishi *et al* 1984, Korber *et al* 1991, Ohshima *et al* 1991, Kubota *et al* 1995). Infection by HTLV-I or a related virus may be necessary to initiate oncogenesis but there may not be a need to maintain the complete viral genome for maintenance of the transformed phenotype. When defective viruses have been identified in both ATL and HTLV-associated MF/SS there is preferential retention of *pX* sequences, suggesting that continued expression of genes from this region of the virus may be necessary for oncogenesis. Defective BLV proviruses with preferential retention of the *pX* region are also found in leukaemic B cells from cattle with EBL (Kettmann *et al* 1982) (Section 1.9.2.1).

It is possible that recombination and complementation could occur between defective HTLV-I-related retroviral genomes and other exogenous viruses, endogenous retrovirus-like elements or cellular genes. Recombination and complementation leading to enhanced oncogenicity have been demonstrated in avian, feline and murine retroviruses (Weiss *et al* 1973, Hayward and Hanafusa 1975, Stewart *et al* 1986, Stoye *et al* 1991, Sheets *et al* 1993, Golovkina *et al* 1997). HERV sequences with similarity to HTLV-I genes and thus potential for recombination have been identified (Mager and Freeman 1987, Perl *et al* 1989, Fujihara *et al* 1994). HERV-K sequences, although not homologous to HTLVs, express full-length and spliced mRNA transcripts and form virus particles in human germ cell and trophoblastic neoplasms, indicating that not all endogenous retroviral elements are transcriptionally quiescent (Löwer *et al* 1993, Herbst *et al* 1996).

1.11.1.7 Detection of human T lymphotropic viruses in other cutaneous T cell lymphomas

Anagnostopoulos *et al* (1990) detected HTLV-I by PCR using *pol* region primers in five patients with cutaneous $CD30^+$ ALCL from Germany and one patient from Iran. SB hybridisation demonstrated monoclonal integration of HTLV-I in cutaneous lesions in five patients and incomplete provirus in four (Anagnostopoulos *et al* 1990, Detmar *et al* 1991). The Iranian patient was subsequently reclassified as a case of classical MF (Detmar *et al* 1991). Conversely, in 24 patients with cutaneous $CD30^+$ ALCL from Europe and Japan there was no evidence of HTLV-I infection by SB hybridisation or PCR using *pX* primers (MacGrogan *et al* 1996, Wood *et al* 1997). Serum antibodies against HTLV-I have been reported in three patients with angiocentric TCL and HTLV-I *gag* and *pX* sequences were amplified by PCR from lesional DNA of one (McNutt *et al* 1990, Shimokawa *et al* 1993). Insufficient studies have been done to determine whether HTLV-I or related viruses might be involved in cutaneous $CD30^+$ ALCL or angiocentric TCL.

1.11.2 Human T lymphotropic viruses and large granular lymphocytic leukaemia

There is evidence that HTLV-I and HTLV-II are involved in a small proportion of cases of LGL leukaemia. Seroreactivity against HTLV-I/II has been detected in patients with $CD3^+$ LGL leukaemia (Sohn *et al* 1986, Pandolfi *et al* 1987, Starkebaum *et al* 1987, Levitt *et al* 1988, Loughran *et al* 1994a). Familial cases of $CD3^+$ LGL leukaemia have been reported and one case had antibodies against HTLV-I/II Gag but not HTLV-I $p21^{Env}$ (Loughran *et al* 1994a). HTLV-II infection has been demonstrated by PCR in a small proportion of cases (Loughran *et al* 1992, 1994b). Some patients with $CD3^-$ LGL leukaemia have serological cross-reactivity with epitopes of HTLV-I $p21^{Env}$ (Loughran *et al* 1997b, 1998). HTLV-I or HTLV-II have been isolated or detected by PCR in a small proportion of cases of $CD3^-$ LGL leukaemia, although not necessarily in the leukaemic cells (Martin *et al* 1993, Heneine *et al* 1994, Loughran *et al* 1998). A case of $CD2^+$ $CD3^-$ $CD4^+$ $CD5^+$ $CD7^-$ $CD8^-$ $CD16^-$ $CD25^+$ $CD56^-$ $CD57^-$ leukaemia with LGL morphology ("atypical ATL") and monoclonal integration of HTLV-I has been described (Sakamoto *et al* 1994). Other studies have not demonstrated any association between LGL leukaemia and HTLV-I or HTLV-II (Imamura *et al* 1988, Woessner *et al* 1994, Zambello *et al* 1995). A model for LGL leukaemia in HTLV-I *tax*-transgenic mice is discussed in Section 1.9.1.

1.11.3 Other viruses associated with cutaneous T cell lymphomas and large granular lymphocytic leukaemia

1.11.3.1 Herpesviruses

Epstein-Barr virus (EBV) is able to infect and transform T cells in addition to B cells and epithelial cells, since all these cell types can express the EBV receptor CR2 (CD21) (Stevenson *et al* 1986, Fingeroth *et al* 1988, Watry *et al* 1991). EBV appears to be aetiologically involved in most cases of nasopharyngeal/sinonasal angiocentric T cell lymphoma (lethal midline granuloma) (Harabuchi *et al* 1990, Kanavaros *et al* 1993). An EBV-related virus, HV_{MNE}, has been detected in T cell lines and cutaneous lymphoid infiltrates of a non-human primate, the pig-tailed macaque (*Macaca nemestrina*), with MF (Rivadeneira *et al* 1999). Although antibodies against EBV-associated nuclear antigen (EBNA) have been detected at increased frequency in patients with MF/SS, EBV genomes are rarely detected in neoplastic cells and the evidence for an aetiological role for EBV in most primary CTCLs, including MF/SS, is doubtful (Lee *et al* 1990, Dreno *et al* 1994, Kanavaros *et al* 1994, Anagnostopoulos *et al* 1996, Angel *et al* 1996, Jumbou *et al* 1997). Similarly, most primary cutaneous $CD30^+$ ALCLs are negative for EBV, although EBV is associated with some cases of primary systemic $CD30^+$ ALCL (Anagnostopoulos *et al* 1989, Hamilton-Dutoit and Pallesen 1992, Kanavaros *et al* 1992, Peris *et al* 1995, Anagnostopoulos *et al* 1996, Herbst *et al* 1997). There is little indication that EBV has an aetiological role in angioimmunoblastic TCL, Lennert's lymphoma, lymphomatoid papulosis or intestinal TCL (Weiss *et al* 1992, Kadin *et al* 1993, Khan *et al* 1993, Pan *et al* 1993, Anagnostopoulos *et al* 1994).

Patients with LGL leukaemia usually have antibodies against EBV (Loughran *et al* 1993). EBV in clonal episomal form has been demonstrated in leukaemic cells in some cases of $CD3^+$ and $CD3^-$ LGL leukaemia (Kawa-Ha *et al* 1989, Chan *et al* 1992, Hart *et al* 1992). However, other molecular studies have not confirmed an aetiological relationship between EBV and LGL leukaemia (Loughran *et al* 1993, Pellenz *et al* 1996).

Although human herpesvirus (HHV) 7 is a T lymphotropic virus, it has not been definitively associated with neoplasia (Berneman *et al* 1992, 1998). HHV-6 was detected by PCR in skin biopsies of one of 30 patients with CTCL, whereas all samples were negative by PCR for EBV, HSV-1 and HSV-2 (Brice *et al* 1993). Skin biopsies from patients with CTCL and PBMCs from patients with T cell LGL leukaemia were also negative for HHV-8 (KS-associated herpesvirus) by PCR (Dupin *et al* 1997, Henghold *et al* 1997, Loughran *et al* 1997a). Thus, there is insufficient evidence to incriminate herpesviruses other than EBV in TCLs (Nagore *et al* 2000).

1.11.3.2 Human immunodeficiency virus type 1

T cell neoplasms, including MF, have been observed in HIV-1-infected people with AIDS (Crane *et al* 1991, Nahass *et al* 1991, Kerschmann *et al* 1995, Munoz-Perez *et al* 1999). Most are $CD8^+$, possibly a consequence of depletion of the $CD4^+$ T cell population in AIDS. EBV may be involved in the development of cutaneous $CD30^+$ ALCLs in AIDS patients (Dreno *et al* 1993). A T cell lymphoma with monoclonal integration of HIV-1 in neoplastic

cells has been described in an AIDS patient (Herndier *et al* 1992), but most AIDS-associated TCLs can be attributed to immunosuppression.

1.11.3.3 Hepatitis viruses

In an Italian study, some patients with $CD3^-$ LGL leukaemia had serum antibodies against hepatitis B (HBV) and C (HCV) viruses (Zambello *et al* 1995). HCV was not associated with lymphoproliferative disease in humans in France (Hausfater *et al* 2001). A patient with polyclonal proliferation of $CD3^+$ $CD8^+$ LGLs directed against HBV-infected $CD4^+$ T cells has been reported, supporting the hypothesis that LGLs may be proliferating in response to chronic stimulation by viral antigens (Agostini *et al* 1989).

1.12 Associations between viruses and neoplasia

1.12.1 Viruses and human neoplasia

It has been difficult to establish aetiological associations between viruses and neoplasia in humans (Evans and Mueller 1990, Zur Hausen 1991). The identification of EBV as the cause of Burkitt's lymphoma was the first such association to be established (Henle *et* al 1968). The process of discovery leading to the identification of HTLV-I as the cause of ATL (Section 1.6.1.5) further illustrates the difficulties in confirming causality. Only a small proportion of infected people develop ATL and there is a prolonged phase of induction between infection and the occurrence of disease. The process of oncogenesis in ATL appears to be complex, with multiple stages influenced by viral gene expression (Section 1.8) and host factors such as immune status and point mutations or chromosomal translocations involving oncogenes (Section 1.6). Neoplasms such as CTCLs with similar clinicopathological features to ATL may develop in people that do not appear to be infected with HTLV-I. In other neoplasms of humans, for example carcinomas caused by papillomaviruses, different strains of viruses may have different oncogenic potential (Walboomers *et al* 1999). Cofactors are often required for oncogenic progression in virus-induced neoplasia, for example aflatoxins and alcohol in hepatocellular carcinoma caused by HBV (Monto and Wright 2001) and nitrosamines in nasopharyngeal carcinoma caused by EBV (Liebowitz 1994).

1.12.2 Criteria for establishing aetiological associations

The Henle-Koch postulates provide a clear but limited set of criteria for determining whether an infectious agent is the cause of a disease (Rivers 1936). The agent should be found in all cases of the disease and its location in tissues should correspond with the distribution of lesions. The agent should be isolated from lesions and cultivated to purity outside the host; when inoculated back into the host, the cultivated agent should produce identical disease. However, these criteria cannot be applied to viruses causing human neoplasia. Since viruses are propagated in cell cultures, it is difficult to ensure that inocula are free of other agents that could be alternative sole or cooperating causes of neoplasia. In particular, contamination of cell cultures with non-human viruses has been a major source of confusion in the search for viruses involved in human neoplasia (Reitz *et al* 1976, Smith *et al* 1979, Popovic *et al* 1982). The problem of ensuring purity of viruses has only been overcome by

the use of infectious full-length molecular clones (Kimata *et al* 1994b, Zhao *et al* 1995, Derse *et al* 1997, Robek and Ratner 1999).

An alternative set of criteria for the association of viruses with neoplasia was proposed by Evans (1976) and are developed here. Evidence of exposure to the virus should be demonstrated by detection of infection using virus isolation, molecular assays, antigen assays or tests for other viral markers including serological or cell-mediated immune responses (Section 1.4). The association of a particular virus with a specific neoplasm should be consistent and repeatable by different researchers in different places at different times. Infection should precede the development of neoplasia. However, initial infections with many oncogenic viruses are subclinical and therefore the time of exposure may be difficult to establish. There may be a dose response, with an increased frequency or earlier onset of neoplasia associated with higher levels of infection. Viral markers should be more prominent (higher antibody titres, higher concentration of circulating virus or higher antigenaemia) in cases with neoplasia than in matched controls. It should be possible to demonstrate infection in the neoplasm by virus isolation or molecular, immunological or ultrastructural techniques. However, not all viruses that initiate neoplasia may remain in the affected tissue (hit-and-run mechanism of viral oncogenesis) (Skinner 1976, Smith and Campo 1988, Shen *et al* 1997, Nevels *et al* 2001) or viruses may induce neoplasia indirectly, for example lymphoid neoplasia associated with HIV-1-induced immunosuppression in AIDS patients (Cremer *et al* 1990, Schulz *et al* 1996, Demario and Liebowitz 1998, Smith *et al* 1998).

An apparent association between a virus and a neoplasm would be considered more biologically plausible if the virus is from a taxonomic group that has previously been associated with a similar type of neoplasm in the same or related species (Fredricks and Relman 1996). Oncogenic viruses isolated from humans with neoplasia should be able to transform cells *in vitro*. They may have the capacity to induce comparable neoplasms when inoculated into susceptible non-human animals. Experimental manipulation of the virus or its host may alter the expression of transforming properties. However, suitable *in vitro* and non-human animal models are not always available because of the difficulty in reproducing neoplasia in alternative hosts with human viruses (Section 1.9). Prevention of infection with a candidate oncogenic virus by vaccination or other prophylactic interventions should be associated with a decrease in the incidence of the neoplasm. T cell neoplasia in domestic fowl caused by Marek's disease virus (MDV) can be prevented by vaccination with herpesvirus of turkeys or low virulence strains of MDV (Churchill *et al* 1969, Okazaki *et al* 1970). Anti-viral therapy might increase the frequency or time to development of neoplasia in infected people or cause regression of neoplasms that have already developed.

Many human oncogenic viruses, for example HTLV-I, HHV-8, HBV, HCV and the human papillomaviruses (HPVs), proved difficult to isolate until the development of specialised cell culture systems and there are likely to be other human viruses that have not been identified. Increasingly, viruses are being identified and characterised by molecular techniques before they have been cultivated *in vitro* (Fredricks and Relman 1996, Gao and Moore 1996). A λ bacteriophage cDNA expression library was screened with antiserum from patients with non-A, non-B hepatitis to detect sequences of HCV, an RNA virus associated with hepatocellular carcinoma (Young and Davis 1983, Choo *et al* 1989). HHV-8 was discovered by

amplification of viral sequences from KS, a skin tumour associated with AIDS, using representational difference analysis (RDA) (Lisitsyn *et al* 1993, Chang *et al* 1994). Consensus PCR using group-specific primers has been used to amplify sequences of previously unknown HPV genotypes from patients with cervical neoplasia (Van den Brule *et al* 1992). Novel herpesviruses have been detected by degenerate and consensus PCR in B cell lymphomas of common marmosets (*Callithrix jacchus*) (Ramer *et al* 2000), fibropapillomas of green turtles (*Chelonia mydas*) (Quackenbush *et al* 1998) and genital carcinomas of California sea lions (*Zalophus californianus*) (Lipscomb *et al* 2000).

Fredricks and Reiman (1996) outlined guidelines for establishing an aetiological association between a microorganism and disease based on molecular evidence in the absence of cultivation of the putative pathogen. These guidelines can be applied to novel virus-like sequences and neoplasia. Specific putative viral nucleic acid sequences should be present in neoplastic cells, although this may not apply to hit-and-run agents or those that act indirectly. There should be a lower frequency of detection, a lower copy number or absence of the specific virus-like sequences in unaffected tissues or in regressing neoplasms. Sequences might be detectable in samples that precede the development of neoplasia, indicating prior infection. The taxonomic group to which the putative virus belongs, inferred by sequence analysis, should be one that has known oncogenic members. The type of neoplasm being associated with specific novel virus-like sequences should be consistent with the known biological properties of members of that group. When assessing the significance of retroviral sequences detected in neoplasia, the potential for amplification of endogenous retrovirus-like sequences should be considered, particularly when using primers from regions such as *pol* where there is a high degree of similarity between endogenous and exogenous sequences. In neoplasia induced by retroviruses, each neoplastic cell would be expected to carry at least one integrated provirus but there is also the possibility that cells might lose part or all of the provirus following neoplastic transformation.

1.12.3 Epidemiological studies

Epidemiological criteria are also important in determining whether associations between viruses and neoplasia are likely to be causal (Evans 1976). The geographical distribution of infection with a virus should be similar to that of the neoplasm with which it is associated, adjusting for age of infection and presence of cofactors. The correlation between the distribution of HTLV-I infection and the occurrence of ATL was the most important factor in identifying a causal association. Even when a virus and a neoplasm have a similar geographical distribution, there is still a need for well-designed epidemiological studies. Not all infected people may develop the neoplasm; with viruses such as HTLV-I neoplasia may develop in only a small proportion of infected people and there may be a long delay between infection and development of the neoplasm. Viruses may not be responsible for all cases of a particular neoplasm if there is multifactorial causation. In these cases, it is necessary to study the degree of association between disease and hypothesised causal factors by measuring relative risks or odds ratios in cross-sectional, case-control and cohort studies. A significantly higher proportion of people infected with a candidate oncogenic virus or with specific viral markers (Section 1.4) should develop neoplasia than uninfected people or those without such markers.

1.12.4 Involvement of HTLV-I in cutaneous T cell lymphomas and large granular lymphocytic leukaemia

Evidence for the involvement of HTLV-I or related viruses in MF/SS, other CTCLs and LGL leukaemia is presented in Section 1.11. Seropositivity for HTLV-I structural and regulatory proteins has been demonstrated in many cases. A number of studies have reported detection of HTLV-I sequences by PCR and SB hybridisation in PBMCs, neoplastic infiltrates and cell lines derived from affected patients. There is some evidence that defective HTLV-I or HTLV-II or variant proviruses are integrated in neoplastic cells of some cases of MF/SS and other CTCLs, with preferential retention of the *pX* region. However, the discrepancy in reported frequencies of detection of HTLV-I or HTLV-II markers in patients with these neoplasms must result in serious doubts as to the veracity of many studies. Methodological problems such as PCR contamination have to be excluded before positive results can be accepted. Concerns about previous studies have highlighted the need to establish stringent criteria for determining whether HTLV-I, HTLV-II or related viruses are aetiologically involved in CTCLs and LGL leukaemia.

Clear case definitions for MF/SS and other CTCLs must be established in order to distinguish these T cell neoplasms from ATL and similarly the diagnostic criteria for LGL leukaemia must be well-defined. It is then necessary to obtain unambiguous evidence that patients with non-ATL CTCLs and LGL leukaemia are infected with HTLV-I, HTLV-II or related retroviruses. Isolation and propagation of infectious virus from affected patients would be the most definitive form of evidence, particularly if the virus is shown to be a new species of retrovirus or a distinct strain of HTLV-I or HTLV-II based on sequence information and detailed virus characterisation. Since retroviruses establish persistent infections, patients with neoplasms caused by defective integrated proviruses would be expected to be co-infected with intact infectious virus that can be isolated from PBMCs or other non-neoplastic cells. It is conceivable that cells containing intact proviruses could be eliminated by the host immune response, leaving only cells infected by defective viruses that fail to express antigens. In these cases it would be necessary to rely on molecular techniques that detect the remnant sequences. Stringent precautions would have to be taken to eliminate contamination by PCR products. The sequences of amplified products would have to be determined. An additional level of proof would be to accept results only when sequences of PCR products differ by statistically significant percentages from prototypical strains of HTLV-I and HTLV-II, particularly strains that might be handled by the same group in the same laboratory and used as positive controls. Since the level of sequence variation among HTLV-I isolates is low, criteria for interpretation of sequence results might be different to that of more variable viruses such as HIV-1. Sequences should be reproducible when obtained from different PCR products from the same and possibly different patients at different times. Only the study by Manca *et al* (1994) has so far met these criteria for MF/SS.

Studies conducted at the LRF Virus Centre from 1992 to 1995 to search for HTLV-I-related viruses in MF/SS, LGL leukaemia and other neoplasms are described in Chapters 2 to 4 of this thesis. The results of this work are discussed in Chapter 5 and compared with the findings of other studies.

Chapter 2

Detection of Retroviruses in Human T Cell Leukaemias and Lymphomas by Cell Culture

2.1 Introduction

2.1.1 Approach to isolation of retroviruses from T cell neoplasms

2.1.1.1 Role of human T lymphotropic retroviruses in T cell neoplasia

Human T lymphotropic virus type I (HTLV-I) is the cause of adult T cell leukaemia/lymphoma (ATL), a neoplasm of $CD4^+$ T cells (Section 1.6.1). HTLV-II has been isolated from cases of $CD8^+$ T cell variant hairy cell leukaemia (HCL) but has not been definitively associated with neoplasia (Section 1.7.1). The related retrovirus bovine leukaemia virus (BLV) causes B cell leukaemia and lymphoma in cattle (Section 1.9.2.1). Retroviral aetiologies have been proposed for a variety of diseases of humans for which the cause is currently unknown, including T and B cell lymphoid neoplasms (Sections 1.10.5 and 1.11) and autoimmune diseases (Section 1.6.4). The hypothesis tested in this study was that HTLV-I, HTLV-II, BLV or related viruses are associated with other T cell leukaemias and lymphomas. Combinations of cell and molecular biology techniques were used to search for retroviruses in patients with these neoplasms.

2.1.1.2 Spectrum of clinical diseases investigated

The emphasis in this study was on cutaneous T cell lymphomas (CTCLs), including mycosis fungoides (MF) and its leukaemic variant Sézary syndrome (SS). These neoplasms have similar clinicopathological features to some forms of ATL and have been the subject of investigations by many groups in an attempt to confirm a retroviral aetiology (Section 1.11.1). Cases of large granular lymphocytic (LGL) leukaemia were examined for the presence of HTLV-related retroviruses because of reports associating this T or natural killer (NK) cell lymphoproliferative disorder with HTLV-I and HTLV-II (Section 1.11.2). Samples from a cluster of adult acute lymphoblastic leukaemia (ALL) cases in butchers and abattoir workers were also examined for the presence of HTLV-I, HTLV-II and BLV, the latter virus because of occupational exposure to cattle (Whittaker 1991) (Sections 1.9.2.1 and 1.10.5).

2.1.1.3 Outline of cell culture approach

The aim of the cell biology section of this project was to isolate retroviruses from human T cell neoplasms by cultivating T cells from peripheral blood mononuclear cells (PBMCs), skin and lymph nodes of affected patients. Cultivated T cells would act as host cells for the propagation of T lymphotropic retroviruses. Protocols for the long-term cultivation of T cells from patients with T cell neoplasia and patients with retroviral infections were designed.

Techniques for the establishment of cultures included stimulation with concanavalin A (ConA) or phytohaemagglutinin (PHA) then interleukin (IL) 2 (Gazdar *et al* 1980, Poiesz *et al* 1980b, Ho *et al* 1990), IL2 alone, IL2 in combination with IL4 (Uchiyama *et al* 1988, Kaltoft *et al* 1992, Mori *et al* 1993b), IL7 (Dalloul *et al* 1992) and granulocyte-macrophage colony stimulating factor (GMCSF) (Zucker-Franklin *et al* 1991). Conditioned medium (CM) was prepared by collecting cell culture supernatants from PHA-stimulated umbilical cord blood mononuclear cells (UCMCs), providing a source of T cell growth factor (TCGF) (Poiesz *et al* 1980a, Hoshino *et al* 1983). TCGF is an heterogeneous medium containing IL2 and other soluble growth factors (Morgan *et al* 1976, Ruscetti *et al* 1977, Gazdar *et al* 1979, 1980, Mier and Gallo 1980). In addition, CM was prepared by ConA stimulation of PBMCs from a patient with SS, producing Sézary T cell activating factor (SAF) CM (Abrams *et al* 1991a, b).

Lymphocytes from patients with T cell neoplasia were also cocultivated with UCMCs that would act as recipients for infection with retroviruses released from primary neoplastic cells or other infected cells from the same patient (Miyoshi *et al* 1981b, Markham *et al* 1983, Popovic *et al* 1983). Cultured cells were examined for evidence of retroviruses by electron microscopy (EM) and cell pellets were collected for analysis by the polymerase chain reaction (PCR) (Chapter 3). Culture supernatants were collected for detection of reverse transcriptase (RT) activity. The immunophenotypes of cells from long-term cultures were determined by flow cytometry.

In this chapter the origins and properties of T cell lines established from patients with ATL (Section 2.1.2) and CTCLs (Section 2.1.3) are reviewed to provide a background to the approaches used at the Leukaemia Research Fund (LRF) Virus Centre to establish similar continuous T cell lines. The sources of clinical samples are described in Section 2.2. Techniques for the cultivation of lymphocytes from clinical samples are described in Section 2.3.5 and 2.3.6. All methods relating to collection of samples for subsequent molecular studies are also included in this chapter. The results of investigations for evidence of retroviruses in cultured T cells are described in Section 2.4.

2.1.2 Establishment of adult T cell leukaemia/lymphoma cell lines

2.1.2.1 ATL cell lines and isolation of HTLV-I and HTLV-II

The discovery of HTLV-I and HTLV-II is described in Section 1.3.2.1. HTLV-I (type isolate $HTLV_{CR}$) was first detected in two $CD4^+$ T cell lines established from a patient with a form of ATL initially diagnosed as MF (Gazdar *et al* 1979, 1980, Poiesz *et al* 1980a, b). These cell lines were HUT102, derived from lymph node cells, and CTCL-3, established from PBMCs. A second isolate ($HTLV_{MB}$) was obtained from a $CD4^+$ T cell line (CTCL-2) derived from PBMCs of another patient with a form of ATL initially diagnosed as SS (Poiesz *et al* 1981). HTLV-I (ATLV) was also identified in $CD4^+$ T cell lines established from patients with ATL in Japan by direct cultivation of PBMCs (cell line MT1) (Miyoshi *et al* 1979a, 1980, Hinuma *et al* 1981) and by cocultivation of UCMCs with ATL cells (cell line MT2) (Miyoshi *et al* 1981a, b). Numerous other $CD4^+$ T cell lines have been established subsequently from ATL patients (Gootenberg *et al* 1981, Hinuma *et al* 1982a, Yoshida *et al* 1982, Hoshino *et al* 1983, Markham *et al* 1983, Sugamura *et al* 1984a) (Sections 1.3.2 and 1.4.1).

In most cases the cell lines established from PBMCs of patients with ATL are not derived from the neoplastic clone but represent non-leukaemic cells infected *in vitro* (Miyoshi *et al* 1981a, Nowell *et al* 1984, Maeda *et al* 1985, 1987). HTLV-II ($HTLV_{MO}$) was first isolated from a $CD8^+$ T cell line derived from a patient with a T cell variant of HCL (Saxon *et al* 1978a, b, Kalyanaraman *et al* 1982b). The key to identifying these viruses was the establishment of $CD4^+$ or $CD8^+$ T cell lines from patients with neoplasia and such an approach continues to be important in attempts to isolate new human T lymphotropic viruses.

2.1.2.2 Methods for establishing HTLV-I-infected T cell lines from ATL patients

HTLV-I-infected T cell lines have been established from ATL patients by stimulation of PBMCs with ConA or PHA and continued cultivation in the presence of CM (TCGF) or IL2 (Gazdar *et al* 1979, 1980, Poiesz *et al* 1980a, b, Gootenberg *et al* 1981, Poiesz *et al* 1981, Hinuma *et al* 1982a, Hoshino *et al* 1983, Sugamura *et al* 1984a, Maeda *et al* 1987). Activation of T cells by ConA or PHA increases the efficiency of infection and transformation (Merl *et al* 1984). CM and IL2 may be withdrawn as cell lines attain IL2 independence (Markham *et al* 1983, Yssel *et al* 1989, Höllsberg *et al* 1992, Rohwer *et al* 1994) (Section 1.3.2.3). Cultured neoplastic T cells containing integrated defective HTLV-I from a patient with $CD8^+$ ATL also proliferated in response to IL2; these cells were shown to express the IL2 receptor (IL2R) (Matsushita *et al* 1994).

Occasionally, HTLV-I-infected T cell lines have been established from ATL patients without the use of stimulants (Katoh *et al* 1986). Cocultivation of PBMCs from ATL patients with UCMCs is a frequently used method for establishing HTLV-I-infected T cell lines (Miyoshi *et al* 1981a, b, Yoshida *et al* 1982, Markham *et al* 1983, Popovic *et al* 1983, Hjelle *et al* 1992b).

2.1.2.3 Proliferative responses of ATL cells

ATL-derived neoplastic T cells proliferate in response to IL2, IL4, IL7 and IL15 (Uchiyama *et al* 1988, Umadome *et al* 1988, Mori *et al* 1993b, Persaud *et al* 1995, Yamada *et al* 1998). The receptors for these cytokines share the common γ (γ_c) chain and this component may be a mutual element in the signalling pathway for stimulation of HTLV-I-infected T cells by these exogenous cytokines during the process of transformation (Persaud *et al* 1995) (Section 1.8.1.1). IL4 and IL7 can substitute for IL2 in immortalising some HTLV-I-infected T cells (Uchiyama *et al* 1988, Umadome *et al* 1988, Mori *et al* 1993b, Persaud *et al* 1995, Asadullah *et al* 1996b, Mastino *et al* 1997).

ATL cells also proliferate in response to GMCSF (Matsushita *et al* 1997). The receptors for GMCSF, IL3 and IL5 share a common β (β_C) receptor but the responses of ATL cells to IL3 and IL5 have not been studied in detail (Guthridge *et al* 1998).

2.1.2.4 Infection of T and B cell lines with HTLV-I and herpesviruses

Epstein-Barr virus (EBV)-infected B lymphoblastoid cell lines (B-LCLs) coinfected with HTLV-I are occasionally established from patients with ATL (Yamamoto *et al* 1982a, Hirose *et al* 1984, Koyanagi *et al* 1984). These cell lines express IL2R and proliferate in response to IL2 (Sugamura *et al* 1984b). Since HTLV-I Tax up-regulates complement receptor 2 (CD21), the receptor for EBV (Section 1.8.1.2), HTLV-I-infected T cell lines established from patients with ATL could also be susceptible to coinfection with EBV (Koizumi *et al* 1992, Kuraya *et al* 1995, Ohtsubo *et al* 1997). However, this appears to be an infrequent event (Ohtsubo *et al* 1997, 1999).

2.1.2.5 Infection of T cell lines with other T lymphotropic retroviruses

Cultivation of T cells from patients with T cell neoplasia might occasionally result in propagation of other human T lymphotropic viruses, such as HIV-1, HIV-2, human herpesvirus (HHV) 6 and HHV-7 (Barré-Sinoussi *et al* 1983, Levy *et al* 1984, Popovic *et al* 1984, Clavel *et al* 1986, Salahuddin *et al* 1986, Downing *et al* 1987, Tedder *et al* 1987, Frenkel *et al* 1990, Berneman *et al* 1992a, Zhou *et al* 1999).

2.1.3 Cultivation of T cells from patients with cutaneous T cell lymphomas

2.1.3.1 T cell lines established from patients with mycosis fungoides/Sézary syndrome

The growth requirements of representative MF/Sézary T cell lines are summarised in Table 2.1. MF/SS-derived $CD4^+$ T cell lines that do not require stimulants for growth include Hut78 (Gazdar *et al* 1980, Poiesz *et al* 1980a, b), HH (Starkebaum *et al* 1991) and L-726A (Crae *et al* 1991). L-726A was established at the LRF Virus Centre from skin lesions of a patient with SS. TCGF (IL2)-dependent $CD4^+$ MF/Sézary T cell lines include two cell lines from skin and one from a lymph node of patients with MF (Kaltoft *et al* 1984), the Se-Ax cell line cultivated from PBMCs of a patient with SS (Kaltoft *et al* 1987), a T cell line from PBMCs of a patient with CTCL established by Kadin *et al* (1988), nine MF/Sézary cell lines (SZ series) from PBMCs of patients with SS (Abrams *et al* 1991a, b) and a T cell line from a patient with CTCL established by Boehncke *et al* (1993). The $CD4^+$ T cell line My-La, from the skin of a patient with MF (Kaltoft *et al* 1992), and two T cell lines established from patients with CTCL by Boehncke *et al* (1993) required IL2 and IL4. Another T cell line established from a CTCL patient by Boehncke *et al* (1993) was dependent on IL4 alone. Three IL7-dependent T cell lines (CHA, BEL and RIC) were established from MF/SS patients by Dalloul *et al* (1992).

In most cases T cell clones cultured from MF lesions are not derived from neoplastic MF/Sézary cells but arise from tumour infiltrating lymphocytes (TILs) (Ho *et al* 1990, Harwix *et al* 2001).

Table 2.1: Growth requirements of representative HTLV-I and HTLV-II-negative $CD4^+$ T cell lines established from patients with mycosis fungoides/Sézary syndrome.

Cell line	T cell neoplasm	Source of cells	Reference
Independent of exogenous growth stimulants			
Hut78	CTCL	PBMCs	Gazdar *et al* 1980
L-726A	SS	Skin	Crae *et al* 1991
HH	CTCL	PBMCs	Starkebaum *et al* 1991
Interleukin 2-dependent			
Se-Ax	SS	PBMCs	Kaltoft *et al* 1987
SZ-4	SS	PBMCs	Abrams *et al* 1991a, b
Interleukin 2 and interleukin 4-dependent			
My-La	MF	Skin	Kaltoft *et al* 1992
Interleukin 7-dependent			
CHA	SS	PBMCs	Dalloul *et al* 1992

CTCL Cutaneous T cell lymphoma
MF Mycosis fungoides
SS Sézary syndrome
PBMCs Peripheral blood mononuclear cells

2.1.3.2 T cell lines established from patients with other T cell lymphomas

Selected $CD4^+$ T cell lines established from patients with T cell lymphomas (TCLs) other than MF/SS are listed in Table 2.2. These cell lines are not dependent on growth factors and do not appear to be infected with HTLV-I or HTLV-II. As discussed above, a number of T cell lines established from patients initially diagnosed with MF or SS (for example HUT102, CTCL-2, CTC-16 and Sez 627), were found to be infected with HTLV-I and these patients would be regarded as having ATL (Poiesz *et al* 1980b, Uchiyama *et al* 1980, Poiesz *et al* 1981, Reitz *et al* 1983, Namiuchi *et al* 1986, Detmar *et al* 1991). Zucker-Franklin *et al* (1991) provided equivocal evidence for HTLV-related viruses in cell lines established from PBMCs of 17 patients with MF and one patient with SS using GMCSF and IL2 as growth stimulants (Section 1.11.1.4). An IL2-dependent $CD4^+$ T cell line WHN2 has been established from a patient with HTLV-I-negative leukaemia resembling ATL (Kagami *et al* 1993).

2.1.3.3 Methods for establishing T cell lines from patients with mycosis fungoides/Sézary syndrome

$CD4^+$ T cell lines have been established from patients with MF/SS and other CTCLs by *in vitro* propagation of lymphocytes extracted from skin, lymph nodes or blood. To establish these cell lines, growth of T cells has been stimulated with mitogens such as ConA or PHA, along with various combinations of IL2, IL4, IL7 and GMCSF (Gazdar *et al* 1980, Ho *et al* 1990, Abrams *et al* 1991a, Starkebaum *et al* 1991, Zucker-Franklin *et al* 1991, Dalloul *et al* 1992, Kaltoft *et al* 1992, Boehncke *et al* 1993).

CM prepared from PHA-stimulated PBMCs or UCMCs has been used to provide a source of TCGF (Mier and Gallo 1980, Poiesz *et al* 1980b, Kaltoft *et al* 1984, 1987). SAF CM produced by ConA-stimulated PBMCs from a patient with SS was used to establish T cell lines from other patients with MF/SS (Abrams *et al* 1991a, b). MF/SS patient-derived lymphocytes have also been cocultivated with UCMCs and other cells (Detmar *et al* 1991). Kaltoft *et al* (1984) found that depletion of $CD8^+$ T cells (TILs) permitted outgrowth of neoplastic $CD4^+$ T cell clones from skin-derived lymphocyte cultures. Adaptations of these methods to the cell culture studies at the LRF Virus Centre are described in more detail in Section 2.3.6.

2.1.3.4 Proliferative responses of mycosis fungoides/Sézary cells

The *in vitro* proliferative responses of PBMCs and skin-derived lymphocytes to mitogens are reduced in most patients with MF/SS (Burg *et al* 1978, Golstein *et al* 1986, Ho *et al* 1990, Wood *et al* 1990b) (Section 1.10.1.3). Although MF/Sézary cells usually express the T cell receptor (TCR)/CD3 complex, they can more readily be activated through antigen-independent pathways such as those involving CDw60, CD2, and CD28 (Hansen *et al* 1993). Proliferative responses have been demonstrated to IL2 alone or in combination with IL4, IL7 and GMCSF (Kaltoft *et al* 1987, Zucker-Franklin *et al* 1991, Dalloul *et al* 1992, Kaltoft *et al* 1992, Foss *et al* 1994), as well as to CM (TCGF) with IL2 activity (Poiesz *et al* 1980a, Kaltoft *et al* 1984, Abrams *et al* 1991b, 1993). In some studies MF/Sézary cells have proliferated strongly in response to IL7 and IL15 and less strongly to IL2 and IL4 (Kaltoft *et al* 1987, Dalloul *et al* 1992, Kaltoft *et al* 1992, Foss *et al* 1994, Döbbeling *et al* 1998).

Table 2.2: HTLV-I and HTLV-II-negative $CD4^+$ T cell lines established from patients with T cell leukaemias and lymphomas other than mycosis fungoides/Sézary syndrome.

Cell line	T cell neoplasm	Reference
CCRF-CEM	T cell ALL	Foley *et al* 1965
MOLT-3	T cell ALL	Minowada *et al* 1972
MOLT-4	T cell ALL	Minowada *et al* 1972
H-SB2	T cell ALL	Royston *et al* 1974
JURKAT (JM)	T cell ALL	Schneider *et al* 1977
RPMI 8402	T cell ALL	Srivastava *et al* 1975
TALL-1	T cell ALL	Hiraki *et al* 1978
HPB-ALL	T cell ALL	Morikawa *et al* 1978
SUP-T3	T cell ALL	Smith *et al* 1984
Kit 225	T cell CLL	Hori *et al* 1987
HPB-MLT	T cell lymphoma	Morikawa *et al* 1978
SUP-T1	T cell lymphoma	Smith *et al* 1984
IARC 301	T cell lymphoma	Duprez *et al* 1985
HPB-MLp-W	T cell lymphoma	Morikawa *et al* 1991

ALL Acute lymphoblastic leukaemia
CLL Chronic lymphoblastic leukaemia

Cytokines produced by MF/Sézary cells and other cells in MF lesions are listed in Section 1.10.1.5. Although many MF/SS-derived T cell lines express IL2Rα (CD25) and require IL2 for growth, most MF/Sézary cells in lesions do not express CD25 (Boehnke *et al* 1993) (Section 1.10.1.3). Therefore, any response to IL2 *in vivo* is likely to be mediated by a less strongly responsive receptor complex composed of the β_c (CD122) and γ_c (CD132) subunits of the IL2R.

The use of TCGF and IL2 for cultivation of primary T cells from skin lesions or PBMCs of patients with MF/SS could select for T cells that express the high affinity IL2R but are not derived from the neoplastic clone. Mitogen-activated PBMCs from SS patients produce SAF, a 28 kDa protein that induces IL2Rα on MF/Sézary cells, rendering them responsive to IL2 (Abrams *et al* 1993). This autocrine loop of stimulation could account for the successful propagation of the SZ series of T cell clones from patients with SS by Abrams *et al* (1991b).

As discussed above, some MF/SS-derived T cell lines require IL4 alone or in combination with IL2 for growth (Kaltoft *et al* 1992, Boehncke *et al* 1993). IL4 expression is low in early MF lesions, although it may increase in later stage MF (Vowels *et al* 1994, Asadullah *et al* 1996a).

IL7 and IL7 receptor (IL7R) α (CD127) are expressed by a proportion of MF/Sézary cells in cutaneous lesions (Foss *et al* 1994, Asadullah *et al* 1996b, Bagot *et al* 1996, Döbbeling *et al* 1998). IL7 produced by keratinocytes or MF/Sézary cells could act in an autocrine or paracrine fashion to induce expression of IL7Rα and IL2Rα on MF/Sézary cells (Dalloul *et al* 1992, Heufler *et al* 1993, Foss *et al* 1994, Bagot *et al* 1996). The presence of such a stimulatory pathway would be consistent with the establishment of IL7-dependent T cell lines from some MF/SS patients (Dalloul *et al* 1992, Foss *et al* 1994, Döbbeling *et al* 1998). However, it has been difficult to demonstrate a role for IL7 in maintaining proliferation of MF/Sézary cells *in vivo* (Asadullah *et al* 1996b).

IL15 prolongs the survival of MF/Sézary cells and is a growth factor for some MF/SS cell lines; IL15 mRNA is expressed by PBMCs, basal cell layer keratinocytes and T cells infiltrating the skin in MF/SS patients (Döbbeling *et al* 1998). These observations strengthen the premise that the γ_c chain of the receptors for IL2, IL4, IL7 and IL15 plays an important role in maintaining proliferation of MF/Sézary cells *in vivo*.

2.1.4 Cell lines derived from patients with LGL leukaemia

Although no fresh PBMCs were submitted for culture from patients with LGL leukaemia in this study, a brief description of established NK or T cell lines derived from affected patients is given here. Five $CD3^+$ $CD8^+$, one $CD3^+$ $CD8^-$ and one $CD3^-$ $CD8^-$ IL2-dependent LGL cell lines were established using PHA and IL2 as growth stimulants (Pistoia *et al* 1986). Three $CD3^+$ $CD8^+$ LGL cell lines were established using IL2 (Loughran *et al* 1988b). TKS-1, a $CD3^-$ $CD56^-$ cell line with LGL morphology, was derived from a patient with $CD3^-$ $CD56^+$ LGL leukaemia using IL2 (Kojima *et al* 1994). NK-92 is an IL2-dependent $CD3^-$ $CD7^+$ $CD56^+$ NK LGL cell line that expresses the IL2Rα (CD25) (Gong *et al* 1994).

Neoplastic LGL cells proliferate in response to IL2 *in vitro* (Koizumi *et al* 1986). NK-YS is an EBV-infected NK cell line established from a nasal angiocentric NK cell lymphoma with LGL morphology by cocultivation with a murine stromal cell line in the presence of IL9 (Tsuchiyama *et al* 1998). The requirements for establishment of T cell LGL cell lines thus appear to be similar to those for other T cell lines.

2.1.5 Proliferation of normal T cells in culture

2.1.5.1 *In vitro* activation and proliferation of normal T cells

T cells are normally activated through binding of specific antigens to the TCR (Minden and Mak 1986, Davis and Bjorkman 1988). The lectins ConA and PHA are non-specific (polyclonal) activators of T cells that bind to the TCR-CD3 complex (Palacios 1982, Chilson and Kelly-Chilson 1989). Monoclonal antibodies (MAbs) against accessory cell surface molecules such as CD2, CD3, CD28 and CDw60 will also activate T cells (Meuer *et al* 1984, Hansen *et al* 1993). T cells from healthy people will not respond to IL2 unless activated. Resting T cells express the low affinity β_c and γ_c subunits of the IL2R but have limited expression of the high affinity α subunit (CD25) (Minami *et al* 1993). In addition, the amount of IL2 secreted by resting T cells is very low. Following activation, T cells transiently produce IL2 and express higher levels of IL2Rα, which combines with the β_c and γ_c subunits to form the high affinity IL2R complex, allowing the cells to respond strongly to IL2. Activated T cells will proliferate for 1 to 2 days by this IL2-IL2R autocrine mechanism but will not usually persist in culture, even in the continued presence of exogenous IL2, and rarely become immortalised spontaneously (Section 2.1.5.2). In contrast, T cells from patients with some T cell leukaemias and lymphomas, including HTLV-I infected patients with ATL, often respond directly to IL2 and are more readily immortalised (Section 2.1.2.3). There are differences in the *in vitro* proliferative properties of helper (Th) and suppressor (Ts) T cells (Taylor *et al* 1987). Th cells are able to produce IL2 whereas Ts cells require an exogenous source to maintain viability.

The use of cytokines such as IL2, IL4, IL7 and IL15 to stimulate the growth of MF/Sézary cells in culture has been described in Section 2.1.3.2. These four cytokines are important in preventing the death of activated normal T cells *in vitro*, similar to their effects on MF/Sézary cells, although IL4 and IL15 are more effective than IL2 or IL7 (Kaltoft 1998, Vella *et al* 1998). Many other cytokines, such as IL6, IL9, IL12 and interferon (IFN) γ, are involved in growth and differentiation of normal T cells (Lanzavecchia and Sallusto 2000).

2.1.5.2 Longevity of normal T cells *in vitro*

The longevity of normal human T cells in culture is limited but varies according to the type of medium and conditions of cultivation (Perillo *et al* 1989). In standard cell cultures, unstimulated primary T cells derived from PBMCs usually survive for 20 to 30 days in the absence of exogenous growth stimulants before undergoing death by apoptosis. Activated T cells proliferate more strongly and will persist in culture for longer; death of most or all members of the population ("crisis") usually occurs at 30 to 60 days. Under such conditions,

normal human T cells are able to undergo 50 ± 10 population doublings before their proliferative capacity is lost, similar to human fibroblasts (Hayflick 1965, Houck *et al* 1971, Perillo *et al* 1989, Goletz *et al* 1994).

2.1.5.3 Role of the cellular environment in maintaining proliferation of T cells

The compartments of the body in which neoplastic cells proliferate in MF/SS are complex and dynamic microenvironments. Cutaneous lesions in MF contain a diverse range of cells, including keratinocytes, fibroblasts, Langerhan's cells, interdigitating cells, melanocytes and TILs, in addition to MF/Sézary cells. Cell-cell contact and secretion of cytokines by these cells is likely to be important in maintaining survival and growth of neoplastic T cells (Section 1.10.1.5). Antigen presenting cells (APCs) are particularly important in activating T cells *in vivo* (Johnson and Jenkins 1993). T cell-T cell interactions are also significant and can result in activation through costimulatory pathways such as those mediated by CD28-B7 and CD40-CD40 ligand (Grewal and Flavell 1996, Greenfield *et al* 1998). However, the limited lifespan of T cells *in vitro* suggests that these interactions cannot maintain the viability of T cell populations in the absence of other proliferative signals. Maintaining the survival of T cells *in vitro* in the absence of these supporting elements is one of the challenges in attempts to establish continuous T cell lines.

The presence of mixed cell populations in cultures of lymphocytes from skin lesions, PBMCs and lymph nodes could maintain the survival of MF/Sézary cells and promote the outgrowth of continuous T cell lines derived from neoplastic clones. Cultures of adherent cells derived from the peripheral blood were able to support the growth of normal $CD4^+$ and $CD8^+$ T cells in the absence of exogenous cytokines for up to 3 months (Sutkowski *et al* 1995). Apoptosis that follows withdrawal of IL2 from activated normal T cells can be prevented by cultivation in the presence of fibroblasts (Scott *et al* 1990) (Section 1.8.7.2). These stromal cells produce growth factors and could have an effect similar to that of exogenous CM.

2.1.6 Detection of retroviral infection in cultured cells

Methods for detecting infection with HTLV-I and HTLV-II in cultured T cells are described in Section 1.4.1. With varying degrees of specificity, these methods are applicable to the detection of other retroviruses, including novel T lymphotropic retroviruses. Virions can be visualised by EM in most productive retroviral infections and RT activity is detectable in culture supernatants, but these assays do not permit specific identification. Budding retroviruses in cultured T cells may be difficult to distinguish from cell membrane blebs. The RTs of HTLV-I, HTLV-II and BLV have stronger activity with magnesium (Mg^{2+}) than with manganese (Mn^{2+}) as cofactors (Gilden *et al* 1975, Graves *et al* 1977, Poiesz *et al* 1980a, Rho *et al* 1981, Kalyanaraman *et al* 1982b, Hoffman *et al* 1985). Similarly, HIV-1 and HIV-2 exhibit Mg^{2+}-dependent RT activity (Barré-Sinoussi *et al* 1983, Popovic *et al* 1984). RT activity due to the presence of an exogenous retrovirus has to be distinguished from that produced by endogenous retroviruses and from non-specific cellular RT activity. Density gradient centrifugation can be used to associate RT activity with a particular density of particle.

The use of immunological techniques for detection of virus-specific antigens is usually based on prior knowledge and isolation of the virus of interest or closely related viruses in order to generate specific reagents. Sera from patients with ATL were used to detect immunological reactivity in T cell lines infected with HTLV-I before a retrovirus had been identified in these cultures (Miyoshi *et al* 1980, Hinuma *et al* 1981). Cross-reactivity between antibodies to virus structural group antigens (Gag) has been a useful technique for detecting novel retroviral infections in cultivated cells. The use of molecular techniques can be highly specific, for example using unique primers for amplification of HTLV-I sequences by PCR. Alternatively, group-specific consensus and degenerate primers can be used to amplify retroviral sequences from a number of related viruses, as well as potentially from novel viruses (Chapter 3). In this study EM, RT assays and PCR were used in attempts to identify retroviruses in T cells cultured from patients with T cell neoplasia. A wide range of serological reactivity against HTLV-I/II has been reported in MF/SS patients by different groups (Section 1.11.1.3). Serological testing conducted previously at the LRF Virus Centre had demonstrated that most patients with MF/SS were negative for HTLV-I/II by ELISA and WB ELISA, with only a few patients exhibiting seroindeterminate reactivities. Therefore, serological testing was not performed routinely on samples from MF/SS patients in this study.

2.2 Sources of clinical samples

Samples for testing in this project were obtained from patients with MF/SS and other cutaneous lymphoid infiltrates, LGL leukaemia and ALL in the United Kingdom (UK).

2.2.1 Cutaneous T cell lymphomas and other cutaneous lymphoid infiltrates

To study the involvement of retroviruses in CTCLs, samples of skin, lymph nodes and blood were collected from patients with cutaneous lymphoid infiltrates in the West of Scotland and other parts of the UK. Most samples were submitted by Dr D Tillman, Western Infirmary and Department of Dermatology, University of Glasgow Medical School. Other samples were submitted by Professor R Mackie and Dr D Bilsland, Western Infirmary and Department of Dermatology, University of Glasgow Medical School, Dr D Ellis, Inverclyde Royal Hospital, Greenock, Dr J Norris, Dumfries and Galloway Royal Infirmary, Dumfries, Dr E Spilg, Victoria Royal Infirmary, Glasgow, Dr F Humphreys, Dr J Hunter and Dr M Tidman, Department of Pathology, University Medical School, Edinburgh, and Dr N Kirkham, Department of Histopathology, Royal Sussex County Hospital, Brighton.

Samples were received from 36 patients with cutaneous lymphoid infiltrates during the period 1987 to 1995. Patients in this study were 20 to 91 years of age (mean 58 years, median 61 years). Histological, haematological and immunohistochemical examinations demonstrated that most cases were MF or SS but there were also cases of cutaneous (peripheral) T cell lymphoma (CTCL), angiocentric T cell lymphoma, small plaque parapsoriasis, Langerhan's cell histiocytosis (LCH) and CBCL (Table 2.3).

The clinicopathological features of these cutaneous lymphoproliferative disorders are described in Section 1.10. Most patients with MF/SS conformed to the case definitions for these diseases and the neoplastic population in all cases consisted of $CD4^+$ T cells. One patient with SS (patient 1198) had a marked $CD3^+$ $CD4^+$ lymphocytosis with aberrant expression of CD25 (Section 1.11.1.2). Samples of whole blood (10 ml) were diluted with an equal volume of transport medium containing heparin without preservative (Appendix 4: A4.2.2) and transported chilled to the LRF Virus Centre. Samples of fresh tissues (skin or lymph nodes) were placed in 10 ml of transport medium also containing heparin without preservative and transported similarly.

2.2.2 Large granular lymphocytic leukaemia

Samples of blood from six patients with LGL leukaemia in England were submitted by Dr M Bhavnani, Department of Haematology, Royal Albert Edward Infirmary, Wigan. Patients were 67 to 81 years of age and had $CD3^+$ $CD8^+$ LGL leukaemia (Table 2.4). T cell receptor (TCR) rearrangements were detected in one of four patients tested by SB hybridisation. Whole blood (10 ml) was diluted with an equal volume of transport medium containing heparin without preservative and transported chilled to the LRF Virus Centre.

2.2.3 Adult acute lymphoblastic leukaemia in butchers from Cardiff

PBMCs and bone marrow from five patients with ALL were kindly provided by Dr J Whittaker, Department of Haematology, University Hospital of Wales, Heath Park, Cardiff (Whittaker 1991). All patients were male butchers from Cardiff, 21 to 31 years of age (age of one patient unknown). They had ALL of common, pre-B cell or T cell type (Section 1.10.5) (Table 2.5). PBMCs had been harvested from heparinised blood samples at the University Hospital of Wales by Ficoll density gradient centrifugation (Section 2.2.2) and pellets of 1×10^5 cells stored frozen at -80°C. Bone marrow had also been stored frozen at -80°C. PBMCs and bone marrow were packed with solid CO_2 (dry ice) pellets for transportation to the LRF Virus Centre.

2.2.4 Samples from people without neoplasia

Umbilical cord blood was collected at the Southern General Hospital, Glasgow, and Royal Alexandria Hospital, Paisley. The blood was mixed with an equal volume of transport medium with heparin at the time of collection and transported chilled to the LRF Virus Centre. These samples were not screened for viruses but the population was considered to be at low risk for infection with HTLV-I or HTLV-II, since Scotland is not an endemic region for these viruses. UCMCs were used for cocultivation with cells derived from patients with neoplasia and as a source of CM for cell cultures (Section 2.3.6.10). Samples of human placenta were also obtained from the same sources for extraction of DNA. Peripheral blood was collected by venipuncture from adults working in the Department of Veterinary Pathology, University of Glasgow Veterinary School, and placed in transport medium with heparin. PBMCs from adult peripheral blood were used as negative control samples for PCRs.

Table 2.3: Patients with cutaneous lymphoid infiltrates.

Patient number	Age (years) at first submission	Sex	Clinical diagnosis	Date samples received	Samples	Cell culture	Molecular analysis
397	75	M	MF	2/3/87	Skin	+	+
				2/3/87	PBMCs	+	+
				17/10/91	PBMCs	+	+
				17/10/91	PBMCs	+	+
436	64	M	MF	5/5/88	Skin	+	+
				5/5/88	PBMCs	+	+
				19/9/88	Skin	+	+
				19/9/88	PBMCs	+	+
				21/1/94	Skin	+	+
				21/1/94	PBMCs	+	+
461	66	M	MF	29/6/88	Skin	+	+
				29/6/88	PBMCs	+	+
				6/10/88	Skin	+	+
				6/10/88	PBMCs	+	+
				21/2/91	Skin	–	+
				21/2/91	PBMCs	+	+
				4/6/91	Skin	+	+
				4/6/91	PBMCs	+	+
685	39	F	MF	21/8/89	Skin	+	+
726	64	F	SS	27/11/87	Skin	+	+
805	66	M	ATCL	28/3/90	Skin	+	+
1023	56	F	CTCL	29/11/90	Skin	+	+
1093	27	F	MF	4/3/91	Skin	+	+
				4/3/91	PBMCs	+	+
				5/12/91	Skin	+	+
				10/2/92	PBMCs	+	+
1096	24	M	SPP	12/3/91	Skin	–	+
1150	34	M	LCH	21/6/91	Skin	+	+
				21/6/91	PBMCs	+	–

Table 2.3: (continued).

Patient number	Age (years) at first submission	Sex	Clinical diagnosis	Date samples received	Samples	Cell culture	Molecular analysis
1162	56	M	SS	13/8/91	PBMCs	+	–
				13/8/91	Lymph node	+	–
				23/4/92	Skin	+	+
				23/4/92	PBMCs	+	+
1198	81	M	SS	1/10/91	PBMCs	+	–
				16/2/93	PBMCs	+	+
				3/6/93	PBMCs	+	+
				4/8/93	Skin	+	+
				4/8/93	PBMCs	+	+
1204	72	F	ATCL	4/10/91	Skin	–	+
1302	77	M	MF	7/11/91	Skin	+	+
1303	61	M	MF	7/11/91	Skin	+	–
				7/11/91	PBMCs	+	–
				18/5/93	Skin	+	+
				18/5/93	PBMCs	+	+
1312	20	F	MF	26/11/91	PBMCs	–	+
1356	54	M	SS	21/2/92	PBMCs	+	+
1357	69	M	MF	27/2/92	Skin	+	+
				27/2/92	PBMCs	+	+
1359	31	M	CBCL	28/2/92	Skin	+	+
				28/2/92	PBMCs	+	+
				18/11/93	Skin	+	+
				18/11/93	PBMCs	+	+
1360	68	M	ATCL	3/3/92	Skin	+	...
				3/3/92	PBMCs	+	–
1365	62	M	MF	4/3/92	Skin	+	+
				4/3/92	PBMCs	+	–
2116	63	F	CBCL	9/7/92	Skin	+	+
				9/7/92	PBMCs	+	+
2132	40	M	CBCL	21/8/92	Skin	+	+
				21/8/92	PBMCs	+	+

Table 2.3: (continued).

Patient number	Age (years) at first submission	Sex	Clinical diagnosis	Date samples received	Samples	Cell culture	Molecular analysis
2140	42	F	MF	8/9/92	PBMCs	+	+
2522	41	M	MF	18/2/93	Skin	+	+
				18/2/93	PBMCs	+	+
2531	47	M	MF	4/3/93	Skin	+	+
				4/3/93	PBMCs	+	+
2547	53	M	CBCL	26/3/93	PBMCs	+	+
2551	77	F	MF	1/4/93	Skin	+	+
				1/4/93	PBMCs	+	+
2552	54	M	MF	1/4/93	Skin	+	+
				1/4/93	PBMCs	+	+
2560	90	F	MF	15/4/93	Skin	+	+
				15/4/93	PBMCs	+	+
2578	86	F	MF	13/5/93	Skin	+	+
				13/5/93	PBMCs	+	+
2600	61	M	MF	16/6/93	Skin	+	+
				16/6/93	PBMCs	+	+
2669	91	M	SS	14/10/93	Skin	+	+
				14/10/93	PBMCs	+	+
				14/10/93	Lymph node	+	+
2746	64	M	MF	17/12/93	Skin	+	+
				17/12/93	PBMCs	+	+
2757	54	F	SS	29/12/93	PBMCs	+	+
2862	57	M	MF	15/4/94	Skin		

ATCL	Angiocentric T cell lymphoma
CBCL	Cutaneous B cell lymphoma
CTCL	Cutaneous T cell lymphoma
LCH	Langerhan's cell histiocytosis
MF	Mycosis fungoides
SPP	Small plaque parapsoriasis
PBMCs	Peripheral blood mononuclear cells
SS	Sézary syndrome

Table 2.4: Patients with large granular lymphocytic leukaemia.

Patient number	Age (years)	Sex	Immunophenotype	Date samples received	Samples	Cell culture	Molecular analysis
1119	74	F	$CD3^+$ $CD8^+$	9/5/91	PBMCs	+	+
1125	74	M	$CD3^+$ $CD8^+$	16/5/91	PBMCs	–	+
1126	76	M	$CD3^+$ $CD8^+$	16/5/91	PBMCs	–	+
1148	71	M	$CD3^+$ $CD8^+$	20/6/91	PBMCs	+	+
1195	81	M	$CD3^+$ $CD8^+$	29/8/91	PBMCs	–	+
1196	67	M	$CD3^+$ $CD8^+$	4/9/91	PBMCs	–	+

LGL leukaemia Large granular lymphocytic leukaemia
PBMCs Peripheral blood mononuclear cells

Table 2.5: Cases of acute lymphoblastic leukaemia in butchers from Cardiff.

Patient number	Age (years)	Sex	Immunophenotype	Date samples received	Samples	Cell culture	Molecular analysis
1128	21	M	Common ALL	16/5/91	PBMCs	–	+
				16/5/91	Bone marrow	–	+
1129	U	M	Pre-B cell ALL	12/5/89	PBMCs	–	+
				12/5/89	Bone marrow	–	+
1130	25	M	T cell ALL	16/5/91	PBMCs	–	+
				16/5/91	Bone marrow	–	+
1131	22	M	Common ALL	13/6/91	PBMCs	–	+
				13/6/91	Bone marrow	–	+
1158	31	M	T cell ALL	2/8/91	PBMCs	–	+
1167	21	M	Common ALL	5/8/91	PBMCs	–	+

ALL Acute lymphoblastic leukaemia
PBMCs Peripheral blood mononuclear cells
U Unknown

2.3 Materials and methods

2.3.1 Containment facilities for handling clinical samples

2.3.1.1 Level II containment facility

Containers with clinical samples were opened in class II microbiological safety cabinets (MSCs) in the level II containment facility at the LRF Virus Centre. The containment facility operated under negative pressure. Air extracted from MSCs and from the main containment room passed through independent high efficiency particulate air (HEPA) filters. Clean laboratory coats and surgical gowns were worn. MSCs and the main containment room were disinfected with formaldehyde gas every 6 months. Laboratory personnel were routinely vaccinated against hepatitis B virus (HBV) and tuberculosis. Samples entering the laboratory were assigned unique patient and sample identifying numbers. Accompanying documents were filed in the laboratory and sample details recorded in a computerised database.

2.3.1.2 Microbiological safety cabinets

Handling procedures and the laminar flow of air in the class II MSCs prevented cross-contamination of samples and reduced the risk of infection of personnel. Two latex gloves were worn on each hand when working in MSCs. The outer gloves were discarded and replaced every time hands were withdrawn from the operating environment. Sample containers and the interiors of MSCs were sprayed with 1% Virkon-S or 70% ethanol before and after samples were handled. Disinfection was maintained for at least 15 minutes between uses of MSCs by different operators or for different purposes. Disposable plastic pipettes, sample containers, bottles for solutions and media, centrifuge tubes and tissue culture flasks were used for most work. The use of glass was minimised. Sterile round-nosed scissors and rats-toothed forceps were used for manipulation of tissues, including cutting tissues into pieces for extraction of DNA. Solutions were transferred by pipette using battery-operated devices. Components of pipetting devices potentially in contact with tissue culture fluids were dismantled and soaked in 1% Virkon-S between uses and filters were changed.

The three MSCs in the level II containment facility were used for handling different material. MSC 1 was used for handling known HTLV-I, BLV, EBV, human herpesvirus (HHV) 6 or HHV-7-infected cell lines. MSC 2 was used for handling clinical specimens and tissue cultures derived from those specimens. MSC 3 was used for preparing sterile medium and solutions and for handling cell cultures demonstrated to be free of specified exogenous retrovirus or herpesvirus infections. Viable cells were stored in liquid nitrogen (-195.8°C). Samples of serum and cells and tissue samples for DNA extraction were stored at -80°C in freezers in a room adjacent to the containment laboratory. Handling of potentially infectious material outside MSCs was undertaken with appropriate precautions. Cell culture flasks were sealed when in incubators and during handling in the main containment room. Cells were inactivated before removal from MSCs for quantification by haemocytometer counts (Section 2.3.3.3). During centrifugation tubes were placed in sealed screw-top cannisters (Aerosolve, Beckman).

2.3.1.3 Disposal of waste

Used plasticware and glass heparin containers were soaked in 2% Virkon-S in trays for 1 hour before the trays were placed in plastic autoclave bags and autoclaved at 121°C for 1 hour. Discarded samples, solutions and tissue culture media were diluted with at least an equal volume of 2% Virkon-S in screw-top plastic containers and subjected to the same autoclave protocol. All other laboratory waste was placed in double autoclave bags and autoclaved at 121°C for 1 hour before removal from the laboratory and disposal by incineration.

2.3.2 Materials

The sources of frequently used materials are listed in Appendix 3 and the compositions of routinely used solutions for cell biology are listed in Appendix 4. Additional reagents and buffers are specified in individual sections.

2.3.2.1 Cell culture

Cells were cultivated in 50 cm^3 offset screw-top tissue culture flasks containing 5 to 10 ml medium (Costar) or in 250 cm^3 flasks containing 25 to 50 ml medium (Appendix 4: A4.1). Some cultures were performed in 96-well microtitre plates containing 50 to 200 µl medium per well or 12-well tissue culture plates containing 1 to 2 ml medium per well.

2.3.2.2 Materials for manipulation of fluids

Disposable pipettes (Costar) suitable for handling 1, 5, 10 and 25 ml volumes were used for transferring fluids (Appendix 4: A4.1). Blood samples, cell culture media and other fluids were centrifuged in 15 and 50 ml screw-top polypropylene centrifuge tubes (Falcon, Becton Dickinson and Sterilin, J Bibby). These tubes, as well as 5 ml screw-top polycarbonate bijous and 20 ml Universal tubes, were used for storing fluids and tissues. Small quantities of media and additives for cell culture were sterilised by filtration through 0.2 and 0.45 µm cellulose acetate filters (Acrodisc, Gelman Sciences) attached to syringes. Larger volumes were sterilised by vacuum filtration using 250 and 500 ml bottles with 0.2 or 0.45 µm cellulose acetate filters (Costar).

2.3.2.3 Centrifuges

The main centrifuge used for cell culture work was the GPR bench centrifuge (Beckman) with a GH 3.7 swinging bucket rotor. This held 15 and 50 ml screw-top polypropylene centrifuge tubes in racks inside screw-top cannisters (Aerosolve). Microtitre (96-well) plates were centrifuged using microplate carriers. A bench microcentrifuge (Micro Centaur, MSE) was used for centrifuging 0.6 ml flip-top PCR tubes and 1.5 ml flip-top and screw-top prolypropylene microcentrifuge tubes.

2.3.2.4 Cell culture medium

Standard tissue culture medium consisted of RPMI 1640 medium nominally containing 10 or 20% foetal bovine serum heat-inactivated at 56°C (hi-FBS), 400 mg/ml streptomycin, 400 U/ml penicillin and 2 mM L-glutamine (RPMI 1640 + 10 or 20% hi-FBS + 2-ME) (Moore *et al* 1967) (Appendix 4: A4.2.1). Medium with 10% hi-FBS was used for most established cell lines and medium with 20% hi-FBS for cultivation of cells derived from clinical samples. Phenol red was included as an indicator of changes in pH. Gentamicin was sometimes added at a final concentration of 50 to 70 μg/ml if bacterial contamination developed. Amphotericin B was added to cultures at risk of fungal contamination at a final concentration of 1 μg/ml. However, cultures that developed fungal growth were discarded without opening the contaminated flasks.

2.3.3 Manipulation of cells

2.3.3.1 Standard procedure for cultivation of eucaryotic cells

Established cell lines were usually split at a ratio of 1:4 to 1:6 twice weekly to densities ranging from 5×10^4 to 5×10^6 cells/ml. The tissue culture flasks were aerated with filtered 5% CO_2 in air for 30 sec then sealed and incubated at 37°C. Cells in suspension were diluted with an appropriate quantity of standard tissue culture medium and unwanted cells and medium were discarded by dilution with at least an equal volume of 2% Virkon-S.

Cells that formed adherent monolayers, including BLV-FLK and fibroblast cultures derived from skin samples of patients, were separated from the plastic of the tissue culture flasks by incubation with trypsin or dispase (Appendix 4: A4.2.5). For trypsinisation, the cell culture medium was removed and replaced with 1.5 to 3 ml 0.25% crude trypsin in 1 M ethylenediaminetetraacetic acid (EDTA) (trypsin-EDTA). The flask was incubated at 37°C for 5 to 15 min with occasional gentle tilting. When most cells appeared separated by light microscopy, the flask was tapped firmly to dislodge remaining adherent cells. Alternatively, the fluid was flushed up and down several times using a pipette. Tissue culture medium containing 20% hi-FBS (10 ml) was added to the flask and mixed to dilute and inactivate the trypsin. The cells were then centrifuged at 270 *g* (1,000 rpm) for 5 min and the supernatant was discarded. The cell pellet was resuspended in an appropriate volume of tissue culture medium, returned to a tissue culture flask and aerated with 5% CO_2 in air before incubation. Dispase was used at a concentration of 1 U/ml for passaging cultures of skin from clinical samples that had adherent fibroblasts underlying lymphocytes in suspension.

When the quantity of cells extracted from a clinical sample was low, 12-well plates or 96-well microtitre plates were used to maintain the density of cells in culture. Cells in 96-well microtitre plates were usually cultured at 1×10^6 to 3×10^6 cells/ml in 200 μl medium. N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer, gentamicin and amphotericin B were added to the standard tissue culture medium. Approximately 200 μl of autoclaved ultrapure water were added to unused wells to maintain humidity. The lids were secured with tape.

2.3.3.2 Light microscopy

An inverted light microscope (Labovert, Leitz) was used for examination of cells in culture flasks and for haemocytometer counts at powers of 40x, 100x or 200x.

2.3.3.3 Quantification of cells

The concentrations of PBMCs, UCMCs and cultured cells were determined by counting cells in known volumes using an improved Neubauer haemocytometer chamber (Fisons). Cells in even suspension (50 µL) were stained with 0.1% crystal violet in 1% acetic acid (50 µl) for 3 to 5 min in a 5 ml screw-top polycarbonate bijou. Erythrocytes in preparations of PBMCs were lysed by the acetic acid. The stained cells were pipetted under the coverslip of the haemocytometer chamber and the numbers of viable cells were counted in each of four 1 mm x 1 mm x 0.1 mm (0.1 mm^3) chambers. The concentration of cells in the original volume was calculated by multiplying the total number of viable cells by 1 x 10^4. The haemocytometer chamber and glass cover slips were stored in 1% Virkon-S between uses.

2.3.3.4 Cryopreservation of viable cells and cell cultures

Viable cells for cryopreservation were suspended at a density of 1 x 10^6 to 1 x 10^7 cells/ml in tissue culture medium containing 20 to 50% hi-FBS and 10% dimethyl sulphoxide (DMSO). Alternatively, cells were suspended in hi-FBS containing 8% DMSO. Aliquots of 1 ml were transferred to screw-top polypropylene low temperature storage vials (Cryotube, Nunc). These were placed in a freezing container (Nalgene) filled with isopropanol and held at -80°C for 4 hours for a controlled decrease (1°C/min) in temperature. The vials were then transferred to a liquid nitrogen ($liqN_2$) container for storage at -195.8°C.

2.3.3.5 Cultivation of cryopreserved cells

Cryopreservation tubes were removed from liquid nitrogen and placed in a screw-top metal cannister to prevent dissemination of contents should the tubes explode as they thawed. The metal cannister was placed in a MSC for 5 min until the contents were thawed. The cryotube was removed from the cannister, opened and the contents were pipetted drop by drop into a 15 ml screw-top polypropylene centrifuge tube containing 5 ml of the appropriate tissue culture medium. The 15 ml tube was centrifuged at 270 *g* for 5 min and the supernatant was discarded. The cell pellet was resuspended in 2 to 5 ml of standard tissue culture medium and the concentration of viable cells was determined (Section 2.3.3.3). Cells were diluted to 5 x 10^5 to 5 x 10^6 cells/ml in appropriate tissue culture medium, aerated with 5% $C0_2$ in air and incubated at 37°C. Cells were examined daily and subcultured as required. If many cells died in the first week of culture, these were removed by centrifuging through a Ficoll gradient (Section 2.3.5.1). The purified viable cells were washed by centrifuging and discarding the supernatant, then were resuspended in fresh medium, aerated with 5% $C0_2$ in air and returned to cultivation.

2.3.3.6 Collection of cell culture supernatants

Supernatants were collected from selected cell cultures for use as CM and for assay of RT activity after centrifuging at 270 *g* for 5 min.

2.3.3.7 Cytospins for Epstein-Barr virus latent membrane protein staining

Cultured cells for cytospin preparations for EBV latent membrane protein (LMP) 1 staining were centrifuged to remove the medium and washed three times in PBS 1x. After counting using an haemocytometer (Section 2.3.3.3), cells were resuspended at a concentration of 1 x 10^6 cells/ml. Volumes of 100 µl containing 1 x 10^5 cells were centrifuged onto 3-aminopropyltriethoxysilane (APES)-coated glass cytospin slides (Fisons) at 400 rpm in a cytospin centrifuge (Shandon Cytospin 2) for 5 min. The cytopsin preparations were air dried, fixed in ice-cold acetone or methanol for 30 min and stored at -20°C. Staining for EBV LMP-1 with MAbs CS1-4 was performed by Dr A Armstrong at the LRF Virus Centre using techniques established in the laboratory (Armstrong *et al* 1992).

2.3.3.8 Testing for mycoplasma contamination

Testing for contamination with mycoplasma was performed by June Freeland and Linda Andrew at the LRF Virus Centre. Sub-confluent normal rat kidney (NRK) cells were grown with 0.5 ml test culture supernatant in slide flasks (Nunclon, Nunc) for 3 days at 37°C. The cells were fixed with ice cold methanol:glacial acetic acid (3:1), washed twice with distilled water, then stained with 50 ng/ml Hoescht 33258 (Sigma) for 10 min at room temperature (Appendix 4: A4.7). Stained cells were washed with distilled water and examined under a wet-mounted cover slip using a fluorescent microscope (Laborlux K, Leitz). Mycoplasma typing was performed using a commercial PCR kit (Boehringer-Mannheim).

2.3.4 Established cell lines

2.3.4.1 C8166

C8166 (C81-66-45) is an HTLV-I-infected human $CD4^+$ T cell line derived from the productive HTLV-I-infected cell line HUT102 (Salahuddin *et al* 1983). HUT102 (ATCC TIB 162) was the first HTLV-I-infected cell line established (HTLV-I type strain $HTLV_{CR}$) and was derived from lymph node cells of a patient in the USA with cutaneous ATL resembling MF (Gazdar *et al* 1979, 1980, Poiesz *et al* 1980a, b, Hay *et al* 1988) (Section 1.3.2.1). C8166 contains one full length defective and two incomplete integrated HTLV-I proviruses, the incomplete proviruses having deletions of most of their regions from *gag* to *env* (Bhat *et al* 1993). All three proviruses are transcriptionally active and C8166 expresses Tax and $p21^{III}$ but has no expression of Rex, Gag or Env, no detectable RT activity and no production of virions (Salahuddin *et al* 1983, Bhat *et al* 1993). It is thus a suitable cell line for handling at containment level II. DNA and cell pellets derived from C8166 were used as positive controls for HTLV-I and HTLV-I/II PCR analyses.

2.3.4.2 J.JHAN

J.JHAN is a derivative of the human $CD4^+$ T cell line Jurkat (JM) (ATCC TIB-152), cultured from neoplastic T cells of a child with ALL and not known to be infected with any exogenous retrovirus (Schneider *et al* 1977, Gillis and Watson 1980, Hay *et al* 1988). It was provided to the LRF Virus Centre by Dr M Steele, Western General Hospital, Edinburgh. DNA and cell pellets from this cell line were used as negative controls for HTLV-I and HTLV-I/II PCR analyses and culture supernatants were collected for use in the RT assay.

2.3.4.3 L-726A

L-726A is an IL2-independent $CD4^+$ T cell line established at the LRF Virus Centre from skin lesions of a patient with SS (Crae *et al* 1991) (Section 2.1.3.1). No viruses have been demonstrated in this cell line. DNA and cell pellets from L-726A were tested for HTLV-I and HTLV-I/II by PCR and culture supernatants were tested for evidence of RT activity.

2.3.4.4 HH

HH is an IL2-independent $CD4^+$ T cell line derived from PBMCs of a patient with CTCL and was kindly provided by Dr G Starkebaum, Department of Medicine, Seattle Veterans Affairs Medical Center, Seattle, Washington, USA (Starkebaum *et al* 1991) (Section 2.1.3.1). This cell line is not known to be infected with HTLV-I. DNA and cell pellets from HH were tested for HTLV-I and HTLV-I/II by PCR and culture supernatants were tested for evidence of RT activity.

2.3.4.5 My-La and My-La MC

My-La is an IL2 and IL4-dependent $CD4^+$ T cell line established from the skin of a patient with MF (Kaltoft *et al* 1992) (Section 2.1.3.1). My-La marker chromosomes (MC) is a derivative of My-La with a 46XY≡ karyotype. These cell lines were kindly provided by Dr K Kaltoft, Department of Medicine, Institute of Human Genetics, Aarhus University, Denmark. They were grown in standard tissue culture medium containing 10% hi-FBS, 50 U/ml IL2 and 30 U/ml IL4. DNA and cell pellets were tested for HTLV-I and HTLV-I/II by PCR and culture supernatants were tested for evidence of RT activity.

2.3.4.6 BLV-FLK

BLV-FLK is a BLV-infected foetal lamb kidney (FLK) fibroblast cell line (Astier *et al* 1978, Onuma *et al* 1981, Rhim *et al* 1983, Hay *et al* 1988). It has one or a few intact copies of the BLV provirus and produces infectious virions (Marbaix *et al* 1981). Cell culture supernatants from this cell line were used as positive controls in RT assays and DNA and cell pellets were used as positive controls in BLV PCR analyses (Chapter 3).

2.3.4.7 FLK

FLK is an ovine fibroblast cell line not known to be infected with exogenous retroviruses (Astier *et al* 1978). Infection of this cell line with BLV resulted in the persistently infected cell line BLV-FLK described above. Cells obtained from this cell line were used as negative controls in BLV PCR analyses.

2.3.5 Processing of clinical samples

2.3.5.1 Purification of mononuclear cells from peripheral blood

Blood samples had been diluted with an equal volume of heparinised transport medium at the time of collection (Section 2.2.1) (Appendix 4: A4.2.2). On receipt at the LRF Virus Centre they were diluted with an equal volume of Hanks' balanced salt solution (HBSS + 2% hi-FBS) to one quarter of their original concentration. The diluted blood (20 to 25 ml) was carefully layered with a pipette over 15 ml of metrizoate-Ficoll (Ficol 400 solution, Ficoll-Paque, Pharmacia LKB or J.Prep, J.Bio), an aqueous solution of density 1.077 g/ml containing polymerised sucrose and sodium metrizoate/diatrizoate, in each of two 50 ml screw-top polypropylene centrifuge tubes (Falcon). The gradient was centrifuged at 1,500 *g* for 20 to 30 min at 18°C with no braking. Erythrocytes passed through the layer of Ficoll to sediment in the bottom. PBMCs remained at the interface with the Ficoll layer. PBMCs were removed with a polypropylene bulb pipette, transferred to a 50 ml centrifuge tube containing 30 ml HBSS + 2% hi-FBS and centrifuged at 270 *g* for 5 min. The PBMCs, forming a pellet at the bottom of the centrifuge tube, were retained and the supernatant was discarded. If the supernatant was red due to lysed erythrocytes, the washing step was repeated by resuspending the cells in 30 ml HBSS and centrifuging. The pelleted PBMCs were resuspended in 10 ml medium and centrifuged again. The final cell pellet was resuspended in 1 to 3 ml medium and the concentration of cells was determined using an haemocytometer (Section 2.3.3.3). PBMCs were diluted to 1×10^5 to 2.5×10^5 cells/ml in appropriate medium with selected stimulants for cultivation of T cells (Section 2.3.6). Volumes containing 1×10^6 to 1×10^8 PBMCs in PBS 1x were transferred to 15 ml screw-top polypropylene centrifuge tubes to prepare cell pellets for DNA extraction (Section 3.3.5.1). Cell pellets for PCR were prepared with 1×10^5 PBMCs in PBS 1x in 0.6 ml flip-top PCR tubes (Section 3.3.5.1).

2.3.5.2 Processing of tissue samples

Tissue samples (skin, lymph nodes and human placenta) were processed on the day of receipt for tissue culture and storage at -80°C for later DNA extraction. Tissues were dissected into pieces approximately 1 mm^3 in sterile plastic Petri dishes using sterile round-nosed scissors and rats-toothed forceps. Approximately 1 ml of standard tissue culture medium was added to prevent desiccation. Half of each tissue was stored frozen at -80°C for DNA extraction and half was cultivated in an attempt to establish T cell lines (Section 2.3.6). Dispase II was added to the medium at a concentration of 1 U/ml and incubated at 37°C for 10 to 15 min to assist in the dissociation of cells from samples of skin and lymph nodes.

2.3.6 Cultivation of lymphocytes from clinical samples

PBMCs and cells from skin and lymph nodes of patients with MF/SS and other cutaneous lymphoid infiltrates were cultivated in an attempt to establish T cell lines. Cells and tissues were suspended in standard tissue culture medium (RPMI 1640 + 20% hi-FBS + 2-ME) containing selected exogenous cytokines (Appendix 4: A4.2.6), CM or no stimulants, or were cocultivated with UCMCs. Specific protocols were adapted from those used by other groups to establish $CD4^+$ cell lines from patients with MF/SS (Sections 2.1.3.3 and 2.1.3.4) (Table 2.6). Once or twice weekly cells were centrifuged and resuspended in fresh medium according to the selected protocol.

2.3.6.1 Unstimulated cultures

Cells harvested from blood, skin and lymph nodes were cultivated in flasks at a density of 1 x 10^6 cells/ml. Half of the culture volume was removed and centrifuged at 270 *g* for 5 min once weekly or as required. The cells were resuspended in an equal volume of fresh medium and returned to the same flask. The remaining supernatant was stored for assay of RT activity, as were supernatants collected from other cultures (Section 2.3.3.6).

2.3.6.2 Phytohaemagglutinin and interleukin 2

Cells were cultivated at a density of 1 x 10^6 cells/ml with 5 μg/ml PHA-P for 48 to 72 hours. The medium was then replaced with medium containing 50 U/ml IL2.

2.3.6.3 Concanavalin A and interleukin 2

Cells were cultivated at a density of 1 x 10^6 cells/ml with 5 μg/ml ConA for 48 to 72 hours. This was replaced with medium containing 50 U/ml IL2.

2.3.6.4 Interleukin 2

Cells were cultivated at a density of 1 x 10^6 cells/ml with 50 U/ml IL2.

2.3.6.5 Interleukin 7

The protocol for use of IL7 for cultivation of T cells from patients with MF/SS was adapted from Dalloul *et al* (1992) and Foss *et al* (1994). Cells were cultivated at a density of 1 x 10^6 cells/ml with 10 ng/ml IL7.

2.3.6.6 Interleukin 2 and interleukin 7

Cells were cultivated at a density of 1 x 10^6 cells/ml with 50 U/ml IL2 and 10 ng/ml IL7.

Table 2.6: Protocols for cultivation of T cells from cutaneous T cell lymphoma patients.

PHA → IL2	5 μg/ml phytohaemagglutinin then 50 U/ml interleukin 2
ConA → IL2	2 μg/ml concanavalin A then 50 U/ml interleukin 2
IL2	50 U/ml interleukin 2
IL2 + GMCSF	10 U/ml interleukin 2 + 100 U/ml granulocyte macrophage colony stimulating factor
IL2 + IL4	50 U/ml interleukin 2 + 30 U/ml interleukin 4
IL2 + IL7	50 U/ml interleukin 2 + 10 ng/ml interleukin 7
IL7	10 ng/ml interleukin 7
UCMC CM	25% umbilical cord blood mononuclear cell conditioned medium
IL2 + SAF CM	15 U/ml interleukin 2 + 0.2% Sézary activating factor conditioned medium
Cocultivation	Cocultivation with umbilical cord blood mononuclear cells
Unstimulated	No exogenous growth factors

2.3.6.7 Interleukin 2 and interleukin 4

The protocol for use of IL2 and IL4 to stimulate MF/Sézary cells was adapted from Kaltoft *et al* (1992). Cells were cultivated at 1 x 10^6 cells/ml with 50 U/ml IL2 and 30 U/ml IL4.

2.3.6.8 Interleukin 2 and granulocyte-macrophage colony stimulating factor

GMCSF was used according to the method described by Zucker-Franklin *et al* (1991). Cells were cultivated at a density of 2.5 x 10^6 cells/ml with 10 U/ml IL2 and 100 U/ml GMCSF.

2.3.6.9 Interleukin 2 and Sézary T cell activating factor conditioned medium

SAF CM was prepared according to the method described by Abrams *et al* (1991b, 1993). PBMCs from a patient with SS (patient 1198) were cultivated at 4 x 10^6 cells/ml with 2 μg/ml ConA for 48 hours. The supernatant was harvested by centrifugation at 270 *g* for 5 min. Residual ConA was inactivated with 1 mg/ml α-methyl mannoside and the supernatant was filtered into a 20 ml screw-top Universal container through a 0.45 μm cellulose acetate filter (Acrodisc, Gelman Sciences) attached to a syringe. This supernatant, designated SAF CM, was stored at -20°C until required. Cultures were initiated from other patients with MF/SS at densities of 1 x 10^6 cells/ml with 0.2% SAF CM and 15 U/ml IL2 for 3 days. The cultures were then maintained with 15 U/ml IL2 (without SAF CM), medium being replaced two to three times per week. After 6 weeks, cultures were re-stimulated with 0.2% SAF CM and 15 U/ml IL2 for 3 days before reverting to 15 U/ml IL2 alone.

2.3.6.10 Umbilical cord blood mononuclear cell conditioned medium

UCMC CM, an unmodified culture supernatant also known as lymphocyte conditioned medium, was prepared from UCMCs stimulated with PHA-P. UCMCs were cultivated at a density of 1 x 10^6 to 2.5 x 10^6 cells/ml with 5 μg/ml PHA-P for 48 hours. The supernatant was harvested after 3 days by centrifuging the cells at 270 *g* for 5 min and stored at -20°C. UCMC CM was added at a concentration of 25% to cultures of cells from clinical samples containing 1 x 10^6 cells/ml that had been stimulated with 5 μg/ml PHA-P for 48 hours. The supernatant was replaced with a mixture of 75% medium and 25% UCMC CM once per week.

2.3.6.11 Cocultivation with umbilical cord blood mononuclear cells

UCMCs were cocultivated with cells from patients with neoplasia to act as recipient cells for infection with retroviruses released from patient cells and to produce growth stimulants that might promote outgrowth of continuous T cell lines. UCMCs were cultivated at 1 x 10^6 cells/ml with 5 μg/ml PHA-P for 48 to 72 hours. These cells were centrifuged then resuspended at 2 x 10^6 cells/ml. Fresh or cultured cells (donor cells) from patients with neoplasia were suspended at a density of 2 x 10^6 cells/ml. Equal volumes of suspensions of donor and recipient cells were mixed in the same flask and cultivated with 50 U/ml IL2. A control flask containing 2 x 10^6 UCMCs/ml in the same medium was maintained in parallel. A

different sex was chosen for recipient UCMCs and donor cells from the patient with neoplasia so that any cell line established from cocultures could be karyotyped to determine the origin of the cells.

2.3.7 Immunophenotyping of cultured lymphocytes by flow cytometry

The immunophenotypes of cultured lymphocytes were determined by flow cytometry using an EPICS Elite II (Coulter). Cells were labelled with MAbs to determine the expression of surface antigens with specificity for different cell types (Table 2.7) (Appendix 4: A4.3.2). Cells removed from cultures were centrifuged in 15 ml screw-top polypropylene tubes at 270 *g* for 5 min. The supernatant was discarded and the cells were resuspended in 5 ml HBSS + 2% hi-FBS. After counting using an haemocytometer (Section 2.3.3.3), aliquots of 1 x 10^6 cells were transferred to 5 ml polypropylene cytometer tubes (Sarstedt) and centrifuged again at 270 *g* for 5 min. The supernatant was removed and the cells were resuspended in PBS 1x containing 0.1% sodium azide and 2% bovine serum albumin (PAB). The tubes were centrifuged and the supernatant was removed. The cells were resuspended in 50 µl PAB containing 20% rabbit serum and held on ice for 30 to 60 min to block non-specific binding sites on cells. The cells were again washed with 1 ml PAB, centrifuged and the supernatant discarded. This solution was replaced with 10 µl of a solution containing 20% rabbit serum in PAB and 5 µl/ml fluorescein isothiocyanate (FITC) or R-phycoerythrin (RPE)-conjugated murine MAbs against CD2, CD4, CD8, CD15, CD19, CD30, CD33 or CD45. FITC and RPE-conjugated murine MAbs against *Aspergillus niger* glucose oxidase were used as negative controls. Cells were double-labelled with anti-CD2-FITC + anti-CD19-RPE and anti-CD8-FITC + anti-CD4-RPE for two-colour EPICS analysis. Binding of MAbs to cells was allowed to proceed for 20 to 30 min on ice. Unbound MAbs were removed by centrifuging three times at 270 *g* for 5 min, the supernatant being removed and the cell pellets resuspended in 1 ml PAB each time. After the last wash step, the cells were fixed by adding 1 ml 1% paraformaldehyde in PBS 1x and held at 4°C until analysed by flow cytometry. EPICS analysis was performed by Linda Andrew in the LRF Virus Centre.

2.3.8 Electron microscopy

Cells from four long-term cultures of T cells (≥ 6 months) established from three patients with MF, as well as lymphocytes from five other patients with MF or SS after 21 to 50 days in culture, were processed for electron microscopy (EM) in an attempt to observe intracellular and budding retrovirus particles. After determining the density of cells by counting using an haemocytometer (Section 2.3.3.3), aliquots of 5 x 10^6 cells were placed in 15 ml screw-top polypropylene centrifuge tubes. The tubes were centrifuged at 270 *g* for 5 min and the supernatant was discarded. Cell pellets were resuspended in 5 ml of 2% glutaraldehyde in 0.05 M sodium cacodylate pH 7.2 to 7.3 and the tubes were centrifuged at 900 *g* (2,000 rpm). The supernatant was removed and carefully replaced with 1 ml glutaraldehyde solution. Glutaraldehyde-fixed cell pellets were submitted to the EM unit in the Department of Veterinary Pathology for post-fixing, epoxy embedding and examination by thin section transmission EM (Zeiss 109). Processing and examination of EM samples were performed by Ross Blackley, with additional interpretation by Dr Helen Laird.

Table 2.7: Specificity of selected haematopoietic cell surface markers.

Cell surface markers are indicated by their cluster of differentiation (CD) nomenclature.

Surface marker	Predominant cell types
CD45	Haematopoietic cells
CD2	T cell
CD19	B cell
CD3	Mature T cell
CD4	$CD4^+$ helper/inducer T cell
CD8	$CD8^+$ cytotoxic/suppressor T cell
CD15	Myeloid lineage cells
CD33	Myeloid lineage cells
CD30	Activation marker

2.3.9 Reverse transcriptase assay

2.3.9.1 Principle of reverse transcriptase assay

Reverse transcriptase (RT) is essential for retroviral replication and present in all replication competent retrovirus virions (Baltimore 1970, Temin and Mizutani 1970) (Section 1.2.3). An assay based on detection of RT activity can be used as a general technique for revealing the presence of retroviruses. RT assays are based on incorporation of nucleosides into DNA using RNA as a template by utilising the RNA-dependent DNA polymerase activity of viral RTs. The gold standard is the conventional RT assay based on incorporation of radioactive nucleosides into the cDNA product and measurement of radioactivity (Kacian 1977). The inclusion of Mg^{2+} or Mn^{2+} as cofactors in the assay is used to determine the relative Mg^{2+} or Mn^{2+}-dependence of any RT activity present. Use of MAbs and biotinylated or digoxigenin-labelled nucleosides has allowed enzyme-linked immunosorbent assay (ELISA)-based assays for RT activity to be developed, increasing sensitivity (Eberle and Seibl 1992, Suzuki *et al* 1993). Sensitivity has been further increased with PCR-based RT assays that allow exponential amplification of cDNA produced from an RNA template, with detection of product by Southern blot (SB) hybridisation or ELISA (Silver *et al* 1993, Pyra *et al* 1994). RNA viruses are used as templates in these assays because they do not have a DNA phase that could contaminate the RT-PCR reaction and are utilised less efficiently than synthetic templates for reverse transcription by cellular DNA polymerases, reducing non-specific reactivity (Robert *et al* 1972).

2.3.9.2 Conventional reverse transcriptase assay

The conventional reverse transcriptase assay was performed according to the method of Klement and Nicolson (1977). Supernatants collected from cell cultures and stored at -20°C (Section 2.3.3.6) were thawed and clarified by centrifugation at 10,000 *g* (13,000 rpm) for 20 min in a J2-21 centrifuge (Beckman) with a JA-20 fixed angle rotor. Clarified supernatants were transferred to 10 ml plastic centrifuge tubes (Ultra-Clear, 14 mm x 89 mm, Beckman) in SW41 Ti buckets. The tubes were filled to within 0.5 cm of the top with Tris-NaCl-EDTA (TNE) 1x and opposing buckets were balanced to within 50 mg. The samples were centrifuged at 210,000 *g* (35,000 rpm) in an L8-60M ultracentrifuge (Beckman) with an SW41 Ti swinging bucket rotor at 4°C for 1 hour. Alternatively, they were centrifuged at 274,000 *g* (40,000 rpm) for 30 min. Retrovirus virions would be expected to form part of a pellet including some cellular debris at the bottom of the tubes. The supernatants were removed, the tubes were allowed to drain and the inside surfaces were dried, taking care not to dislodge the pellet. Virus disruption buffer, containing 20 mM dithiothreitol (DTT), 0.1% Nonidet P-40 (NP-40), 40 mM Tris pH 8.1 and 50m M KCl (Appendix 4: A4.4.2), was added with a pipette (170 µl for each 10 ml of original supernatant volume) and the bottom of each tube was scraped with the pipette tip to dislodge adherent debris and virus particles. The tubes were placed on ice for 10 to 15 min to allow virions to be disrupted by the buffer. The solution was assayed for RT activity immediately or stored at -80°C.

Reaction solutions (labelled X, Y and Z) were prepared for testing for Mg^{2+} and Mn^{2+}-dependent RT activity (Table 2.8). In the presence of RT responsive to either cofactor, tritiated thymidine triphosphate (^{3}H-TTP) (Appendix 4: A4.4.3) would be incorporated by reverse transcription into radiolabelled poly-thymidine deoxyribonucleic acid (poly-dT) that could be quantified in a scintillation counter. Solution X contained poly-adenosine ribonucleic acid (poly-rA) and oligo-thymidine deoxyribonucleic acid (oligo-dT) (Poly(rA).p(dT)$_{12\text{-}18}$, Pharmacia); the poly-rA acted as a template and the oligo-dT acted as a primer for RNA-dependent DNA polymerase (RT) activity in the presence of Mg^{2+} or Mn^{2+}. Solution Y contained poly-adenosine deoxyribonucleic acid (poly-dA) and oligo-dT (Poly(dA).p(dT)$_{12\text{-}18}$, Pharmacia); the poly-dA acted as a template for DNA-dependent DNA polymerase activity and the oligo-dT similarly acted as a primer. Since DNA polymerases of cellular origin may have a degree of RNA-dependent DNA polymerase activity, the quantification of DNA-dependent DNA polymerase activity in a sample gives an indication as to whether RT activity in a sample is likely to be due to the presence of a retrovirus or to contamination by cellular material (Goodman and Speigelman 1971). Solution Z did not contain either synthetic oligonucleotide reagent and acted as a negative control for ^{3}H-TTP retention within the assay system.

In each assay, samples of supernatant were labelled 1, 2, 3 ... n. Assays for both Mn^{2+} and Mg^{2+}-dependent RT activities were performed in duplicate. For each cofactor, 25 µl of reaction solution X, Y or Z were added to duplicate 15 ml screw-top polypropylene tubes labelled X_1, Y_1, Z_1; X_2, Y_2, Z_2; X_3, Y_3, Z_3; ... X_n, Y_n, Z_n (Table 2.9). Disrupted virus (25 µl) was added to each of these volumes and the mixtures were incubated in a water bath at 37°C for 1 hour. The tubes were placed on ice and mixed with approximately 10 ml of an aqueous solution containing 10% trichloroacetic acid (TCA) and 1% w/v sodium pyrophosphate ($NaPP_i$) that had been cooled to 4°C. This mixture was placed on ice for 10 to 20 min to precipitate DNA, including ^{3}H-TTP-labelled products of the RT reaction.

A 1225 sampling manifold (Millipore) was used to collect precipitated DNA onto filters (GF/C glass microfibre filters, 2.5 cm, Whatman) by vacuum filtration (OM109 vacuum/pressure pump, Millipore). The top plate had 12 cups, each holding up to 15 ml. It was screwed onto a unit in which 12 glass microfibre filters were placed over polypropylene support screens on a support plate. Fluid passed through the filters under vacuum and was collected into a reservoir. The glass microfibre filters were moistened with a few ml of 10% TCA, 1% $NaPP_i$ before the RT assay samples (approximately 10 ml) were poured into the cups and vacuum filtration commenced. The RT assay reaction tubes were rinsed with approximately 10 ml of 10% TCA, 1% $NaPP_i$; this was poured into the cups on the sampling manifold top plate and the vacuum filtration repeated. The filters were rinsed twice with 10 to 15 ml 5% TCA under vacuum, then with 10 ml 98% ethanol (absolute alcohol) under vacuum. The glass microfibre filters were removed from the apparatus with plastic forceps and allowed to dry on blotting paper at room temperature. The sampling manifold was dismantled and soaked in decontaminating solution (Decon) overnight, then rinsed thoroughly with water and allowed to dry.

Table 2.8: Reaction solutions for reverse transcriptase assay.

Reagent	X (µl)	Y (µl)	Z (µl)
Mn^{2+}-dependent reverse transcriptase assay			
0.8 M Tris pH 8.1	20	20	20
Tris pH 8.1, 0.15 M NaCl	–	–	160
1 M KCl	20	20	20
20 mM $MnCl_2$	40	40	40
5 U/ml Poly(rA).$p(dT)_{12-18}$	160	–	–
5 U/ml Poly(dA).$p(dT)_{12-18}$	–	160	–
^3H TTP	40	40	40
Ultrapure water	120	120	120
Total	400	400	400
Mg^{2+}-dependent reverse transcriptase assay			
0.8 M Tris pH 8.1	20	20	20
Tris pH 8.1, 0.15 M NaCl	–	–	160
1 M KCl	20	20	20
0.1 M $MgCl_2$	80	80	80
5 U/ml Poly(rA).$p(dT)_{12-18}$	160	–	–
5 U/ml Poly(dA).$p(dT)_{12-18}$	–	160	–
^3H TTP	40	40	40
Ultrapure water	80	80	80
Total	400	400	400

Table 2.9: Preparation of samples for reverse transcriptase assay.

Cofactor	Sample code	RNA activity (rA)	DNA activity (dA)	Negative activity (N)
		X	Y	Z
Mn^{2+}	1	X_1	Y_1	Z_1
	2	X_2	Y_2	Z_2
	3	X_3	Y_3	Z_3
	⋮	⋮	⋮	⋮
	n	X_n	Y_n	Z_n
Mg^{2+}	1	X_1	Y_1	Z_1
	2	X_2	Y_2	Z_2
	3	X_3	Y_3	Z_3
	⋮	⋮	⋮	⋮
	n	X_n	Y_n	Z_n

The dry glass microfibre filters containing RT assay products were placed in 18 ml Type II polyethylene scintillation vials (Poly-Q vials, Beckman) with 5 ml scintillation liquid (Ecoscint A, National Diagnostics). Vials were loaded onto racks in a scintillation counter (LS1701 Liquid Scintillation System, Beckman) and the ^{3}H counts per minute (cpm) were determined by exposure of the detector for 10 min per vial. After use, scintillation vials, fluid and filters were place in sealed plastic bags and sent for disposal through the same system as for other radioisotopes in use in the Department of Veterinary Pathology.

The RNA-dependent DNA polymerase (RT) activity (rA) in each sample, determined by quantification of ^{3}H-TTP-labelled product transcribed from the poly-rA template, was compared to the DNA-dependent DNA polymerase activity (dA) in the same sample, determined by quantification of ^{3}H-TTP-labelled product transcribed from the poly-dA template, after correction for background ^{3}H-TTP activity with no template (N). Evidence for RT activity was an elevated value of rA divided by N (rA/N) with either Mn^{2+} or Mg^{2+}, while the values of dA divided by N (dA/N) in the same sample remained low.

2.3.9.3 Positive and negative control samples

Positive control samples for testing in the RT assay were supernatants obtained from known retrovirus-infected cell lines that are productive for retrovirus virions. Supernatants from the BLV-infected fibroblast cell line BLV-FLK grown at the LRF Virus Centre were used as positive controls for Mg^{2+}-dependent RT activity (Section 2.3.4.6). Supernatants from FL4, an IL2-dependent feline cell line infected with the Petaluma strain of feline immunodeficiency virus (FIV) were used as an additional positive control for Mg^{2+}-dependent RT activity (Yamamoto *et al* 1991). Supernatants from this cell line were provided by Dr M Hosie, Department of Veterinary Pathology. SSN-1 is a fish cell line derived from the striped snakehead (*Channa striatus*) and persistently infected with snakehead fish retrovirus (SnRV) (Frerichs *et al* 1991, Hart *et al* 1996). Supernatants from this cell line, used as a positive control for Mn^{2+}-dependent RT activity, were kindly provided by Dr D Hart, Department of Veterinary Pathology.

2.3.9.4 Product-enhanced reverse transcriptase (PERT) assay

The product-enhanced RT (PERT) assay is a PCR-based method for detection of RT activity that uses brome mosaic virus as the RNA template (Pyra *et al* 1994). PERT allows detection of 1×10^{-9} U (2.1×10^{2} molecules) of murine leukaemia virus (MuLV) RT, equivalent to the activity present in 3 to 11 MuLV virions. This is 1×10^{6} to 1×10^{7} times more sensitive than conventional RT assays. Samples of supernatants from cell cultures of selected patients with CTCL were submitted to Dr J Schüpbach, Swiss National Center for Retroviruses, University of Zurich, Switzerland, for testing in the PERT assay for RT activity, with detection and quantification by ELISA. Positive and negative controls were as described by Pyra *et al* (1994).

2.4 Results

Whole blood, skin and lymph nodes submitted to the LRF Virus Centre from patients with cutaneous lymphoid infiltrates during the period 1992 to 1995 were processed for cultivation of T cells and for molecular analyses (Sections 2.2.1 and 2.4.1). Samples received at the LRF Virus Centre from 1987 to 1992 had been cultivated mainly by Shauna Crae and resulted in the establishment of one continuous $CD4^+$ T cell line, L-726A (Section 2.1.3.1). Cells derived from skin and PBMCs during this period had been treated with PHA, IL2, CM, polybrene, 12-o-tetradecanoyl phorbol-13 acetate (TPA, phorbol-12-myristate 13-acetate), anti-CD3 and anti-IFNα. Cells had also been cocultivated with UCMCs and depleted of $CD8^+$ T cells. Serum samples had tested negative for HTLV-I by ELISA and WB ELISA, except for a few patients who had seroindeterminate bands by WB ELISA.

Results of investigations prior to 1992 will not be presented. However, samples stored from this period were used in molecular analyses described in Chapter 3 (Table 2.3). Cell pellets for PCR and DNA extraction, as well as some viable cells, had been stored from samples submitted from patients with LGL leukaemia and butchers from Cardiff with ALL in 1991 (Sections 2.2.2 and 2.2.3). These included PHA-stimulated PBMCs that had been cultured from one patient with LGL leukaemia. Samples from these patients were processed for molecular analyses (Chapter 3) (Tables 2.4 and 2.5). The results of cell biology studies are described here.

2.4.1 Cultivation of lymphocytes from patients with cutaneous lymphoid infiltrates

PBMCs and cells derived from skin and lymph nodes were cultivated from 15 patients with CTCL and three patients with CBCL (Table 2.10). Most samples of PBMCs were stimulated with PHA or ConA then IL2, IL2 alone or were unstimulated (Table 2.11). PBMCs were also cultivated with IL2 + SAF CM, IL2 + GMCSF, IL2 + IL4, IL2 + IL7, IL7 alone or UCMC CM. PBMCs from eight patients were cocultivated with UCMCs. Skin samples were cultivated with PHA or ConA then IL2, IL2 alone, IL2 + GMCSF, IL2 + IL7 or IL7 alone or were unstimulated. Lymph node samples from two patients were cultivated with ConA then IL2, IL2 alone, IL2 + SAF CM, IL2 + GMCSF, IL2 + IL4, IL7, UCMC CM or with no stimulants and were also cocultivated with UCMCs.

Within 1 week of stimulation with PHA, ConA, IL2 alone or in combination with other cytokines and UCMC CM, lymphocytes in culture formed dense clumps of proliferating cells, between which were many single cells exhibiting morphological changes suggestive of activation. These proliferating cells were larger than non-proliferating cells and had protrusions from the cell membrane. The clumps of proliferating cells dispersed within a few weeks and most cells died within 1 to 2 months of initiation of cultures. The use of cytokines did not appear to result in extended survival of most cultured lymphocytes.

Four long-term T cell cultures (greater than 6 months duration in culture) were established from skin samples of patients with MF (patients 2531, 2600 and 2862) following stimulation with PHA then IL2, IL2 alone or IL2 + IL4 (Table 2.12). These cultures were maintained for 6 to 8 months and remained dependent on IL2 for the duration of cultivation. No viruses were detected by EM in any of these long-term cultures (Section 2.4.2) and supernatants were negative for RT activity (Section 2.4.3). All four long-term cultures were immunophenotyped as $CD8^+$ T cell populations by flow cytometry. Since the source neoplasms comprised cutaneous infiltrations of $CD4^+$ T cells, as is typical for MF, the proliferating $CD8^+$ T cells in these cultures were thought to represent long-lived IL2-driven cytotoxic T cell clones derived from non-neoplastic infiltrates (TILs) in the skin.

One EBV-infected B-LCL was cultivated for 5 months from a skin sample from patient 2132 who had CBCL (Table 2.12). This culture had been stimulated with PHA then IL2. EBV infection was demonstrated by staining for LMP-1 (Section 2.3.3.7).

In skin cultures from patients 2116, 2132, 2552 and 2578, adherent fibroblasts grew out from pieces of tissue, forming monolayers that could be grown for more than 6 months. In several cultures, lymphocytes and other mononuclear cells appeared to have enhanced survival when cultivated in suspension above the fibroblast monolayers, remaining viable for 2 to 4 months.

Testing for mycoplasma revealed infection with *Mycoplasma arginini* in the $CD8^+$ T cell line established from patient 2600, as well as in short-term cultures from patients 2116, 2560 and 2669.

2.4.2 Electron microscopy

Cells were submitted for EM examination from four $CD8^+$ T cell cultures that had been grown from three patients with MF for 6 to 8 months, as well as selected cultures from skin and PBMCs of five other patients with MF or SS that had been maintained for 21 to 50 days. EM examinations did not reveal any evidence of retrovirus-like particles (Table 2.12). Representative electron micrographs are shown in Figures 2.1 and 2.2.

Table 2.10: Clinical diagnoses of cutaneous T cell lymphoma cases from which viable cells were submitted for culture 1992 to 1995.

Clinical diagnosis	Number of patients
Mycosis fungoides	11
Sézary syndrome	3
Cutaneous T cell lymphoma	1
Cutaneous B cell lymphoma	3
Total	18

Table 2.11: Methods used for cultivating T cells from patients with cutaneous T cell lymphoma.

Protocol	Number of PBMC samples	Number of skin samples	Number of lymph node samples
PHA → IL2	13	8	0
ConA → IL2	11	5	2
IL2	18	10	2
IL2 + SAF CM	8	0	2
IL2 + GMCSF	9	2	2
IL2 + IL4	8	0	2
IL2 + IL7	2	1	0
IL7	8	2	2
UCMC CM	7	0	2
Cocultivation with UCMCs	8	0	2
Unstimulated	13	7	2

CM	Conditioned medium
ConA	Concanavalin A
IL2	Interleukin 2
IL4	Interleukin 4
IL7	Interleukin 7
GMCSF	Granulocyte macrophage colony stimulating factor
PHA	Phytohaemagglutinin
SAF	Sézary T cell activating factor
UCMC	Umbilical cord blood mononuclear cell

Table 2.12: Features of long-term cultures of cells from patients with cutaneous lymphoid infiltrates.

Patient number	Age (years)	Sex	Clinical diagnosis	Sample number	Sample type	Protocol	Duration in culture (months)	Type of cell line	Electron microscopy
2132	40	M	CBCL	14671	Skin	PHA → IL2	5	EBV LMP^+ B LCL	ND
2531	47	M	MF	13167	Skin	PHA → IL2	7	$CD8^+$ T cell line	–
2600	62	M	MF	13785	Skin	PHA → IL2	6	$CD8^+$ T cell line	–
				13786	Skin	IL2	8	$CD8^+$ T cell line	–
2862	58	M	MF	14679	Skin	IL2 + IL4	6	$CD8^+$ T cell line	–

B-LCL	B lymphoblastoid cell line
CBCL	B cell lymphoma
EBV	Epstein-Barr virus
IL2	Interleukin 2
IL4	Interleukin 4
LMP^+	Latent membrane protein positive
MF	Mycosis fungoides
ND	Not done
PHA	Phytohaemagglutinin
–	No viruses detected

Figure 2.1: Electron micrograph of lymphocytes from a $CD8^+$ T cell line cultivated from a patient with mycosis fungoides.

Culture derived from the skin of patient 2600 following stimulation with IL2. Photograph taken after 49 days in culture. One large lymphocyte (25 μm x 40 μm) exhibits features of activation, including dispersed chromatin and a prominent nucleolus in a slightly indented nucleus. There is abundant cytoplasm with moderately abundant rough endoplasmic reticulum, numerous mitochondria and multiple processes of up to 5 μm length protruding from the cell membrane. One small lymphocyte (10 μm x 12 μm) appears to be relatively inactive, with a small, condensed, indented nucleus and a narrow rim of cytoplasm containing few mitochondria. There are only a few relatively blunt protrusions from the cell membrane. 3,000x. Bar = 10 μm. Courtesy of Ross Blackley.

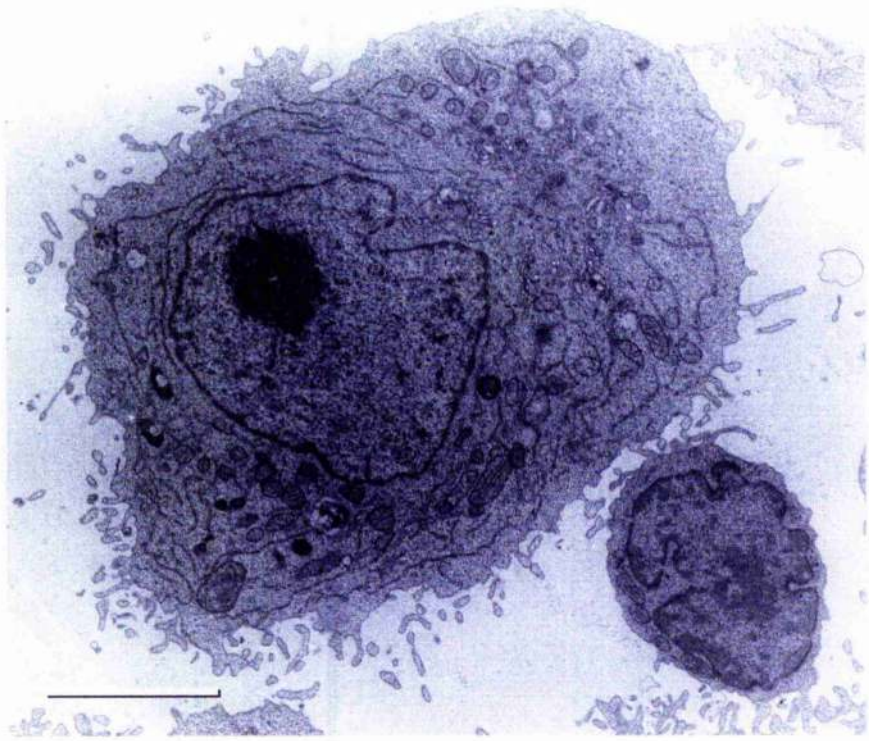
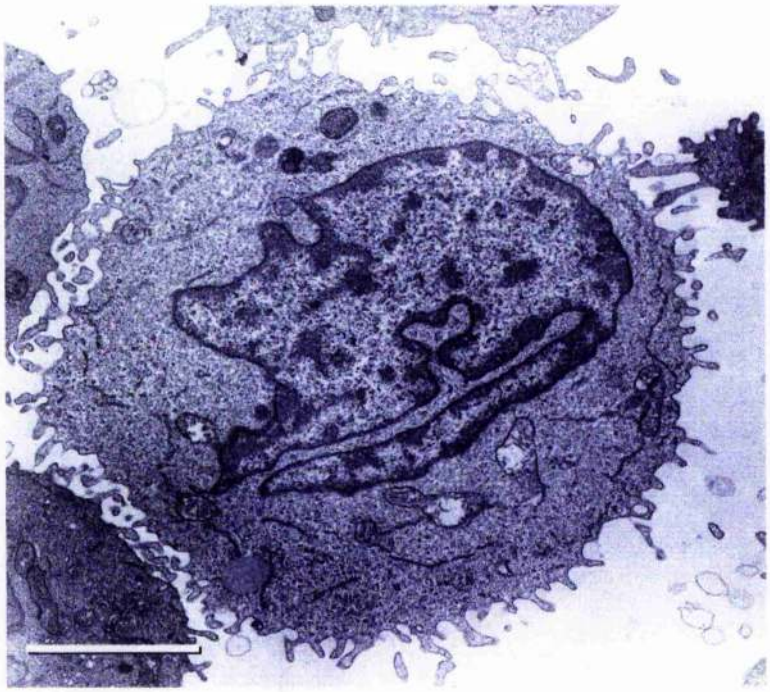


Figure 2.2: Electron micrograph of lymphocytes cultivated from peripheral blood mononuclear cells of a patient with Sézary syndrome.

Culture derived from PBMCs of patient 2669 following stimulation with IL2 and IL4. Photograph taken after 28 days in culture. One medium-sized lymphocyte (20 μm x 20 μm) has moderately condensed chromatin in a lobulated nucleus, relatively inconspicuous endoplasmic reticulum and few mitochondria in the cytoplasm. There are multiple processes up to 3 μm in length protruding from the cell membrane. 4,400x. Bar = 5 μm. Courtesy of Ross Blackley.



2.4.3 Reverse transcriptase assay

2.4.3.1 Standard reverse transcriptase assay

Results of RT assays are shown in Tables 2.13 to 2.15. All scintillation counts were taken from the average of two readings. RT assays were performed on 88 cell culture supernatants in 29 pools from 15 patients. Most samples were negative for RNA-dependent DNA polymerase (RT) activity (rA:N ≤10) with Mn^{2+} and Mg^{2+} as cofactors and also had negligible DNA-dependent DNA polymerase activity (dA:N ≤10) (Table 2.13). A pool of three supernatants collected 23 days after initiation of cultures of PBMCs and skin from patient 436 had an elevated rA:N value of 87.9 with Mg^{2+} as a cofactor. However, cultured cells from this patient did not survive longer than 1 month, the positive supernatants were derived from cultures containing mostly necrotic cells and no RT activity was detected in fresh cultures of PBMCs inoculated with the positive supernatants.

Supernatants from positive control cultures exhibited Mn^{2+} or Mg^{2+}-dependent RT activity corresponding to the known properties of the retroviruses with which they were infected (Table 2.14). Consistently high Mn^{2+}-dependent RT activity was detected in SSN-1 supernatants (rA/N >200), whereas this cell line exhibited low Mg^{2+}-dependent RT activity (rA/N ≤10). A wide variation in Mn^{2+}-dependent and Mg^{2+}-dependent RT activity was detected in BLV-FLK supernatants among assays, even using supernatants collected on the same date, but the level of Mg^{2+}-dependent RT activity was always at least five times the level of Mn^{2+}-dependent RT activity. There was an increased level of DNA-dependent DNA polymerase activity in some BLV-FLK supernatants with Mn^{2+} as a cofactor and this correlated with increased rA/N values in the same samples tested on the same date. The FIV-infected cell line FL4 exhibited high Mg^{2+}-dependent RT activity (rA/N 1,601.4), consistent with the known properties of lentivirus RTs.

The non-productive HTLV-I-infected $CD4^+$ T cell line C8166, as well as three continuous T cell lines not known to be infected with HTLV-I or other exogenous retroviruses (J.JHAN, L-726A, HH and My-La MC), had low Mn^{2+}-dependent and Mg^{2+}-dependent RT activity (rA:N ≤10) (Table 2.15). Normal tissue culture medium (RPMI 1640 + 20% hi-FBS + 2-ME) did not exhibit RT activity with either Mg^{2+} or Mn^{2+} as cofactors (rA/N ≤10).

2.4.3.2 Product-enhanced reverse transcriptase (PERT) assay

All of five samples of supernatants from cell cultures of patients with CTCL had negligible reactivity for Mg^{2+}-dependent RT activity and low Mn^{2+}-dependent RT activity by ELISA (optical densities at 405 nm and 630 nm, $OD_{405/630}$ <2.0) in the PERT assay (Böni and Schüpbach 1993, Pyra *et al* 1994) (Table 2.16).

Table 2.13: Results of reverse transcriptase assay on supernatants from cultured cells of patients with cutaneous lymphoid infiltrates.

Patient number	Source of supernatants (number pooled)	Date of RT assay	Mn^{2+}					Mg^{2+}				
			rA (cpm)	dA (cpm)	N (cpm)	rA/N	dA/N	rA (cpm)	dA (cpm)	N (cpm)	rA/N	dA/N
436	PBMCs/Skin (3)	13/2/94	866	1489	1137	0.8	1.3	507	433	386	1.3	1.1
	PBMCs/Skin (3)	25/2/94	678	481	320	2.1	1.5	489	882	378	1.3	2.3
	PBMCs/Skin (3)	10/3/94	439	891	8059	0.1	0.1	7295	460	83	87.9	5.5
1198/2	PBMCs (2)	10/3/94	598	539	613	1.0	0.9	840	768	169	5.0	4.5
1359/2	PBMCs/Skin (4)	10/3/94	464	973	470	1.0	2.1	518	846	107	4.8	7.9
2116	PBMCs/Skin (3)	10/3/94	1936	760	276	7.1	2.7	401	403	81	5.0	5.0
2132	Skin (3)	10/3/94	467	690	405	1.2	1.7	399	817	88	4.5	9.3
2475	LN (B-LCL) (1)	13/2/94	666	651	502	1.3	1.3	398	550	519	0.8	1.1
	LN (B-LCL) (1)	25/2/94	380	656	450	0.8	1.5	279	266	252	1.1	1.1
2522	PBMCs (5)	13/2/94	1421	720	772	1.8	0.9	215	159	235	0.9	0.7
	PBMCs (5)	10/3/94	678	868	399	1.7	2.2	376	492	298	1.3	1.7
2531	PBMCs (6)	14/5/93	1493	2392	1277	1.2	1.9	717	2091	719	1.0	2.9
	PBMCs (5)	14/5/93	803	1709	2088	0.4	0.8	1447	1592	1423	1.0	1.1
	PBMCs (2)	14/5/93	696	575	836	0.8	0.7	445	805	236	1.9	3.4
	PBMCs (1)	14/5/93	743	605	136	5.5	4.4	900	1389	139	6.5	10.0
	PBMCs (4)	14/5/93	732	2167	1282	0.6	1.7	986	2061	322	3.1	6.4
	PBMCs (4)	14/5/93	1370	1810	884	1.5	2.0	392	697	284	1.4	2.5
	PBMCs (3)	14/5/93	335	245	398	0.8	0.6	256	289	201	1.3	1.4
	PBMCs (5)	13/2/94	804	1603	722	1.1	2.2	240	196	354	0.7	0.6
2551	PBMCs (4)	13/2/94	401	807	614	0.7	1.3	436	204	178	2.4	1.1
	PBMCs (4)	25/2/94	322	470	352	0.9	1.3	353	334	548	0.6	0.6
2600	Skin (4)	13/2/94	280	424	393	0.7	1.1	135	203	300	0.4	0.7
	Skin (3)	10/3/94	406	619	508	0.8	1.2	439	515	129	3.4	4.0
2668	LN (1)	13/2/94	388	421	435	0.9	1.0	523	348	300	1.7	1.2
2669	LN/PBMCs (4)	13/2/94	590	1362	771	0.8	1.8	278	405	367	0.8	1.1
2746	PBMCs (2)	25/2/94	361	300	285	1.3	1.1	300	256	332	0.9	0.8
	PBMCs (1)	10/3/94	355	503	428	0.8	1.2	386	430	88	4.4	4.9
2757	PBMCs (1)	10/3/94	434	503	626	0.7	0.8	387	447	91	4.3	4.9

Table 2.13: (continued)

B-LCL	Epstein-Barr virus-infected B lymphoblastoid cell line
LN	Lymphocyte cultures derived from lymph node cells
PBMCs	Lymphocyte cultures derived from peripheral blood mononuclear cells
Skin	Lymphocyte cultures derived from skin
RT	Reverse transcriptase
cpm	Counts per minute
rA	RNA-dependent DNA polymerase activity (poly rA template)
dA	DNA-dependent DNA polymerase activity (poly dA template)
N	No template
dA/N	dA divided by N
rA/N	rA divided by N
Mg^{2+}	Magnesium cofactor
Mn^{2+}	Manganese cofactor

Table 2.14: Results of reverse transcriptase assay on supernatants from retrovirus infected cell lines and negative control cell culture medium.

Source of test sample	Date supernatant collected	Date of RT assay	Mn^{2+} rA (cpm)	Mn^{2+} dA (cpm)	Mn^{2+} N (cpm)	Mn^{2+} rA/N	Mn^{2+} dA/N	Mg^{2+} rA (cpm)	Mg^{2+} dA (cpm)	Mg^{2+} N (cpm)	Mg^{2+} rA/N	Mg^{2+} dA/N
SSN-1	28/5/93	2/2/94	130491	3272	483	270.4	6.8	900	558	234	3.8	2.4
	28/5/93	13/2/94	141692	1861	226	628.3	8.3	719	330	236	3.0	1.4
	28/5/93	25/2/94	129851	3206	550	236.1	5.8	1130	987	517	2.2	1.9
	28/5/93	10/3/94	91699	463	209	439.8	2.2	327	215	46	7.2	4.7
BLV-FLK	3/1/93	2/2/94	23971	8822	369	65.0	23.9	144318	1510	312	463.3	4.8
	3/1/93	13/2/94	1042	416	259	4.0	1.6	8191	509	298	27.5	1.7
	3/1/93	25/2/94	14330	4855	240	59.7	20.2	65128	985	297	219.6	3.3
	21/2/94	25/2/94	3534	2339	534	6.6	4.4	37586	1838	424	88.8	4.3
	4/3/94	10/3/94	19160	1100	161	119.0	6.8	115005	248	36	3239.6	7.0
FL4	20/5/93	2/2/94	181125	69438	2243	80.8	31.0	1209836	12264	756	1601.4	16.2
RPMI	22/1/94	2/2/94	821	1422	613	1.3	2.3	671	3264	537	1.2	6.1

SSN-1	Striped snakehead fish mixed cell line infected with snakehead fish retrovirus (Mn^{2+}-dependent RT activity)
BLV-FLK	Foetal lamb kidney fibroblast cell line infected with bovine leukaemia virus (Mg^{2+}-dependent RT activity)
FL4	Feline T cell line infected with Petaluma strain of feline immunodeficiency virus (Mg^{2+}-dependent RT activity)
RPMI	RPMI 1640 medium with 20% foetal bovine serum + 2-mercaptoethanol
RT	Reverse transcriptase
cpm	Counts per minute
rA	RNA-dependent DNA polymerase activity (poly-rA template)
dA	DNA-dependent DNA polymerase activity (poly-dA template)
N	No template
dA/N	dA divided by N
rA/N	rA divided by N
Mg^{2+}	Magnesium cofactor
Mn^{2+}	Manganese cofactor

Table 2.15: Results of reverse transcriptase assay on supernatants from established T cell lines.

Cell line	Date supernatant collected	Date of RT assay	Mn^{2+} rA (cpm)	Mn^{2+} dA (cpm)	Mn^{2+} N (cpm)	Mn^{2+} rA/N	Mn^{2+} dA/N	Mg^{2+} rA (cpm)	Mg^{2+} dA (cpm)	Mg^{2+} N (cpm)	Mg^{2+} rA/N	Mg^{2+} dA/N
C8166	14/2/94	25/2/94	543	959	220	2.5	4.4	231	239	136	1.7	1.8
J.JHAN	9/4/93	14/5/94	3042	1627	2963	1.0	0.5	4282	6750	2567	1.7	2.6
	14/2/94	25/2/94	1313	37647	239	5.5	157.8	487	16675	231	2.1	72.3
L-726A	15/10/92	2/2/94	969	26946	518	1.9	52.0	736	3196	1114	0.7	2.9
	15/10/92	13/2/94	384	6458	239	1.6	27.0	441	940	568	0.8	1.7
HH	14/2/94	25/2/94	981	2572	514	1.9	5.0	378	541	462	0.8	1.2
My-La MC	14/2/94	25/2/94	1460	1820	499	2.9	3.6	447	510	402	1.1	1.3

C8166	IL2-independent non-productive HTLV-I-infected $CD4^+$ T cell line (Salahuddin *et al* 1983)
J.JHAN	IL2-independent $CD4^+$ T cell line from patient with T cell acute lymphoblastic leukaemia (Schneider *et al* 1977)
L-726A	IL2-independent $CD4^+$ T cell line from patient with Sézary syndrome (Crae *et al* 1991)
HH	IL2-independent $CD4^+$ T cell line from patient with cutaneous T cell lymphoma (Starkebaum *et al* 1991)
My-La MC	IL2 and IL4-dependent $CD4^+$ T cell line from patient with mycosis fungoides (Kaltoft *et al* 1992)
RT	Reverse transcriptase
cpm	Counts per minute
rA	RNA-dependent DNA polymerase activity (poly-rA template)
dA	DNA-dependent DNA polymerase activity (poly-dA template)
N	No template
dA/N	dA divided by N
rA/N	rA divided by N
Mg^{2+}	Magnesium cofactor
Mn^{2+}	Manganese cofactor

Table 2.16: Results of product-enhanced reverse transcriptase (PERT) assay.

Patient	Clinical diagnosis	Source of supernatant	Optical density ($OD_{405/630}$)	
			Mn^{2+}	Mg^{2+}
1198	SS	Skin	1.7	0.0
2522	CTCL	PBMCs	1.9	0.1
2531	MF	PBMCs	0.0	0.0
2600	MF	Skin	1.7	0.2
2669	SS	PBMCs	1.7	0.0

2.5 Discussion

In the cell biology studies conducted at the LRF Virus Centre from 1992 to 1995, attempts were made to establish continuous $CD4^+$ T cell lines from patients with cutaneous lymphoid infiltrates, particularly MF and SS. The rationale for this approach was that HTLV-I had been isolated from continuous $CD4^+$ T cell lines established from patients with ATL and other diseases associated with HTLV-I (Poiesz *et al* 1980a, b, Miyoshi *et al* 1980, Hinuma *et al* 1981, Poiesz *et al* 1981, Jacobson *et al* 1988). T cell lines from patients with other forms of $CD4^+$ T cell neoplasia, particularly MF and SS, might also propagate HTLV-I or related retroviruses, allowing a viral aetiology to be established for these conditions. However, in this study no $CD4^+$ T cell lines were established from 158 cultures initiated from PBMCs, skin and lymph nodes of 18 patients (Tables 2.10 and 2.11). Most cells from clinical samples of patients with MF/SS, as well as cells from patients with other cutaneous lymphoid infiltrates, died within 3 months of initiation of cultures. This relatively short duration of survival is consistent with the findings of several other groups studying MF/SS (Capésius *et al* 1991, Bazarbachi *et al* 1997) (Section 1.11.1.4). The observation that the longevity of some lymphocyte cultures *in vitro* is increased in the presence of fibroblasts is also consistent with the findings of other groups (Scott *et al* 1990).

In contrast to the findings in this study, Abrams *et al* (1991b) established 25 T cell lines from patients with SS using SAF CM. GMCSF and IL2 were used as growth stimulants by Zucker-Franklin *et al* (1991) to establish 18 cell lines from patients with MF/SS but the immunophenotype of these cell lines was not reported. Few studies that have identified HTLV-related retroviruses in cell cultures from patients with CTCL have confirmed that the propagated cells are of T cell lineage (Kaltoft *et al* 1987, 1988); most of the cell lines from CTCL patients in which there is evidence for infection with a retrovirus are B cell lines (Manzari *et al* 1987, Hall *et al* 1991) or are uncharacterised (Zucker-Franklin *et al* 1991, 1992). In an HTLV-II-infected cell line established by Zucker-Franklin *et al* (1992) from a patient with MF, 80% of cells were $CD2^+$ and only 50% were $CD4^+$ after 71 days in culture, so this culture may contain a mixed population of cells.

HTLV-II has been isolated from $CD8^+$ T cell lines established from patients with T cell variants of HCL, but its role as an aetiological agent in T cell or other neoplasia has not been confirmed (Kalyanaraman *et al* 1982b) (Section 1.7.1). The techniques used in this study were also suitable for cultivating $CD8^+$ T cells and four long-term $CD8^+$ T cell cultures were established from skin samples of three patients with MF. However, these cultures were not derived from neoplastic cells, which in all MF/SS cases consisted of $CD4^+$ T cells. Instead, the $CD8^+$ T cells would have expanded from TILs accompanying the neoplastic infiltrate. Other studies have found that most T cell clones cultured from MF lesions are not derived from neoplastic MF/Sézary cells but originate from TILs (Ho *et al* 1990, Harwix *et al* 2001). Depletion of $CD8^+$ T cells (TILs) may permit the outgrowth of neoplastic $CD4^+$ T cell clones from MF lesions (Kaltoft *et al* 1984). This approach was used frequently at the LRF Virus Centre from 1987 to 1991 but did not result in the establishment of any continuous $CD4^+$ T cell lines.

EM, RT assays and PCR were used for detection of retroviruses in cultured cells in this study. No retroviruses were detected by EM in four continuous $CD8^+$ T cell lines established from three patients with MF or in short term cell cultures from five other patients with MF or SS. RT activity was detected in one pool of supernatants from degenerating cells of one patient, but this result could not be reproduced because all cultured cells from this patient died within one month. The results of PCR analyses conducted on cultured cells are presented in Chapter 3.

In this study one EBV-infected B-LCL was established from the skin of a patient with CBCL, consistent with the *in vitro* transforming properties of EBV (Pattengale *et al* 1973). The B-LCL is more likely to be derived by EBV infection of bystander lymphocytes than from neoplastic B cells, since most primary CBCLs are not associated with EBV, except in immunocompromised patients (Dupin *et al* 1997, Beylot-Barry *et al* 1999, Nagore *et al* 2000). However, detailed molecular genetic, viral and immunophenotypic analyses were not undertaken on the B-LCL or CBCL in this case.

Mycoplasma infection was detected in several cell cultures established from clinical samples and was though to be derived from infected source material rather than from contamination in the laboratory.

The results of these studies and the use of cell culture for isolation of novel retroviruses are discussed further in Chapter 5.

Chapter 3

Use of Molecular Techniques to Detect Human T Lymphotropic Virus-Related Retroviruses in Human T Cell Leukaemias and Lymphomas

3.1 Introduction

3.1.1 Molecular approach to detection of human T lymphotropic viruses in T cell neoplasms

The aim of the molecular biology part of this project was to detect proviral sequences of human T lymphotropic virus type I (HTLV-I), HTLV-II or related retroviruses in human T cell neoplasms using the polymerase chain reaction (PCR) in combination with Southern blot (SB) hybridisation. HTLV-I causes adult T cell leukaemia/lymphoma (ATL) (Section 1.6.1), whereas HTLV-II has not been confirmed as the cause of neoplasia (Section 1.7.1). The hypothesis tested in this study was that HTLV-I, HTLV-II or related retroviruses are associated with other T cell leukaemias and lymphomas, particularly mycosis fungoides (MF) and Sézary syndrome (SS), in the United Kingdom (UK). These neoplasms have similar clinicopathological features to some forms of ATL (Section 1.11.1). However, the prevalence of HTLV-I and HTLV-II infection in the UK is low, except in populations derived from endemic regions (Section 1.5.2.1). Cases of large granular lymphocytic (LGL) leukaemia were also studied because HTLV-I and HTLV-II have been identified in a small proportion of cases of this disease (Section 1.11.2).

Bovine leukaemia virus (BLV) causes B cell leukaemia and lymphoma in cattle and is endemic in the UK (Chasey *et al* 1978) (Section 1.9.2.1). Although BLV is not known to be a zoonosis, samples from cases of acute lymphoblastic leukaemia (ALL) in butchers from Cardiff were examined for this virus because of their occupational exposure to cattle (Whittaker 1991) (Section 1.10.5). Samples were also tested for HTLV-I and HTLV-II because these viruses are related to BLV.

The molecular techniques used in attempts to identify HTLV-I and related retroviruses in samples from patients with cutaneous T cell lymphoma (CTCL), LGL leukaemia and adult ALL at the Leukaemia Research Fund (LRF) Virus Centre are described in this chapter. PCRs were designed to amplify HTLV-I, HTLV-I/II and BLV proviral DNA sequences from clinical samples and cultured cells from these patients. PCR products were hybridized to HTLV-I or BLV-specific nucleotide probes and products of interest were cloned and sequenced. Results of these studies were used to assess the role of HTLV/BLV group viruses in selected cases of CTCL, LGL leukaemia and adult ALL in the UK.

3.1.2 Detection of retroviral sequences in T cell neoplasms by the polymerase chain reaction

PCR is a method for amplifying specific DNA sequences from target DNA using primer extension and a thermostable DNA polymerase (Saiki *et al* 1985, Mullis and Faloona 1987, Saiki *et al* 1988). Successive cycles of template denaturation, primer annealing and extension result in exponential amplification of target sequences depending on the efficiency of reaction conditions. The technique is highly sensitive and can detect low copy numbers of target DNA sequences. Thus, it is the tool of choice for detecting integrated proviruses in samples in which infected cells may be rare. The frequency of infected peripheral blood mononuclear cells (PBMCs) in HTLV-I carriers may be low (Section 1.6.3.3). Skin lesions from patients with MF/SS and other CTCLs often have low densities of neoplastic lymphocytes, which form part of a population including non-neoplastic tumour infiltrating lymphocytes (TILs) and other inflammatory cells, as well as keratinocytes, fibroblasts, endothelial cells, melanocytes and other cutaneous cell populations.

The sensitivity of detection can be increased by nested PCR, in which a second round of reactions is performed using primer sets specific for sequences within the first round PCR product. SB hybridisation using sequence-specific nucleotide probes that bind to products amplified by PCR can also be used to increase the sensitivity of detection. The stringency of hybridisation can be adjusted to allow detection of sequences with high or low similarity to that of the probe. Hybridisation under conditions of high stringency provides a measure of confirmation of the specificity of product amplification. Hybridisation under conditions of low stringency can be used to detect target sequences that are similar but not identical to the sequence of the probe and could allow detection of novel retroviruses. PCR is also a potentially useful tool for detecting novel retroviral sequences, since primers can be selected from conserved regions of known viruses (Section 3.1.2.3).

In this study, careful attention was paid to preparation of clinical samples, design of PCR primers, standardisation and optimisation of PCRs, determining the sensitivity of detection of target sequences and preventing PCR contamination.

3.2 Sources of clinical samples

Details of patients from whom clinical samples were obtained are given in Section 2.2. The methods for processing clinical samples and cultured cells are described in Chapter 2.

3.2.1 Cutaneous T cell lymphomas and other cutaneous lymphoid infiltrates

Samples of cultured and uncultured PBMCs and skin from 36 patients with cutaneous lymphoid infiltrates, predominantly MF and SS, were examined by PCR for HTLV-I and HTLV-I/II sequences (Tables 2.3 and 3.1).

Table 3.1: Clinical diagnoses of patients with cutaneous lymphoid infiltrates from whom samples were analysed by the polymerase chain reaction.

Clinical diagnosis	Number of patients
Mycosis fungoides	21
Sézary syndrome	5
Cutaneous T cell lymphoma	1
Angiocentric T cell lymphoma	3
Small plaque parapsoriasis	1
Langerhan's cell histiocytosis	1
Cutaneous B cell lymphoma	4
Total	36

3.2.2 Large granular lymphocytic leukaemia

Cultured and uncultured PBMCs from six patients with $CD3^+$ $CD8^+$ LGL leukaemia were tested for HTLV-I and HTLV-I/II sequences by PCR (Table 2.4).

3.2.3 Adult acute lymphoblastic leukaemia in butchers from Cardiff

PBMCs and bone marrow from six patients with ALL, all male butchers from Cardiff, were tested for HTLV-I, HTLV-I/II and BLV sequences by PCR (Table 2.5).

3.3 Materials and methods

3.3.1 Molecular biology facilities

The molecular biology laboratory in the LRF Virus Centre was used for gel electrophoresis of PCR products and restriction endonuclease (RE)-digested DNA, Southern blotting and electroblotting, molecular cloning, purification of plasmids and most other molecular work. A separate room was used for bacterial culture work and a dark room was used for ultraviolet (UV) transilluminaton, photography and exposing autoradiographic film. Radioisotopes were handled in a dedicated radioactivity room in the Department of Veterinary Pathology.

3.3.2 Materials

The sources of frequently used materials are listed in Appendix 3 and the compositions of routinely used stock solutions and buffers for molecular biology are listed in Appendix 5. Additional materials and chemicals are specified elsewhere in the text of individual sections. All chemicals used were of Analytical (Analar) or Molecular Biology grade and, unless otherwise stated, were obtained from Sigma or BDH. Most restriction and DNA modifying enzymes were obtained from Life Technologies. *Taq* polymerase (Amplitaq) and other PCR reagents were obtained from Perkin-Elmer. Ultrapure deionised water obtained from a reverse osmosis filtration system (Millipore) was used in enzymatic manipulations and to dissolve DNA. PCR grade sterile ultrapure water (Sigma) was used for PCRs. Deionised water (Millipore) was used in other general laboratory solutions.

3.3.3 Extraction of high molecular weight DNA from tissues and cells

3.3.3.1 Enzymatic digestion of tissues and cells

High molecular weight (HMW) DNA was extracted in class II microbiological safety cabinetes (MSCs) in the level II containment facility (Section 2.3.1). Cells for DNA extraction were collected from established cell lines (Section 2.3.4), cultured clinical samples (Section 2.3.6) and uncultured PBMCs and lymph node cells. Purification of uncultured PBMCs from blood by metrizoate-Ficoll density gradient centrifugation is described in Section 2.3.5.1. The densities of cells in suspension were determined by counting using an haemocytometer (Section 2.3.3.3). Aliquots containing 1×10^6 to 1×10^8 cells were transferred to 15 ml screw-

top polypropylene tubes and centrifuged at 1,500 *g* (2,700 rpm) in a GPR bench centrifuge (Beckman) with a GH 3.7 swinging bucket rotor for 5 min at room temperature. The supernatant was discarded, cells were resuspended in phosphate-buffered saline (PBS) 1x and the centrifugation was repeated. Cell pellets were stored at -80°C until processed for PCR. Pellets of fresh PBMCs and cultured cells were resuspended in Tris-NaCl-EDTA (TNE) 1x then centrifuged at 270 *g* for 5 min. The supernatant from each pellet was discarded and the cells were resuspended in 5 ml TNE 1x containing 100 μg/ml proteinase K, 0.2 M NaCl and 0.5% sodium dodecyl sulphate (SDS). The mixture was incubated at 55°C for 1 hour or at 37°C overnight (16 to 20 hours).

Preparation of tissues for DNA extraction is described in Section 2.3.5.2. Frozen tissue was cut into small pieces with sterile scissors, placed in sterile plastic bags with 5 to 25 ml of TNE 1x and mechanically disrupted using a Stomacher 80 (Colworth). The contents were transferred to 15 or 50 ml screw-top polypropylene centrifuge tubes and incubated with proteinase K, NaCl and SDS as above.

3.3.3.2 Phenol-chloroform extraction

After SDS-proteinase K digestion, protein and lipids were separated from dissolved DNA by phenol-chloroform extraction. An equal volume of phenol equilibrated with Tris pH 8.0 (Rathburn Chemical Company) was added to each tube at room temperature and the tube was gently inverted to mix the aqueous and organic phases. Tubes were centrifuged at 484 *g* (2,000 rpm) for 10 min in a JS-21 centrifuge (Beckman) using a JA-20 fixed angle rotor to separate the aqueous and organic phases. The upper aqueous phase containing dissolved DNA was transferred to a fresh tube using a wide-bore polypropylene bulb pastette to prevent shearing. Phenol extraction was performed twice then followed by extraction with chloroform or isoamyl alcohol:chloroform (24:1) by the same method.

3.3.3.3 Precipitation and dissolution of DNA

After chloroform extraction, the aqueous phase was transferred by pastette into approximately 2.5 times the volume of 99.7% ethanol (absolute alcohol) to precipitate DNA. HMW DNA, which formed a flocculent suspension, was spooled onto a polypropylene pastette or sealed glass pipette and transferred to a sterile 1.5 ml screw-top prolypropylene microcentrifuge tube. If the concentration was too low for the precipitate to be visualised, the ethanol solution was transferred to a 30 ml glass centrifuge tube (Corex, Sarstedt) and centrifuged at 12,100 *g* (10,000 rpm) for 30 min in the JS-21 centrifuge using a JA-20 rotor. DNA pellets were washed in 1 ml 70% ethanol and air dried at room temperature. The DNA was dissolved in an appropriate volume of ultrapure water or Tris-EDTA (TE) 1x by incubating at 37°C overnight on a roller mixer.

3.3.3.4 Quantification of genomic DNA

The concentration of double-stranded genomic DNA was determined by measuring the optical densities at 260 nm (OD_{260}) and 280 nm (OD_{280}) in a spectrophotometer

(GeneQuant). Calculations were made on the basis that 50 µg/ml of double-stranded DNA has an OD_{260} of approximately 1.0. The ratio of the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) was used to estimate the purity of the nucleic acid; pure preparations of DNA have an OD_{260}/OD_{280} ratio of 1.6 to 1.8 (Sambrook *et al* 1989).

3.3.3.5 Restriction endonuclease digestion of genomic DNA

Genomic DNA was digested with restriction endonucleases (REs) for gel electrophoresis and Southern blot hybridisation (Section 3.3.10). RE digests were performed with 10 µg HMW DNA incubated with 30 U of the appropriate enzyme (Life Technologies) in a 50 µl volume containing proprietary buffer at 1x concentration and 3 mM spermidine (GeneBloc, International Laboratory Services). To prevent inhibition by glycerol in RE storage buffers, the volume of RE solutions added was kept below 10% of the total reaction volume. Reactions were incubated at 37°C overnight. DNA from the C8166 cell line was digested with *Eco*RI, *Pst*I, *Sst*I, *Sst*I/*Hin*dIII, *Sst*I/*Xho*I and *Sst*I/*Bam*HI for assessment of proviral integrations by SB hybridisation (Section 3.3.11.2).

3.3.4 Processing of cells for the polymerase chain reaction

3.3.4.1 Preparation of cells for PCR

Cell pellets for PCR were prepared by diluting cultured and uncultured cells to 1 x 10^5 cells/ml in PBS 1x in the level II containment facility (Sections 2.3.3.3 and 2.3.5.1). An appropriate number of 0.5 ml aliquots containing 1 x 10^5 cells were transferred to 0.6 ml flip-top PCR tubes and centrifuged at 10,500 *g* (13,000 rpm) for 5 min in a bench microcentrifuge (MicroCentaur, MSE). Supernatants were discarded, leaving the pelleted cells in the bottom of each tube. These cell pellets were stored frozen at -80°C until used for PCR analysis. Some cryopreserved cells were prepared for PCR by thawing as described in Section 2.3.3.5. After centrifuging and washing cells with PBS 1x, the density of viable cells was determined (Section 2.3.3.3) and pellets of 1 x 10^5 cells for PCR were prepared as above. Serial dilutions of positive and negative control cells for use in determining PCR sensitivity are described in Section 3.3.8.2.

3.3.4.2 Non-ionic detergent lysis and proteinase K digestion of cells for PCR

To disrupt cells and inactivate proteins in preparation for PCR, 25 µl of non-ionic detergent buffer (NIB) consisting of 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris pH 8.2, 100 µg/ml gelatin, 0.45% Nonidet P-40 (NP-40), 0.45% Tween-20 and 60 ng/µl proteinase K were added to pellets of 1 x 10^5 cells in PCR tubes in the LRF Virus Centre level II containment facility (Appendix 5: A5.6). The buffer was overlain with two drops (approximately 25 µl) of mineral oil (Sigma) using a polypropylene pastette. Tubes were sealed with parafilm (Nesco sealing film, Bando) or placed in a locking rack (Treff) and incubated at 55°C for 1 hour in a heated water bath (Techne). The proteinase K was inactivated by heating at 95°C in a thermal block (Techne) or boiling for 10 min. Tubes containing lysed cells were then placed on ice and 25 µl of PCR master mix were added in preparation for thermal cycling.

3.3.5 Design of oligonucleotide primers for the polymerase chain reaction

3.3.5.1 Principles of primer selection

Sets of oligonucleotide primers for PCR were designed for annealing to specific sequences of target DNA to act as primers for incorporation of oligonucleotides by *Taq* DNA polymerase. Forward (sense) primers allowed extension from 5' to 3' on the negative strand of DNA, while complementary reverse (antisense) primers allowed extension from 5' to 3' on the positive strand. The size of the intervening sequence for efficient amplification by PCR was between 100 and 400 base pairs (bp). Primer sets for PCR were selected with a preference for oligonucleotides of length 17 to 21 bases with similar melting temperatures (T_m) within the range 55 to 65°C. Strings of identical nucleotides were avoided, as were excesses of G and C residues. The sequence at the 3' end of each primer was considered most important for specificity of annealing and a G or C was selected for the 3' terminal residue. Primers were checked for the absence of complementary sequences that might permit self-annealing and amplification of non-specific products.

Theoretical values for annealing temperatures of oligonucleotide primers can be determined using formulae for calculating the T_m of double-stranded DNA. The following formula is applicable to oligonucleotides of 11 to 23 bases, which includes most PCR primers used in this study (Suggs *et al* 1981):

Formula 3.1: $T_m = 4\,(G+C) + 2(A+T)$ °C

Where: A, C, G and T are the number of bases of each nucleotide in the primer. Primers with degenerate bases such as K, M, R or Y were assumed to have been synthesised with an equal proportion of purine and pyrimidine residues and their number was multiplied by three (Appendix 2).

This formula was used to compare the T_m values of forward and reverse primers in a set, with the aim to select primers with similar annealing temperatures. Formula 3.2 was used to calculate the T_m of the 29 base primer 3 (Section 3.3.12.1):

Formula 3.2: $T_m = 81.5 + 16.6 \log_{10}[M^+] + 0.41(\%G+C) - 500/L - P - 0.63(\%\text{Formamide})$ °C

Where: $[M^+]$ = Adjusted molar Monovalent cation concentration: $\dfrac{[Na^+]}{1 + 0.7\,[Na^+]}$

%G+C = Percentage of G and C nucleotides in the DNA

L = Length of the DNA:DNA duplex in bp

P = Percentage base mismatch

However, such theoretical values do not reliably predict the compatibility of any pair of primers. Similarly, although an annealing temperature 5°C below the calculated T_m of the primers is often used as a guide to selecting PCR thermal cycling conditions, it is usually necessary to determine the optimal annealing temperature empirically (Section 3.3.7.3).

3.3.5.2 Design of primers for amplification of HTLV-I, HTLV-II and BLV by PCR

Primer sets for amplification of HTLV-I, HTLV-I/II and BLV sequences by PCR were designed at the LRF Virus Centre based on alignments of viral sequences and on previously published information (Appendices 6 to 8). Incomplete proviruses with preferential retention of the *pX* region are frequent in neoplastic cells of patients with ATL (Section 1.6.1.4) and have been suspected in patients with MF/SS (Section 1.11.1.6). The possibility that proviruses with deletions are involved in the aetiology of CTCLs was addressed by selecting PCR primers from the LTR, *gag*, *pol*, *env* and *pX* regions of the HTLV-I genome (Section 1.3.3). Use of the full panel of primer sets on any infected sample would allow detection of some viral sequences by PCR even if other regions of the proviral genome were absent due to deletions. The use of primer sets with different sensitivities could lead to false deductions that incomplete proviruses are present and therefore it was important to optimise and quantify the sensitivity of PCR reactions.

Published sets of primers for PCR that reliably amplified HTLV-I *pol* and HTLV-I/II *pX* sequences were used as standards for detection of HTLV-I and HTLV-II (Kwok *et al* 1988). LTR, *gag* and *pX* primers that had been used to detect defective HTLV-I proviruses in patients with MF were adopted from Hall *et al* (1991). Additional HTLV-I *gag* primers were those used to detect variant (Melanesian strain) HTLV-I in Papua New Guinea (Sherman *et al* 1992). Primers for *env* had previously been used in attempts to detect HTLV-I sequences by PCR in patients with multiple sclerosis (MS) (Greenberg *et al* 1989a) (Section 1.6.4.6). Two additional sets of unpublished HTLV-I *pol* and *pX* PCR primers that had been used at the LRF Virus Centre previously were also included in the study (primer sets 3 & 4 and 67 & 68). Primers for amplification of BLV *gag* sequences were adapted from a published study (Murtaugh *et al* 1991).

Amplification of a sequence of β-globin was used as a test for the integrity of DNA (Saiki *et al* 1988) (Appendix 9). These PCR primer sets are shown in Figure 3.1. Accepted letter codes for nucleotides and amino acids, including nomenclature for incompletely specified bases, are listed in Appendix 2.

3.3.5.3 Design of consensus retroviral PCR primers

There is a high degree of sequence divergence over most of the genome of known retroviruses and thus few regions are available with a high degree of similarity from which consensus PCR primers can be selected. However, regions of nucleotide conservation in the retroviral *pol* gene and amino acid conservation in the translated product of this gene, reverse transcriptase (RT), offer potential for the design of consensus primers that can be used for detection of novel retroviruses. Consensus PCR primers with generic specificity for the HTLV/BLV (*Deltaretrovirus*) group were derived by alignment of regions of similarity

among the HTLV-I, HTLV-II and BLV virus LTR and *gag* nucleotide sequences (Figure 3.2). Alignments of sequences in the GenEMBL database were made using the Genetics Computer Group (GCG) programmes on the University of Glasgow VAX and UNIX mainframe computers. Consensus primers were selected from these regions of homology on the assumption that they are conserved in other, as yet unidentified, HTLV-related viruses. Where regions of homology did not provide for a perfect match of bases among the viruses, degenerate primers were used. This resulted in the identification of one LTR sense primer (174) that could be used in conjunction with either of two antisense primers to yield PCR products of 491 to 522 bp (LTR primer 175) or 1823 to 1858 bp (*gag* primer 176) from HTLV/BLV group viruses (Figure 3.2).

Consensus primers with potential to detect a wide range of retroviruses and hepadnaviruses were also designed by alignment and reverse translation of Pol amino acid sequences (Mack and Sninsky *et al* 1988, Donehawer *et al* 1990). Two regions of conservation in the retroviral RT protein have been identified that are sufficiently close together for amplification of a product of suitable size by PCR (Appendix 10: A10.1). The number of permutations of nucleotide codons for the invariant amino acids in these regions is relatively small, facilitating design of degenerate primers. The 5' conserved region contains the motif LPQG, which is consistent among exogenous retroviruses except for the substitution of R for G in simian retrovirus 1 (SRV-1). Group-specific 5' primers can be derived from a 9 amino acid segment in this region with consistent LPQ and SP residues. The 3' conserved amino acid sequence contains the motifs YMDD or YVDD. The regions between these primer sequences differ among the retroviruses and can be used for specific identification by hybridisation, nested PCR or sequencing.

A potential problem with retroviral consensus primers is that they will bind to endogenous retroviral sequences, resulting in the generation of PCR products that are difficult to distinguish from those of exogenous retroviruses. This can be overcome by the use of RT-PCR to detect viral genomic RNA in purified virions or mRNA transcripts in infected cells. Donehawer *et al* (1990) used RT-PCR with two degenerate oligonucleotide primers to amplify a 117 bp *pol* fragment from genomic RNA of all retroviruses examined.

Consensus primers adapted from Mack and Sninsky (1988), Donehawer *et al* (1990) and designed by aligning retroviral sequences selected from the GenEMBL database using the GCG programmes are shown in Figure 3.3. These primers (296 & 297) have the potential to amplify a retroviral *pol* sequence of approximately 113 bp. *Bgl*II restriction sites for cloning were added at the ends of both of these primers, resulting in a potential PCR product of approximately 133 bp.

Figure 3.1: Oligonucleotide primers for amplification of HTLV-I, HTLV-II, bovine leukaemia virus (BLV) and β-globin sequences by the polymerase chain reaction.

Letter codes for nucleotides, including nomenclature for incompletely specified bases, are listed in Appendix 2.

LRF oligonucleotide number		Primer sequence 5' to 3' with HTLV-I nucleotide position (HTLV-II nucleotide positions in brackets)	Length (b)	T_m (°C)	Size of PCR product (bp)	Reference
HTLV-I LTR						
118	Sense	582 TGACCCTGCTTGCTCAACTCTA 603	22	66	183	(Hall *et al* 1991)
119	Antisense	764 TCTCTCCTGAGAGTGCTATAG 744	21	62		
HTLV-I *gag*						
120	Sense	1388 CTGCAGTACCTTTGCTCCTCCCTC 1411	24	76	273	(Sherman *et al* 1992)
121	Antisense	1660 TTCTACGAAGGCGTGGTAAG 1641	20	60		
122	Sense	1423 CCATCACCAGCAGCTAGATAGC 1444	22	68	134	(Hall *et al* 1991)
123	Antisense	1556 GCTGGTATTCTCGCCTTAATCC 1535	22	64		
HTLV-I *pol*						
1	Sense	3366 CTTCACAGTCTCTACTGTGC 3385	20	60	119	(Kwok *et al* 1988)
2	Antisense	3484 CGGCAGTTCTGTGACAGGG 3466	19	62		
67	Sense	4758 CCMTACAAYCCMACCAGCTCAG 4779	22	69	186	Unpublished
68	Antisense	4943 GTGGTGRAKYTGCCATCGGGTT 4922	22	69		
HTLV-I *env*						
124	Sense	5800 CTCGAGCCCTCTATACCATG 5819	20	62	327	(Greenberg *et al* 1989)
125	Antisense	6126 GGATCCTAGGGTGGGAACAG 6107	20	60		
HTLV-I/II *pX*						
		(7248) (7267)				
50	Sense	7359 CGGATACCCAGTCTACGTGT 7378	20	62	159	(Kwok *et al* 1988)
51	Antisense	7517 GAGCCGATAACGCGTCCATCG 7497	21	68		
		(7406) (7386)				
		(7370) (7398)				
3	Sense	7481 TCACCTGGGACCCCATCGATGGACGCGTT 7509	29	68	112	Unpublished
4	Antisense	7592 GTAAGGACCTTGAGGGTC 7575	18	56		
		(7481) (7464)				
126	Sense	7597 CCAATCACTCATACAACCCCCA 7618	22	66	127	(Hall *et al* 1991)
127	Antisense	7723 CTGGAAAAGACAGGGTTGGGA 7703	21	64		
BLV *gag*						
194	Sense	1109 CTGACCTAGAACAACTTTGC 1128	20	58	247	(Murtaugh *et al* 1991)
195	Antisense	1355 GACGAGTAGGGAGATTTTTCC 1315	21	62		
β-globin						
18	Sense	14 ACACAACTGTGTTCACTAGC 33	20	58	91	(Saiki *et al* 1988)
52	Antisense	123 CAACTTCATCCACGTTCACC 104	20	60		

Figure 3.2: Consensus oligonucleotide primers for amplification of human T cell lymphotropic/bovine leukaemia virus group viral LTR sequences by the polymerase chain reaction.

Letter codes for nucleotides, including nomenclature for incompletely specified bases, are listed in Appendix 2.

HTLV/BLV group viruses	Sequence alignment	Length (b)	T_m (°C)
Sense			
HTLV-I	779 TTGGGGGCTCGTCCGGGAT 797		
HTLV-II	765 TTGGGGGCTCGTCCGGGAT 783		
BLV	532 TTGGGGGCTCGTCCGGGAT 550		
	*******************		
Consensus sense LTR primer 174	5′ > TTGGGGGCTCGTCCGGGAT < 3	19	61
Antisense			
HTLV-I	1267 AGACCTACAGGCCATTAA 1284		
HTLV-II	1268 AGACTTACAGGCCATCAA 1286		
BLV	1005 AGAATTACAAGATATCAA 1022		
	*** **** * ** **		
Consensus sequence	5′ > AGAMYTACARGMYATYAA < 3′		
Consensus antisense LTR primer 175	5′ > TTRATRKCYTGTARKTCT < 3′	18	48
HTLV-I	2611 CCTTTAAACCAGAACGCCTCCAGGCC 2636		
HTLV-II	2585 CCTTTAAACCTGAGCGCCTCCAGGCC 2610		
BLV	2330 CCTTTAAACTAGAACGCCTCCAGGCC 2355		
	********* ** **********		
Consensus sequence	5′ > CCTTTAAACYWGARCGCCTCCAGGCC < 3′		
Consensus antisense *gag* primer 176	5′ > GGCCTGGAGGCGYTCWRGTTTAAAGG < 3′	26	54

Expected PCR product sizes	174 & 175	HTLV-I	506 bp
		HTLV-II	522
		BLV	491
	174 & 176	HTLV-I	1858 bp
		HTLV-II	1846
		BLV	1823

Figure 3.3: Consensus reverse transcriptase (*pol*) primers for amplification of retroviral sequences by the polymerase chain reaction.

Letter codes for nucleotides, including nomenclature for incompletely specified bases, are listed in Appendix 2.

LRF oligonucleotide number		Primer sequence 5' to 3' with HTLV-I nucleotide position	Length (b)	T_m (°C)	Size of PCR product (bp)
Consensus retroviral *pol*					
		BglII			
296	Sense	2976 GCCGAGATCTYTNCCNCARVG 2986	21	51	~133
297	Antisense	3088 GCCGAGATCTTCRTCNAYRTA 3078	21	46	
		BglII			

3.3.6 Preparation of oligonucleotides

3.3.6.1 Oligonucleotide synthesis and purification

Oligonucleotides for use as PCR primers were manufactured on an automated synthesiser and cartridge-purified (Alta Bioscience or Genosys). They were dissolved in ultrapure water and stored external to the Department of Veterinary Pathology to reduce the risk of PCR contamination (Section 3.3.8). An aliquot from each solution was quantified (Section 3.3.6.2) and the remaining volume was diluted to an appropriate concentration (usually 10 μM) after optimisation for PCR (Section 3.3.7.3).

Selected oligonucleotides were produced within the Department of Veterinary Pathology using an automated synthesiser (Model 381A, Applied Biosystems). These were used as primers for PCRs not involving clinical samples, for example for cloning, and for use as probes for hybridisation to PCR products. They were cleaved from the synthesis cartridge by flushing with 2 ml of 30% ammonia over a 2 hour period and then deprotected by incubating at 55°C in the ammonia solution overnight. The solution was divided into aliquots and desiccated by centrifugation under vacuum (Speedvac SC100, Savant) to remove the ammonia before being dissolved in ultrapure water.

3.3.6.2 Quantification of oligonucleotides

Concentrations of oligonucleotides were determined by measuring OD_{260} and OD_{280} in a spectrophotometer (Section 3.3.3.4). Calculations were made on the basis that 30 μg/ml single-stranded DNA has an OD_{260} of approximately 1.0. Pure preparations of single stranded DNA have an OD_{260}/OD_{280} ratio of 1.6 to 1.8 (Sambrook *et al* 1989). The molar concentration was calculated on the basis that 1 pmol of a single-strand of the 4.3 kb plasmid pBR322 contains 1.4 μg DNA.

3.3.7 Polymerase chain reaction

3.3.7.1 Standard PCR master mixes

Standard PCRs were performed in 0.6 ml PCR tubes in 50 μl volumes containing target DNA, 200 μM each deoxynucleoside triphosphates (dATP, dCTP, dGTP and dUTP or dTTP), 50 mM KCl, 10 mM Tris pH 8.2, 0.05% Nonidet P-40 (NP-40), 0.02 U/μl *Taq* thermostable DNA polymerase (Amplitaq DNA Polymerase, Perkin-Elmer) and PCR grade sterile ultrapure water with optimised concentrations of $MgCl_2$ and oligonucleotide primers. Most oligonucleotides were used at a final concentration of 0.5 or 1.0 mM after optimisation (Section 3.3.7.3). The range of $MgCl_2$ concentrations used in PCRs was 1.0 to 3.0 mM, with 1.5 mM being optimal for most primer sets (Section 3.3.7.3). To prevent evaporation during thermal cycling, the reaction was overlaid with two drops (approximately 25 μl) of mineral oil.

PCR master mixes were prepared with concentrations of reagents allowing for the final addition of the DNA template to a total volume of 50 µl. Usually 10 µl of a 100 ng/µl solution of purified DNA (1 µg) were mixed with 40 µl of PCR master mix. In PCRs using cell pellets, 25 µl of PCR master mix with constituents at appropriate concentrations were added to PCR tubes in which 1 x 10^5 cells had been lysed with 25 µl NIB-proteinase K (Section 3.3.4.2).

3.3.7.2 Standard PCR thermal cycling protocol

PCRs were performed by placing tubes containing reaction mixes in a thermal cycling machine (DNA Thermal Cycler, Perkin-Elmer Cetus) using the following protocol: initial denaturing at 94°C for 7 min; then 40 cycles comprising denaturation at 94°C for 10 sec, cooling at 19.5°C/min over 2 min, annealing at an optimised temperature (usually 55°C) for 10 sec, heating at 17°C/min over 1 min, extension at 72°C for 30 sec, then heating at 22°C/min over 1 min to start the next cycle at 94°C; followed by a final extension at 72°C for 7 min. The reaction was then cooled to 6°C. Samples were stored at 4°C in the molecular biology laboratory unless processed immediately.

3.3.7.3 Optimisation of polymerase chain reaction conditions

Optimal concentrations of oligonucleotide primers and $MgCl_2$ were determined for each primer set using the standard PCR thermal cycling protocol with annealing at 55°C and a standard quantity (1 µg) of positive control DNA template. C8166 DNA was the positive control for HTLV-I PCRs and BLV-FLK DNA was the positive control for BLV PCRs. Serial two-fold dilutions of primers were prepared to give a range of final concentrations of each primer in PCRs from 4.0 to 0.125 mM. The concentration of $MgCl_2$ used in standard PCRs was 1.5 mM but some primer sets required different Mg^{2+} concentrations for optimal results. PCR optimisations for Mg^{2+} were performed with 0.5 to 3.0 mM final concentrations of $MgCl_2$ in grades of 0.5 mM. The composition of other constituents was held constant when varying the primer or $MgCl_2$ concentrations. Amplified products were separated by electrophoresis on 8% polyacrylamide gels, stained with ethidium bromide and examined by UV transillumination (Section 3.3.9).

Using optimised concentrations of primers and $MgCl_2$, with all other constituents as standard, PCRs were then performed with a range of annealing temperatures from 50 to 70°C to allow the optimal annealing temperature to be selected. The quantity of template used was 1 µg positive control DNA. Optimal conditions were considered to be those that yielded large amounts of PCR product at the highest possible annealing temperature with a preference for final concentrations of 1.0 to 2.5 mM $MgCl_2$ and 0.5 to 1.0 µM of each primer in a set. An attempt was also made to select PCR conditions that were relatively robust, for example by not using annealing temperatures or concentrations of $MgCl_2$ or primers that would lead to a loss of sensitivity if altered only slightly.

The reproducibility of PCR results was confirmed by preparing uniform batches of PCR master mixes containing C8166 DNA with primers for amplification of β-globin and HTLV-I sequences. These PCR master mixes were prepared on the same day and frozen at -20°C. PCR thermal cycling was then performed on different days in the same week using reagents thawed on the day of use. The uniformity of amplification in each well of the thermal cycler was also tested by placing an array of PCR tubes containing aliquots from a common master mix across the thermal cycling block and comparing PCR products by gel electrophoresis. The efficacy of amplification was compared using 200 μM dTTP, 200 μM dUTP or 400 μM dUTP and found to be similar.

3.3.7.4 Sensitivity of polymerase chain reactions

The sensitivity of each PCR primer set was determined by preparing PCR master mixes containing graded concentrations of template. All other reaction constituents, including optimised concentrations of primers and $MgCl_2$, were held constant for each sensitivity assay (Section 3.3.7.1). A standard PCR programme of 40 thermal cycles was then performed using the optimised annealing temperature for each primer set.

Serial ten-fold dilutions of positive control DNA in negative control DNA were prepared in the molecular biology laboratory to determine the sensitivity of PCRs for detecting template in DNA samples from tissues. C8166 DNA was diluted in DNA extracted from human placenta, reactive lymph node, PBMCs or J.JHAN cells to assess the sensitivity of PCRs for detecting HTLV-I. Serial dilutions of BLV-FLK DNA in FLK DNA were used to determine the sensitivity of BLV PCRs. In a standard serial dilution, 50 μl of 100 ng/μl C8166 DNA were added with a direct displacement pipette to 450 μl of 100 ng/μl J.JHAN DNA in a 1.5 ml tube to give 10% C8166 DNA. This mixture was vortexed thoroughly for 1 min, then 50 μl was transferred to another tube containing 450 μl of 100 ng/μl J.JHAN DNA to give 1% C8166 DNA. The process was repeated to give a series of dilutions containing 10 to 0.001% positive control DNA in negative control DNA. Tubes containing 1 ml 100% C8166 DNA and 1 ml 100% J.JHAN DNA were included in the set. Each PCR tube in the sensitivity assay for a particular set of primers contained 1 μg total DNA in a 50 μl reaction volume, with 1 μg, 100 ng, 1 ng, 0.1 ng, 0.01 ng, 0.001 ng, 0.0001 ng or nil positive control DNA. Sets of tubes prepared in a similar way for each primer set allowed the sensitivities of HTLV-I and BLV PCRs to be determined for DNA templates such as DNA extracted from skin and lymph nodes.

To assess the sensitivity of PCRs for detecting HTLV-I and HTLV-I/II in PBMCs from patients or in cultured cells, tubes containing pellets of 1×10^5 cells were prepared with decreasing proportions of HTLV-I-infected C8166 cells diluted in HTLV-I/II negative PBMCs, UCMCs or J.JHAN cells (Section 2.3.4). Cells were prepared and diluted in the level II containment facility (Section 2.3.1). In a standard serial dilution, 1 ml of C8166 cells at 1×10^5 cells/ml was added to 9 ml of PBMCs at 1×10^5 cells/ml in a 15 ml tube to give 10% C8166 cells. After thorough mixing by inversion, a 1 ml aliquot was removed from the tube and added to a second tube containing 9 ml of PBMCs at 1×10^5 cells/ml to give 1% C8166 cells. This process was repeated to give a panel of seven tubes containing 10, 1, 0.1, 0.01 and 0.001% C8166 cells diluted in PBMCs. Tubes were inverted gently at all stages of

dilution to maintain even suspensions of cells. Two aliquots of 0.5 ml, each containing 0.5 x 10^5 cells, from each mixture were successively transferred to 0.6 ml PCR tubes, centrifuged at 10,500 *g* for 10 min in a bench microcentrifuge and the supernatants discarded to produce cell pellets of 1 x 10^5 cells. In addition, two 0.5 ml aliquots of pure suspensions of 1 x 10^5 cells/ml C8166 and 1 x 10^5 cells/ml PBMCs were centrifuged in 0.6 ml PCR tubes to produce pellets of 1 x 10^5 cells representing 100% C8166 and 100% PBMCs, respectively. These tubes were centrifuged at 10,500 *g* for 10 min in a bench microcentrifuge and the supernatants were discarded. The cell pellets were stored at -80°C until processed for PCR.

3.3.7.5 Nested polymerase chain reaction

Nested PCRs were performed on some samples to increase sensitivity or to re-amplify PCR products for cloning. Nested primers were designed for annealing to sequences internal to primers 50 & 51 for amplification of first round PCR products of HTLV-I (282 & 283) and HTLV-I/II (288 & 289) (Figure 3.4). The HTLV-I inner primer set was optimised as a single round PCR on C8166 DNA and amplified the expected product of 136 bp. Even though no HTLV-II positive control was available for the HTLV-II inner primer set, primers 288 & 289 also amplified a product of similar size from C8166, due to sufficient sequence similarity between the two viral sequences. Second round PCRs were performed using the inner sets of primers at 0.5 μM each and 1.5 mM $MgCl_2$ in a standard PCR reaction with 10 μl of a 1 in 100 dilution of first round product amplified from clinical samples using primers 50 &51.

3.3.7.6 Consensus retroviral PCR

The consensus HTLV/BLV LTR oligonucleotide primers 174 & 175 and 174 & 176 (Figure 3.2) and consensus retroviral primers 296 & 297 (Figure 3.3) were used in PCRs with standard master mixes and thermal cycling conditions (DNA Thermal Cycler, Perkin-Elmer Cetus) in an attempt to amplify HTLV-I PCR products from pMT2 (Section 3.3.11.2), C8166 and BLV-FLK DNA, with DNA from human placenta and the FLK cell line as negative controls. Serial two-fold dilutions of each primer were made to yield final concentrations of 4.0 to 0.125 μM and this titration was tested with 1 μg C8166 DNA in standard PCR mixes using 1.5 or 2.5 mM $MgCl_2$. Thermal cycling protocols with annealing temperatures of 55, 60 and 65°C were compared.

Primer sets were also used in a touchdown PCR protocol in which the annealing temperature is changed decrementally with each cycle to increase the likelihood of amplifying sequences with the highest similarity to the oligonucleotide primers (Don *et al* 1991). The following protocol was used on a 9600 GeneAmp PCR System thermal cycler (Perkin-Elmer): initial denaturing at 94°C (5 min); then 26 cycles with denaturation at 94°C (1 min), annealing initially at 68°C then decreasing by 0.5°C per cycle (2 min) and extension at 72°C (1 min); followed by 14 cycles of denaturation at 94°C (1 min), annealing at 55°C (2 min) and extension at 72°C (1 min); final extension at 72°C for 7 min then the reaction was held at 4°C. Primers 174 & 175 were used at 0.5 μM each with 1.5 and 2.5 mM $MgCl_2$.

Figure 3.4: Nested oligonucleotide primers for amplification of HTLV-I and HTLV-II *pX* sequences.

LRF oligonucleotide number		Primer sequence 5' to 3' with HTLV-I nucleotide position	Length (b)	T_m (°C)	Size of PCR product (bp)
HTLV-I *pX*					
282	Sense	7368 AGTCTACGTGTTTGGAGACT 7387	20	58	135
283	Antisense	7503 TCCATCGATGGGGTCCCAGGT 7483	21	68	
HTLV-II *pX*					
288	Sense	7261 TACGTGTTTGGCGATTGTGT 7280	20	58	131
289	Antisense	7391 CCATCGATGGGGTCCCAGGT 7371	20	66	

3.3.8 Prevention of PCR contamination

3.3.8.1 Preparation of PCR reagents

Since PCR is highly sensitive, contamination of reagents with positive control DNA samples, plasmids containing target sequences and previously amplified PCR products can be a source of false positive results. Stringent precautions were taken at the LRF Virus Centre to minimise the possibility of contamination of PCRs during the preparation and analysis of samples. Reagents for PCR were ordered from sources outside the Department of Veterinary Pathology and divided into aliquots of the required volume at an external location by a person who did not work in the Department. These reagents were delivered as required and temporarily stored at -20°C in a dedicated freezer in a room separate from both the level II containment facility and the molecular biology laboratory.

PCR master mixes for use with clinical samples were prepared in a designated room separate from other buildings in the Department of Veterinary Pathology and not used for any other purpose. Pipettes, pipette tips, PCR tubes, gloves and other equipment were dedicated to this room. PCR master mixes were prepared at the start of the day before all other laboratory work. A new disposable surgical gown, face mask, surgical cap and two pairs of gloves were worn while handling reagents and tubes. Positive (direct) displacement pipettes or air displacement pipettes (Gilson) with plugged tips were used during the preparation of solutions. Gloves were changed between each sample. Reagents and tubes were placed on ice in disposable foam buckets during the preparation of PCR master mixes. Master mixes were then transported on ice to the level II containment facility.

3.3.8.2 Preparation of clinical samples for PCR

The level II containment facility was used for handling all clinical samples, including processing of PBMCs and tissues, cultivation of cells, extraction of DNA and preparation of cell pellets for PCR. PCR master mixes were combined with DNA samples or added to tubes containing NIB-proteinase K-disrupted cell pellets in this laboratory (Section 3.3.4.2). Cell cultures infected with HTLV-I (C8166) or BLV (BLV-FLK) for use as positive controls were grown in the level II containment facility but were always handled in a separate class II MSC (Section 2.3.1.2). Aliquots of cells from these cell lines, including serial dilutions of positive control cells, were prepared in the same MSC (MSC 1). The use of bench centrifuges was common to all cell culture work in the laboratory. Extractions of DNA from positive control cells were performed in the molecular biology laboratory.

3.3.8.3 Positive and negative controls

PCR tubes containing water in place of DNA or disrupted cells were processed in parallel with all clinical samples to act as negative controls. Control tubes were opened and closed at the same time as their matching sample tubes but care was taken to ensure that the clinical sample was not itself a source of contamination.

DNA from virus negative cell lines and cell pellets containing PBMCs from people in the Department of Veterinary Pathology were included in each batch of PCRs as additional negative controls.

Sealed PCR sample and control tubes were transferred a room adjacent to the level II containment facility for thermal cycling. Positive control samples were added to PCR master mixes in the molecular biology laboratory and placed in the thermal cycler after all tubes containing clinical samples had been handled. After completion of the PCR thermal cycle, tubes were transferred to the molecular biology laboratory to be opened for gel electrophoresis, electroblotting and hybridisation of PCR products.

3.3.8.4 Substitution of dUTP for dTTP in PCR reactions

Deoxyuridine triphosphate (dUTP) was substituted for deoxythymidine triphosphate (dTTP) in PCR nucleotide mixes as a further precaution against contamination. Uracil (U) was thus incorporated into PCR products instead of thymidine (T) and contamination could be reduced or eliminated by adding the enzyme uracil N-glycosylase (UNG) (Cambio or Life Technologies) to PCR master mixes to eliminate carry-over PCR products (Longo *et al* 1990). The protocol for prevention of PCR contamination using UNG was to add 0.25 U of enzyme per 100 µl reaction volume of selected PCR mixes prior to amplification. The mixture was incubated at 37°C for 1 hour to break down any U-containing products. The initial 94°C denaturation step in the standard PCR (Section 3.3.7.2) was increased from 7 to 10 min to inactivate the enzyme. Trials using UNG in the molecular biology laboratory showed that it eliminated most potential contamination unless large quantities of PCR products were used.

3.3.9 Polyacrylamide gel electrophoresis and electroblotting of polymerase chain reaction products

3.3.9.1 Polyacrylamide gel electrophoresis

PCR products less than 500 bp were analysed by polyacrylamide gel electrophoresis (PAGE). After removal from the thermal cycling machine, PCR tubes were opened in the molecular biology laboratory and 8 µl of PCR product from each tube were mixed with 2 µl of gel loading buffer/dye 5x. Glycerol in the buffer acted as a carrier for the DNA. Xylene cyanole and bromophenol blue dyes were indicators of electrophoretic migration. Fine pipette tips were used to load the 10 µl mixture into wells of 8% polyacrylamide gels (Appendix 5: A5.6.1) that had been prepared between 10.0 cm x 7.2 cm x 0.1 cm glass plates with a comb inserted at the top to create the wells. A DNA molecular weight (MW) marker, 400 ng ϕX174/*Hae*III (Life Technologies), was loaded on each gel as a size reference. PCR products were separated by gel electrophoresis using a Mini-Protean II gel electrophoresis apparatus (Bio-Rad) for 1 hour at 120 mV (17 mV/cm) with power supplied by a standard electrophoresis unit (Pharmacia). Double-stranded DNA PCR products, as well as single-stranded DNA such as unused oligonucleotide primers, which carry a net negative charge, migrated towards the anode. TBE 1x was used as the buffer in the gel electrophoresis tank.

After electrophoresis, gels were removed from between the glass plates and stained with 0.5 mg/ml ethidium bromide in deionised water for 6 min. Ethidium bromide is a fluorescent agent that intercalates double-stranded DNA. PCR products were viewed using a UV transilluminator (TC-254A, Spectroline or 3-3504 Foto/Prep I, Fotodyne) while wearing a face shield and photographed with a Polaroid MP4 Land camera using Polaroid Type 57 high speed (3,000 ASA/36 DIN) film (Genetic Research Instrumentation).

3.3.9.2 Electroblotting of PCR products

PCR products separated by PAGE were transferred to 0.45 μm nylon membrane (Hybond-N, Amersham) by electroblotting in preparation for hybridisation to sequence-specific probes. Polyacrylamide gels were immersed in alkali buffer for 8 min to denature double-stranded DNA, neutralising buffer (pH 8.0) for 8 min, then TAE 1x for 5 min (Appendix 5: A5.7). Each gel was positioned onto a sheet of nylon membrane moistened with TAE 1x, then sandwiched between two sheets of moistened blotting paper (No. 3, Whatman or GB003, Schleicher & Schuell) and placed in a rack between two gauze pads to maintain apposition. The rack was inserted into the electrode unit of a mini-electroblotting apparatus (Transblot cell, Bio-Rad), the tank was part-filled with TAE 1x and a frozen ice container was added to keep the buffer cool. Applying 15 V for 1 hour from an electrophoresis power unit transferred DNA from the gel to the nylon membrane, while a magnetic stirrer was used to circulate the buffer and prevent overheating. After electroblotting, the apparatus was dismantled and the nylon membrane exposed to 120 J/cm^2 UV light for 48 sec (Stratalinker 1800, Stratagene) to fix the DNA to the substrate by cross-linking.

3.3.9.3 Purification of PCR products from polyacrylamide gels

PCR products to be cloned were purified from polyacrylamide gels by excising ethidium bromide-stained fluorescent bands with a clean scalpel blade while examining gels on a UV transilluminator. Excess polyacrylamide was trimmed and the gel slice was cut into small pieces, then placed in a 1.5 ml tube containing an appropriate volume of elution buffer (usually 50 μl) containing 0.5 M ammonium acetate and 1 mM EDTA (Appendix 5: A5.6.1). The tube was incubated at 37°C for 1 hour then centrifuged at 11,600 *g* (13,000 rpm) in a microcentrifuge. The supernatant was transferred to a fresh 1.5 ml tube and the eluted DNA was precipitated by adding 2.5 volumes (125 μl) of absolute alcohol and holding at -20°C for 2 hours. A carrier such as glycogen (Boehringer Mannheim) was sometimes added to the solution at 20 ng/ml to enhance precipitation of DNA present at low concentration.

After centrifugation, the precipitated DNA was washed in 70% ethanol and air dried, then dissolved in a small volume (10 to 20 μl) of ultrapure water or TE 1x. The concentration of DNA was estimated by electrophoresis of 2 to 5 μl on a 1 or 2% agarose gel next to a MW marker or another DNA sample of known concentration.

3.3.10 Agarose gel electrophoresis and Southern blotting

3.3.10.1 Agarose gel electrophoresis

Electrophoresis of DNA in 1 or 2% agarose gels was used for visualisation of PCR products of 500 bp or more, visualisation of plasmids and products of RE digestion and purification of DNA bands for cloning. Agarose was dissolved in TBE 1x by boiling, allowed to cool to 50 to 60°C, then poured into a Perspex tray and allowed to set at room temperature with a comb to form wells. Ethidium bromide was added to the gel during preparation at a concentration of 5 to 10 μg/ml. The gel was immersed in TBE 1x in an electrophoresis tank (BRL) and the comb was removed. An appropriate volume of gel loading buffer/dye 5x was added to each DNA sample and the mixture was pipetted into the wells. A voltage of 1 V/cm was applied across the electrodes from an electophoresis power unit. λ*Hin*dIII (Life Technologies) or ϕX174/*Hae*III were used as molecular weight markers. The DNA was visualised on a UV transilluminator and gels were photographed as required.

HMW DNA samples that had been digested with REs for SB hybridisation (Section 3.3.3.5) were separated by electrophoresis in a 0.8% agarose gel overnight at 1 V/cm. The gel was immersed in 0.5 mg/ml ethidium bromide on a rocking tray for 30 to 60 min to stain the DNA and then placed in deionised water for 30 to 60 min to destain the background. A photograph of the gel was taken under UV transillumination with a ruler adjacent to the MW marker. This photograph provided a reference for estimating the sizes of positive bands that might be revealed following SB hybridisation (Sections 3.3.12). Test samples included C8166 DNA and DNA from patients with neoplasia. Since negative control tissues were not available from the same patients, placental DNA samples digested with the same REs were usually electrophoresed in parallel with each test sample to provide a reference for hybridisation.

3.3.10.2 Southern blotting

RE-digested DNA for hybridisation was transferred to 0.45 μm nylon membrane (Hybond-N, Amersham) using a modified Southern blotting procedure (Southern 1975). After 0.8% agarose gel electrophoresis, the DNA was denatured by immersing the gel in alkali buffer for 1 hour at room temperature. The gel was then submerged in neutralising buffer for 1 hour and finally placed in sodium saline citrate (SSC 10x) transfer buffer for 30 min (Appendix 5: A5.7). Layers of absorbent paper towels were placed in the bottom of a tray and covered with several layers of thick blotting paper (No. 3, Whatman or GB003, Schleicher & Schuell) followed by thin blotting paper (No. 1, Whatman). A piece of nylon membrane that had been cut to the same size as the gel and rinsed with SSC 10x was placed on the blotting paper. The denatured and neutralised gel was laid on top of this membrane and covered with several layers of thin blotting paper that had been moistened with SSC 10x. Care was taken to exclude bubbles. A sponge soaked with SSC 10x was placed over the top layer of blotting paper. The tray and its contents were sealed inside a plastic bag to reduce evaporation during blotting. This system used gravity-assisted capillary action to transfer the DNA downwards onto the nylon membrane.

After overnight blotting, the nylon membrane was rinsed with SSC 3x and exposed to UV light to cross-link the DNA to the substrate (Section 3.3.9.2).

3.3.10.3 Purification of DNA from agarose gels

PCR products to be cloned and inserts to be excised from plasmids by RE digestion for subcloning were purified from agarose gels using diethylaminoethyl (DEAE) membrane (Schleicher & Schuell) or a liquefactive enzyme (Gelase, Cambio). Migrating fragments were visualised by UV transillumination following gel electrophoresis. Where possible, bands were illuminated using a long wavelength (356 nm) hand-held UV light source (UVGL-58 Mineralight Lamp, UVP) or a UV transilluminator set to preparative mode (3-3504 Foto/Prep I, Fotodyne). Purification with DEAE membrane was performed by placing a piece of membrane into a slit cut in the agarose gel on the anode side of a DNA fragment of interest, then the gel was returned to the electrophoresis tank and 1 V/cm was applied for 10 min. When the DNA had migrated onto the DEAE membrane, the membrane was removed, trimmed, placed in 50 µl of elution buffer in a 1.5 ml tube and incubated at 65°C for 1 hour (Appendix 5: A5.6.1). The tube was centrifuged at 11,600 *g* for 10 min in a microcentrifuge and the supernatant transferred to a fresh tube. After adding 2.5 volumes (125 µl) of absolute alcohol, the tube was held at -20°C for 2 hours to precipitate the eluted DNA. A carrier such as transfer RNA (tRNA) was sometimes added. After centrifugation, the precipitated DNA was washed in 70% ethanol and air dried, then dissolved in a small volume of ultrapure water or TE 1x. The concentration of DNA was estimated by electrophoresis of an aliquot from this volume in parallel with a DNA sample of known concentration (usually serial dilutions of the 3.0 kb plasmid pUC8).

DNA to be purified from agarose gels using Gelase was separated by electrophoresis on a 1% low melting point agarose gel in TAE 1x. The fragment of interest was excised from the gel with a scalpel blade, placed in a 1.5 ml tube and weighed. The Gelase enzyme solution (1 U/ml) was added to the gel slice at 1 U (1 ml) per 100 g agarose. The tube was incubated at 70°C for 20 min or 45°C for 1 hour until the agarose was liquefied, then the liquid was transferred to a 30 ml glass centrifuge tube (Corex). DNA was precipitated by adding a 0.1 times volume of 5 M ammonium acetate, holding at room temperature for 30 min, then centrifuging at 12,100 g for 30 min at room temperature in a JS-21 centrifuge using a JA-20 rotor. After washing with 70% ethanol and drying, the DNA was dissolved in ultrapure water or TE 1x and the concentration was estimated by electrophoresis.

3.3.11 Preparation of radiolabelled probes

3.3.11.1 Applications of radiolabelled probes

Hybridisation using radiolabelled probes was employed as a method of increasing the sensitivity of detection and confirming the specificity of PCRs for HTLV-I and BLV. The stringency of hybridisation was adjusted according to the requirements for sensitivity and specificity. Radiolabelled probes were hybridised to DNA that had been separated by gel electrophoresis, blotted onto nylon membranes and cross-linked (Sections 3.3.9 and 3.3.10).

Probes were either linear double-stranded plasmid inserts radiolabelled with α ^{32}P dCTP by random priming (Section 3.3.11.4) or synthetic oligonucleotides that had been 5' end-labelled with γ ^{32}P ATP (Section 3.3.11.5) and purified by Sephadex column filtration (Section 3.3.11.6).

3.3.11.2 Selection and design of probes

The 11.7 kb plasmid pMT2 contains a full-length molecular clone of the HTLV-I provirus from the cell line MT2 cloned into the 2.7 kb vector pUC (Miyoshi *et al* 1981a, b, Seiki *et al* 1982) (Section 2.1.2.1). pMT2 was grown in *Escherichia coli* (*E coli*) DH5α. A 9 kb viral insert, designated pMT2$_i$, was excised from purified plasmid by RE digestion using *Sst*I (Section 3.3.13.4). *Sst*I cuts pMT2 in the HTLV-I LTR and the 9 kb insert is nearly full-length. pMT2$_i$ was labelled with α ^{32}P dCTP by random priming (Section 3.3.11.4) and used as a probe for hybridisation to HTLV-I and HTLV-I/II PCR products and to SBs of RE-digested genomic DNA from C8166 cells. The same probe was also used for hybridisation to RNA blots (Section 4.2.3.2). HTLV-I and HTLV-II have 60% sequence similarity and pMT2$_i$ has been used as a probe for detection of HTLV-II sequences amplified by PCR (Shaw *et al* 1984) (Section 1.5.3.1); in this study primers were used that amplified HTLV-I and HTLV-II sequences from the *pX* region, which is more highly conserved (Figure 3.1).

Three plasmids containing 2.4, 1.1 and 1.9 kb *Pst*I fragments from pMT2 ligated into the 4.3 kb vector pBR322 were also used as probes for hybridisation to SBs of RE-digested genomic DNA from C8166 cells. These plasmids were designated pMT2-1 (6.7 kb), pMT2-2 (5.4 kb) and pMT2-3 (6.2 kb) and were specific for the *pol-env*, *pol*, and *gag-env* regions, respectively. Hybridisation studies with these probes were used to verify the HTLV-I provirus pattern in C8166, consistent with the findings of Bhat *et al* (1993) (Section 2.3.4.1). The probe used for hybridisation to BLV PCR products was a γ ^{32}P ATP 5' end-labelled synthetic oligonucleotide (Section 3.3.11.5) (Figure 3.5).

3.3.11.3 Procedures for handling radioactive substances

Radioisotopes for labelling probes for hybridisation were handled in the radioactivity room in the Department of Veterinary Pathology with precautions to prevent radioactive contamination. A radioactivity badge was worn and all surfaces and equipment were checked with a Geiger counter before, during and after handling radioisotopes. All manipulations were made behind a Perspex shield wearing double gloves. Tubes containing radioactive solutions were stored in containers with appropriate shielding and were held in Perspex blocks during manipulations. Direct displacement or plugged pipettes were used for transfer of radioactive solutions. Absorbent blotting paper with a plastic under-surface (Bench-cote) was used in case of spillage of radioactive material. Reusable equipment that had been in contact with radioactive substances was soaked in decontaminating solution (Decon). Surfaces suspected of being contaminated with radioactivity were also cleaned with this solution. Radioactive waste was placed in designated containers for disposal and solutions were flushed down a dedicated sink for containment in a holding tank. The disposition of all radioisotopes was recorded.

Figure 3.5: Oligonucleotide probe for hybridisation to polymerase chain reaction products generated using bovine leukaemia virus primers.

LRF oligonucleotide number	Oligonucleotide sequence 5' to 3' with nucleotide position	Length (b)	T_m (°C)
BLV *gag*			
301	2736 AGCACTACAGGAACCTCTTCGCCAAGTTTC 2765	30	48

3.3.11.4 Radiolabelling of linear double-stranded plasmid inserts by random priming

Linear double-stranded DNA inserts purified from plasmids following RE digestion (Section 3.3.13.4) were radiolabelled with α ^{32}P dCTP by random priming to produce radioactive oligonucleotide probes for hybridisation. Double-stranded inserts were denatured by boiling 25 ng linear DNA in 25 µl water for 5 min in a 1.5 ml tube then cooling the tube on ice to maintain separation of DNA strands. Solutions added to this volume for random priming were 10 µl of buffer containing dATP, dGTP and dTTP, 5 µl of primer solution containing random hexanucleotides, 2 µl of enzyme solution containing 1 U/µl DNA polymerase I Klenow fragment (Multiprime Kit, Amersham) and 8 µl (2.96 MBq) of α ^{32}P dCTP with a specific activity of 30 TBq/mmol (R82075, Amersham or Redivue, Amersham) (Appendix 5: A5.8.1). The random priming reaction was allowed to proceed for 4 hours at room temperature (R82075, Amersham) or 30 min at 37°C (Redivue, Amersham). The radiolabelled probe was purified by Sephadex column filtration (Section 3.3.11.6).

3.3.11.5 Radiolabelling of oligonucleotide probes by 5' end-labelling

The oligonucleotide probe for hybridisation to BLV PCR products was synthesised in the Department of Veterinary Pathology (Section 3.3.6.1). The 5' end-labelling reaction mix contained 70 pmol synthetic oligonucleotide, 20 U T4 polynucleotide kinase, T4 kinase buffer 1x (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 100 µM spermidine, 100 µM EDTA pH 8.0), and 70 pmol γ ^{32}P ATP (ICN) (specific activity 111 MBq/mmol) in a total volume of 100 µl (Appendix 5: A5.8.2). The reaction mix was incubated at 37°C for 1 hour then the radiolabelled probe was purified by Sephadex column filtration (Section 3.3.11.6).

3.3.11.6 Purification of radiolabelled probes by Sephadex column filtration

Radioactive oligonucleotide probes generated by random priming and 5' end-labelling were purified from unincorporated nucleotides by column filtration. Washed Sephadex beads (Sephadex-G50, Pharmacia) were pipetted into a glass column and the column was equilibrated with TE 1x. Pre-packed disposable Sephadex columns (Sephadex G-50 NICK column, Pharmacia) were also used. The 50 µl solutions containing products of random priming or 100 µl solutions containing products of 5' end-labelling were pipetted into the top of the equilibrated column and flushed into the Sephadex with TE 1x. Orange-G dye was added as a marker for elution of unincorporated nucleotides.

The descent of radioactivity was monitored with a Geiger counter. The first peak of radioactivity eluted from the column contained the ^{32}P-labelled DNA probe. This was collected into a 1.5 ml tube for addition to hybridisation solutions. Unincorporated nucleotides were eluted as a second peak of radioactivity and discarded by flushing down the liquid radioactive waste sink.

3.3.12 Hybridisation of radiolabelled probes to membrane-bound DNA

3.3.12.1 Principles of hybridisation

Pairing of complementary bases in single-stranded DNA molecules results in the formation of DNA:DNA duplexes with a strength of association that depends on the degree of sequence similarity (percentage base mismatch), nucleotide composition, length of sequence, temperature and chemical properties of the solution in which hybridisation takes place. The temperature of hybridisation, composition of hybridisation solutions and stringency of washing can be adjusted according to the predicted properties of probes used for hybridisation. The melting temperature (T_m) is a measure of the stability of nucleotide association and for DNA:DNA duplexes can be calculated using formula 3.2 (McConaughy *et al* 1969, Wetmur 1991) (Section 3.3.5.1). The T_m for hybridisation of DNA probes in solution to target DNA immobilised on nylon membranes is usually slightly lower than the calculated value (Beltz *et al* 1983). Hybridisations are performed at 20 to 25°C below the calculated T_m to maximise the rate of DNA:DNA association and for aqueous solutions this is usually 60 to 70°C. Washing of blots in aqueous solutions to remove unbound and weakly bound probes is usually conducted at 5 to 25°C below the calculated T_m. The effective T_m can be manipulated by adding formamide to the hybridisation solution; T_m decreases by 0.63% for every 1% increase in formamide (Howley *et al* 1979).

In this study, hybridisations using $pMT2_i$ were carried out in standard hybridisation solution containing 50% formamide with SSC 3x ($[Na^+]$ = 0.59 M, thus $[M^+]$ = 0.42) (Appendix 5: A5.9). Probes produced by random priming have an average length of 100 bp and thus a value L = 100 was used for $pMT2_i$ (Section 3.3.11.4). The full-length 9,068 bp HTLV-I sequence has 53.8% G+C (2,093 A, 3,165 C, 1,720 G, 2,090 T) (Appendix 6). Assuming a 100% match with a PCR product with a similar %G+C, the calculated T_m for randomly primed $pMT2_i$ is 61°C. Hybridisation using $pMT2_i$ was routinely performed at 37°C, which is 24°C below the calculated T_m. Hybridisations with oligonucleotide probes are usually performed at 5 to 10°C below the calculated T_m, since the empirically determined T_m is often up to 10°C higher. The synthetic oligonucleotide (301) used as a BLV probe in this study had 30 bases and 50% G+C (Figure 3.5). This probe was hybridised in standard hybridisation solution containing SSC 3x with 50% formamide (Appendix 5: A5.9) at 37°C; the calculated T_m was 48°C.

3.3.12.2 Prehybridisation

Prehybridisation reduces non-specific binding of radiolabelled probes to nylon membranes during hybridisation. Hybridisation solution was prepared with 50% formamide and SSC 3x to achieve an effective T_m 5 to 10°C below the calculated T_m of the probe, as discussed above (Section 3.3.12.1). This solution was used for both prehybridisation and, following addition of radiolabelled probe, for hybridisation. Nylon membranes with bound DNA were moistened with SSC 3x, 0.1% SDS and inserted into 50 ml screw-top polypropylene tubes with care taken to prevent the formation of bubbles.

Membranes were prehybridised with 2.5 ml hybridisation solution by continuous rotation at 37°C in a hybridisation oven (Hybridiser HB-1, Techne) for at least 4 hours. Alternatively, nylon membranes were placed in glass hybridisation tubes (Techne) with a larger volume of hybridisation solution (10 to 20 ml) depending on the size of the tube and surface area of membrane.

3.3.12.3 Hybridisation

The randomly primed α ^{32}P dCTP radiolabelled probe pMT2$_i$ (Section 3.3.11.4) was boiled for 5 min and cooled on ice to denature any DNA duplexes. Usually 25 µl of radiolabelled probe was added to 2.5 ml of the 50% formamide, SSC 3x hybridisation solution that had been used for prehybridisation to yield approximately 1 x 10^6 counts per ml. Nylon membranes were then hybridised with this buffer by rotating the hybridisation tubes in an oven at 37°C overnight. The oligonucleotide probe for BLV was added to a hybridisation solution of the same composition to approximately 1 x 10^6 counts per ml and hybridised overnight at 37°C.

3.3.12.4 Washing blots to remove unbound probe

After overnight hybridisation, the hybridisation solution was removed and disposed of according to normal radioactivity procedures. The nylon membranes were initially washed twice at low stringency by filling the tubes with SSC 2x, 0.1% SDS and rotating in the hybridisation oven at room temperature for five min per wash. Subsequent washes were carried out under high stringency conditions. Usually, this consisted of immersing membranes in SSC 0.5x, 0.1% SDS in a sealed plastic container and washing twice at 65°C for 30 min per wash on an oscillating tray in a heated water bath (Techne). When a SB hybridisation was to be repeated, the nylon membrane was stripped of probe by agitating in 0.1% SDS at 85°C for 30 min then changing the solution and continuing for a further 10 min.

3.3.12.5 Autoradiography

After the final high stringency wash, each nylon membrane was wrapped in plastic (Saran Wrap, Dow, Genetic Research Instrumentation) and exposed to X-ray film (Hyperfilm-MP, Amersham) in an autoradiograph cassette with an intensifying screen under dark room conditions. Cassettes were held at -80°C during exposure and autoradiographs were developed in an automatic processor in the Department of Veterinary Pathology after exposures of 1 to 21 days.

3.3.13 Manipulation of bacteria

3.3.13.1 Growth and storage of bacteria

Strains of bacteria used for most molecular biology purposes were *E coli* HB101 or DH5α. These were grown at 37°C on Luria-Bertani (LB) agar in Petri dishes in a standard incubator or in flasks containing LB broth in an orbital incubator (Fisons) at 225 rpm (Appendix 5:

A5.10.1). Antibiotics were added according to the resistance genes expressed by plasmids within the bacteria. Ampicillin (50 μg/ml) was used for growth of *E coli* containing pMT2 and TA cloning vectors (Section 3.3.16.2) (Appendix 5: A5.10.2). Tetracycline (12 μg/ml) was used for growth of *E coli* containing pMT2-1, pMT2-2 and pMT2-3 (Section 3.3.11.2).

Stocks of viable bacteria were prepared by mixing 1 ml of bacteria in suspension from overnight LB broth cultures with 500 μl sterile 80% glycerol in cryopreservation tubes (Cryotubes, Nunc). Cryotubes were frozen in liquid nitrogen ($liqN_2$) or a mixture of dry ice and absolute alcohol then transferred to a freezer for long-term storage at -80°C. Unwanted bacterial cultures and supernatants were sterilised by autoclaving or inactivated with disinfectant solutions (sodium dichloroisocyanurate, Presept, Surgikos).

3.3.13.2 Transformation of chemically competent bacteria with plasmid DNA

Plasmids are circular double-stranded DNA molecules that replicate in bacteria and are widely used for manipulation of DNA. Plasmids with antibiotic resistance genes can be used for selection of cloned DNA inserts. Transformation is the method by which plasmid DNA is introduced into bacteria. Competent bacteria (Life Technologies) were stored at -80°C and thawed on ice when required for transformation. Volumes of 50 μl were placed in 15 ml polypropylene tubes (2059, Falcon) on ice and 10 to 100 ng of plasmid DNA was added to each tube. The mixture was held on ice for 10 min, then the tubes were placed in a 42°C water bath for 45 sec to induce passage of plasmid DNA across the bacterial cell wall. After the addition of 900 μl of LB broth or SOC, tubes were incubated in an orbital incubator at 37°C for 1 hour to allow expression of plasmid-encoded antibiotic resistance genes. Aliquots of 50 to 200 μl bacteria were spread onto LB agar plates containing the appropriate antibiotic. These plates were incubated at 37°C overnight to allow the selective growth of transformed bacteria.

3.3.13.3 Transformation of bacteria by electroporation

Bacteria were grown at 37°C in 200 ml LB broth containing antibiotics for approximately 6 hours to an OD_{600} of 0.5 to 0.7 (Hanahan 1983). The cultures were centrifuged at 3,740 *g* (4,500 rpm) for 10 min in the JS-21 centrifuge using a JS-7.5 rotor at 4°C. After removing the supernatant, bacteria were centrifuged and resuspended four times in decreasing volumes (400, 200, 20 and 1 ml) of sterile 10% glycerol in water. Aliquots of 100 μl were stored at -80°C. Bacteria were thawed at room temperature, placed on ice and 40 μl of chilled bacterial suspension was mixed with 1 to 2 μl (5 to 10 ng) plasmid DNA in a 1.5 ml tube on ice. This volume was transferred to an ice cold sterile 0.2 cm electroporation cuvette (Bio-Rad), placed in a chilled Teflon block and electroporated with a voltage of 2.5 kV, charge transfer of 2.4 C (25 μF), resistance of 200 Ω and time constant between 4 and 5 sec (Gene Pulser and Pulse Controller, Bio-Rad). Following electroporation, 1 ml of LB broth was added to the cuvette and transferred to a 15 ml tube (2059) for incubation at 37°C for 1 hour at 225 rpm in an orbital incubator. Bacteria were then spread on LB agar containing the appropriate antibiotic and incubated at 37°C overnight.

3.3.13.4 Selection and growth of transformed bacteria

A sterile flamed loop was used to pick single colonies of transformed bacteria that had grown on selective agar plates after overnight incubation. Colonies were inoculated into 15 ml tubes (2059) containing 5 ml LB broth with an appropriate antibiotic and incubated at 37°C in an orbital incubator at 225 rpm overnight. Small scale preparations of plasmids were made from these cultures (Section 3.3.14.1). Large scale plasmid preparations (Sections 3.3.14.2 and 3.3.14.3) were made by transferring 5 ml of overnight broth culture into flasks containing 500 ml of LB broth and an appropriate antibiotic. These flasks were incubated overnight in an orbital incubator at 37°C. Chloramphenicol (25 μg/ml) was added to overnight cultures containing tetracycline (12 μg/ml) to maximise expression of pMT2, pMT2-1, pMT2-2 and pMT2-3 in *E coli* DH5α for large scale plasmid purification.

3.3.14 Purification of plasmid DNA

3.3.14.1 Small scale purification of plasmid DNA

Small scale preparations of plasmid DNA for RE digestion and screening for inserts by gel electrophoresis were prepared by modified alkaline lysis or boiling methods or by column purification. One or two 1.5 ml aliquots of overnight cultures of bacteria in LB broth containing antibiotics were centrifuged in 1.5 ml tubes at 11,600 g in a microcentrifuge for 5 min. Supernatants were removed, leaving a pellet of bacteria in the bottom of each tube.

Bacteria to be disrupted by alkaline lysis were resuspended by vortexing with 100 μl of Solution I (Birnboim and Doly 1979) (Appendix 5: A5.11.1). This suspension was mixed with 200 μl Solution II, which contained 0.2 M NaOH and 1% SDS to lyse cells, then was vortexed with 150 μl Solution III, containing 3 M potassium acetate to precipitate bacterial proteins and genomic DNA. The tubes were centrifuged at 11,600 *g* for 5 min in a microcentrifuge and the supernatants, containing plasmid DNA, were transferred to fresh tubes. Extractions with 1 ml phenol and 1 ml chloroform were each followed by centrifugation and transfer of supernatants to fresh tubes. Plasmid DNA was precipitated by the addition of 2.5 volumes of absolute alcohol followed by centrifugation and washing in 70% ethanol. The DNA pellet was allowed to dry then dissolved in 30 to 50 μl ultrapure water or TE 1x. Plasmid DNA was visualised by electrophoresis of 10 μl on an agarose gel (Section 3.3.10.1) and RE digests were prepared to check for the presence of inserts by gel electrophoresis (Section 3.3.14.4).

Bacteria were harvested from LB broth cultures by centrifugation in 1.5 ml tubes for extraction of plasmid DNA by the boiling method. After removing the supernatant, pelleted bacteria were vortexed with 150 μl sucrose-Triton X-100-EDTA-Tris (STET) solution and 10 μl 10 μg/ml lysozyme (Boehringer Mannheim) to disrupt cell walls (Appendix 5: A5.11.2). Tubes were boiled for 40 sec, placed on ice for 5 min and then centrifuged at 11,600 *g* for 10 min. The glutinous pellet that formed in each tube was removed by pipetting with a wide-bore micropipette tip and discarded. The plasmid DNA was precipitated by adding 100 μl of isopropanol and placing the tubes at -20°C for 15 to 30 min. Tubes were centrifuged at 11,600 *g* for 5 min, the supernatant was removed and the precipitated DNA was washed with

1 ml 70% ethanol. The tubes were allowed to dry and the plasmid DNA was dissolved in 30 to 50 µl ultrapure water or TE 1x as above. RNase A was added at a final concentration of 50 µg/ml in preparation for gel electrophoresis.

Plasmids were column purified for RE digestion and for sequencing using resin columns (Wizard minipreps DNA purification system, Promega). LB broth cultures of bacteria were centrifuged (1.5 ml twice) in 1.5 ml microcentrifuge tubes at 11,600 *g* for 5 min and the bacterial pellet was resuspended in 200 µl cell suspension solution. Bacteria were disrupted with 200 µl cell lysis solution then treated with 200 µl neutralisation solution. After centrifuging at 11,600 *g* for 10 min the supernatant was transferred to a fresh tube and mixed with 1 ml DNA purification resin. This volume was pipetted into a 5 ml syringe attached to a column inserted into a vacuum manifold. A vacuum was applied to filter the liquid through the column then 2 ml column wash solution was added and drawn through under vacuum. The columns were dried by continued vacuum for 2 min then centrifugation for 1 min. DNA was dissolved by adding 50 µl water at 70°C for 5 to 15 min then eluted from the column by centrifuging at 11,600 *g* for 1 min.

3.3.14.2 Large scale crude extraction of plasmid DNA

Flasks containing 500 ml overnight bacterial cultures were cooled on ice and the contents transferred to 250 ml screw-top centrifuge tubes (Beckman) on ice. The cultures were centrifuged at 9,060 *g* (7,000 rpm) in the JS-21 centrifuge using a JS-7.5 rotor (Beckman) for 10 min at 4°C. The supernatant was discarded, the bacteria were resuspended in 100 ml TNE 1x and the centrifugation was repeated. The bacterial pellet was resuspended in 4 ml 0.5% lysozyme solution and allowed to stand at room temperature for 5 min to disrupt bacterial cell walls. The DNA was denatured by adding 16 ml of alkaline lysis solution, vortexing and holding on ice for 10 min. The solution was neutralised with 12 ml of potassium acetate solution pH 4.8 and placed on ice for a further 10 min to allow plasmid strands to re-anneal. The lysed bacteria were centrifuged at 43,700 *g* (19,000 rpm) for 30 min at room temperature in the JS-21 centrifuge using a JA-20 rotor. The supernatant was divided between two 30 ml glass centrifuge tubes (Corex). Plasmid DNA was precipitated by adding 0.6 volumes of isopropanol followed by thorough mixing. The precipitate was centrifuged at 12,100 *g* (10,000 rpm) for 30 min at room temperature in the JS-21 centrifuge using a JA-20 rotor. The supernatant was removed and 5 ml of 70% ethanol was added to the pellet to remove salt from the precipitated plasmid DNA. This solution was centrifuged at 12,100 *g* for 15 min at room temperature. The pellet was allowed to dry for a maximum of 10 min and then dissolved in 5 ml of TE 1x buffer.

3.3.14.3 Large scale purification of plasmid DNA by caesium chloride density gradient centrifugation

The crude extract of plasmid DNA in 5 ml TE 1x (Section 3.3.13.2) was prepared for density gradient centrifugation by adding 1 g/ml CsCl and 740 mg/ml ethidium bromide. The mixture was transferred to 5 ml ultracentrifuge tubes (Quick-Seal, Beckman), which were balanced, heat sealed and centrifuged overnight at 49,000 rpm in an L-8 60M ultracentrifuge

(Beckman) using a Vti 65.2 rotor at 20°C. The lower band of supercoiled plasmid DNA was visualised using a hand-held ultraviolet lamp (UVGL-58, UVP) in the dark room. This band was removed using a needle and syringe and was diluted with TE 1x to a volume of 5 ml. Ethidium bromide was removed from the solution by repeated mixing with water-saturated isobutanol. When the aqueous phase was colourless, the plasmid DNA was precipitated by adding 2.5 volumes of absolute alcohol and holding the solution at -20°C for 2 hours. The tubes were centrifuged at 12,100 *g* for 30 min in the JS-21 centrifuge using a JA-20 rotor at 4°C. Salt was removed by washing with 70% ethanol then the DNA pellet was allowed to dry before being dissolved in 50 to 200 µl ultrapure water or TE 1x.

3.3.14.4 Restriction endonuclease digestion of plasmid DNA

Plasmids were digested with 5 U of the appropriate RE per mg of plasmid DNA in a solution containing proprietary buffer (Life Technologies) at 1x concentration. The most frequently used REs were *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sst*I and *Xba*I (Appendix 5: A5.3.1). Reactions were incubated at 37°C for 1 to 3 hours. If two REs were required to excise a plasmid insert, both enzymes were added to the same solution if they had similar activities in the same buffer. If buffer requirements were substantially different, the enzymes were used in succession and the plasmid DNA was precipitated with ethanol and washed with 70% ethanol to remove salts between each digest. RNase A was sometimes added to RE digests at a final concentration of 50 µg/ml to remove RNA contamination. Inserts were separated from RE-digested plasmids by agarose gel electrophoresis (Section 3.3.10.1) and bands were purified using DEAE membrane or Gelase (Section 3.3.10.3).

3.3.15 Subcloning plasmid inserts

Subcloning was used to transfer a double-stranded segment of insert DNA from one plasmid to another. Plasmid DNA was purified by agarose gel electrophoresis using DEAE membrane (Section 3.3.10.3). Supercoiled plasmid DNA migrated most rapidly, followed by linear and nicked forms. Inserts were excised from plasmids by digestion with one or two REs followed by gel electrophoresis and gel purification (Section 3.3.14.4). A recipient plasmid vector was prepared by digestion with the same REs and gel-purified similarly. The insert and recipient plasmid were mixed at an appropriate molar ratio (usually insert:plasmid 3:1) and ligated in a solution containing T4 DNA ligase and ligation buffer 1x in a total volume of 10 to 40 µl. Purified plasmid or other DNA that had been dissolved in solutions with a low concentration of salt was concentrated by mixing an aqueous solution with 0.1 volumes of 3 M sodium acetate and 2.5 volumes of absolute alcohol. The mixture was held at -20°C for 1 to 2 hours, centrifuged at 11,600 *g* in a microcentrifuge, then washed in 70% ethanol. The DNA was then dissolved in an appropriate volume of ultrapure water or TE 1x.

3.3.16 Cloning and sequencing PCR products

3.3.16.1 Re-amplification of PCR products

Selected PCR products were cloned into plasmid vectors for sequencing. When dUTP was substituted for dTTP in PCRs, products of amplification could not be readily cloned because UNGs present in standard laboratory strains of bacteria degrade plasmids containing inserts with U residues. Therefore, PCR products for cloning were re-amplified by PCR using dTTP in the dNTP mix with the same primers as those used for the original PCR reaction. An alternative approach would have been to use strains of bacteria that were defective for UNG activity (UNG^- strains) for transformation.

3.3.16.2 TA cloning of PCR products

Non-proof reading DNA polymerases, such as the *Taq* polymerase (Amplitaq DNA Polymerase) used in standard PCRs (Section 3.3.7.1), add single dA residues to the 3' ends of duplex DNA molecules generated by PCR. These PCR products can be cloned by ligation into vectors with single 3' dT residues. The vector used for this purpose was pCRII (TA Cloning System, Invitrogen). PCR products were cloned directly from unpurified reaction mixes or were gel-purified and dissolved in ultrapure water before ligation. The reaction mix for TA cloning using PCRII contained 1 µl of PCR product, 4 U T4 DNA ligase, commercial ligation buffer 1x and 50 ng plasmid vector in a total volume of 10 µl. *E coli* DH5α was transformed with the ligation reaction mix (Sections 3.3.13.2) and plated on LB agar containing 50 µg/ml ampicillin and spread with 1 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Blue-white screening was used for selection of plasmids containing inserts. X-gal is a substrate for β-galactosidase, which turns blue when cleaved by active enzyme produced in plasmids that lack inserts. Bacteria transformed with plasmids containing inserts do not produce active enzyme and colonies remain white. LB agar plates spread with transformed bacteria were incubated at 37°C overnight. White colonies were picked and inoculated into 5 ml LB broth containing 50 mg/ml ampicillin. These cultures were grown overnight at 37°C in an orbital incubator at 225 rpm and small scale preparations of plasmids made using the boiling method (Section 3.3.14.1). The plasmids were digested with REs and separated by agarose gel electrophoresis. Plasmids containing inserts of the expected size were identified and the source broth cultures were regrown. Small scale preparations of plasmids were then made using the alkaline lysis method followed by phenol-chloroform extraction or column purified (Wizard minipreps DNA purification system, Promega) (Section 3.3.14.1). The purified plasmids were quantified by spectrophotometry and submitted for sequencing.

3.3.16.3 Sequencing of plasmid inserts

Some plasmids were sequenced at the University of Birmingham (Alta Bioscience) using standard primer sequences (T3 and T7) present in the cloning vector pCRII (Appendix 5: A5.12.1). Other plasmids were sequenced with the ^{T7}Sequencing Kit (Pharmacia), which uses the dideoxy sequencing method in which primer extension by T7 DNA polymerase is terminated in reactions specific for A, C, G and T bases (Sanger *et al* 1977, Tabor and Richardson 1987) (Appendix 5: A5.12.2).

Plasmids that had been purified for sequencing (2 μg) were denatured in 0.4 M NaOH for 10 min at room temperature in 1.5 ml microcentrifuge tubes. The DNA was precipitated with 0.1 volumes of 3 M sodium acetate and 2.5 volumes absolute alcohol at -20°C for 30 min. The tubes were centrifuged at 11,600 *g* for 15 min then the precipitated DNA was washed with 70% ethanol, vacuum desiccated and dissolved in 10 μl ultrapure water. This volume was mixed with 2 μl (10 pmol) universal primer (M13) and 2 μl annealing buffer and incubated at 65°C for 10 min then 37°C for 10 min. The sequencing enzyme T7 DNA polymerase (2 μl) was mixed with 8 μl enzyme dilution buffer on ice. Each of four labelling reactions consisted of 14 μl annealed template and primer, 3 μl labelling mix (dATP), 1 μl (9.25 MBq) α ^{35}S dATP (Amersham) (Appendix 5: A5.12.3) and 2 μl of diluted T7 DNA polymerase in a total volume of 20 μl. The labelling reactions were incubated at room temperature for 5 min then 4.5 μl was added to each of four 1.5 ml microcentrifuge tubes containing 2.5 μl of one of four sequencing mixes ('A', 'C', 'G' or 'T' mix-short) that had been incubated at 37°C for 2 min. The termination reaction was allowed to proceed for 5 min at 37°C then stopped by the addition of 5 μl of a proprietary solution containing formamide, EDTA, bromophenol blue and xylene cyanole.

Aliquots (3 μl) of each reaction were incubated at 80°C for 3 min and placed on ice then 2.5 μl was loaded into one of four adjacent wells of a 5% polyacrylamide sequencing gel (Long Ranger DNA sequencing gel solution Hydro-Link, AT Biochem) (Appendix 5: A5.12.4). The glass sequencing plates had been coated with silicon (Sigmacote SL-2, Sigma) prior to pouring the polyacrylamide gel. The samples were separated by electrophoresis in a sequencing cell (Sequi-Gen II System, 21 cm x 50 cm, Bio-Rad) at 1,500 V for 90 min with power supplied by an electrophoresis unit (ECPS 3000/150, Pharmacia). After dismantling the apparatus and separating the glass plates, the gel was transferred to blotting paper (Number 3, Whatman) and dried at 80°C for 2 hours in a gel-drier (Bio-Rad). Autoradiograph film was exposed for 4 hours then developed. The A, C, G, T sequence was read manually from the pattern of bands in each lane.

3.4 Results

3.4.1 Sensitivity of detection of HTLV-I and BLV by PCR

The primer sets for amplification of HTLV-I, HTLV-I/II and BLV sequences by PCR are shown in Figure 3.1. The sensitivities of these primer sets for detecting HTLV-I and BLV positive control DNA using optimised PCR conditions are indicated in Table 3.2 and depicted in Figures 3.6 to 3.23. Most primer sets were able to detect 0.1 to 1.0 ng positive control DNA in a total quantity of 1 μg DNA when 8 μl from 50 μl of PCR products were electrophoresed on a polyacrylamide gel and stained with ethidium bromide. The sensitivity was increased to 0.01 to 0.1 ng when these PCR products were electroblotted and hybridised to HTLV-I or BLV-specific probes. Some primer sets were less sensitive. Specific PCR products could be detected in not less than 10 ng C8166 DNA on polyacrylamide gels using primers 120 & 121. Primers 67 & 68 also had relatively low sensitivity (10 ng C8166 DNA); both had degenerate bases, increasing the probability of binding to non-specific DNA sequences. Primers 3 & 4 had different calculated T_m values because primer 3 was substantially larger (29 bases) than primer 4 (18 bases), but this primer set retained good sensitivity (1.0 ng C8166 DNA). The primer set used for amplification of BLV sequences (194 & 195) also had relatively low sensitivity (10 ng BLV-FLK DNA). Another set of primers and oligonucleotide probe used for BLV PCRs was found to lack specificity (Agresti *et al* 1993).

The sensitivities of PCRs for detection of HTLV-I and BLV in serial dilutions of positive control cells in PBMCs or other negative control cells are shown in Table 3.3. On ethidium bromide-stained polyacrylamide gels, PCR product could be detected with most primer sets when 100 to 1,000 positive control cells were present in a total quantity of 100,000 cells. Some primer sets could detect 10 positive cells in this quantity. Hybridisation to HTLV-I-specific probes increased the sensitivity to 1 to 100 positive cells.

Serial dilutions of positive control DNA or cells were included with each batch of clinical samples tested by PCR to ensure that sensitivity was maintained. Sensitivities of most primer sets were highly repeatable. If the titration of positive controls in any batch of PCRs with clinical samples did not show acceptable sensitivity, the PCRs were repeated on the same samples. In interpretation of these sensitivity assays, it is important to note that C8166 cells contain three copies of each LTR and of the *pX* region of HTLV-I, but only one copy of *gag*, *pol* and *env* sequences (Section 2.3.4.1). BLV-FLK cells contain one copy of complete provirus (2.3.4.6).

The β-globin primers 18 & 52 were used with concentrations of 0.5 μM each primer and a concentration of 1.5 mM $MgCl_2$ in each PCR. Serial dilutions showed that β-globin sequences could be detected in 0.01 ng DNA using these primers when PCR products were visualised on polyacrylamide gels (Figure 3.24).

Table 3.2: Optimised conditions and sensitivity of polymerase chain reactions.

The listed concentrations of oligonucleotide primers and $MgCl_2$ were used in PCRs for amplification of HTLV-I, HTLV-I/II and BLV sequences following optimisation. The sensitivity of each PCR primer set is given for detection of template (C8166 DNA for HTLV-I/II PCRs and BLV-FLK DNA for BLV PCRs) in a total of 1 μg DNA. In most cases, hybridisation of the HTLV-I probe $pMT2_i$ to electroblots increased the sensitivity of detection by 10-fold relative to examination of ethidium bromide-stained polyacrylamide gels by UV transillumination.

LRF primer number	Optimum concentration of each primer (μM)	Optimum concentration of $MgCl_2$ (mM)	Quantity of positive control DNA detected in a total quantity of 1 μg DNA (ng)	
			Ethidium-bromide-stained polyacrylamide gels	Hybridisation using probe $pMT2_i$
HTLV-I LTR				
118 & 119	0.5	2.5	0.1	0.01
HTLV-I *gag*				
120 & 121	1.0	1.5	10	0.1
122 & 123	1.0	1.5	1.0	0.1
HTLV-I *pol*				
1 & 2	1.0	1.5	1.0	0.1
67 & 68	0.5	2.0	10	1.0
HTLV-I *env*				
124 & 125	1.0	1.5	1.0	0.01
HTLV-I/II *pX*				
50 & 51	1.0	1.5	0.1	0.01
3 & 4	0.5	2.5	1.0	0.1
126 & 127	1.0	1.5	1.0	0.1
BLV *gag*				
194 & 195	0.5	1.5	10	1.0

Table 3.3: Sensitivity of polymerase chain reactions for detection of HTLV-I, HTLV-I/II and BLV sequences in cell pellets.

The sensitivity of each PCR primer set is given for detection of template (C8166 DNA for HTLV-I and HTLV-I/II PCRs and BLV-FLK DNA for BLV PCRs) in pellets of 1×10^5 cells digested with non-ionic detergent buffer and proteinase K. In most cases, hybridisation of the HTLV-I probe $pMT2_i$ to electroblots increased the sensitivity of detection by 10-fold relative to examination of ethidium bromide-stained polyacrylamide gels by UV transillumination.

LRF primer number	Number of positive cells detected in a total of 1×10^5 cells	
	Ethidium-bromide-stained polyacrylamide gels	Hybridisation using probe $pMT2_i$
HTLV-I LTR		
118 & 119	1×10^2	1×10^1
HTLV-I *gag*		
120 & 121	1×10^3	1×10^1
122 & 123	1×10^2	1×10^0
HTLV-I *pol*		
1 & 2	1×10^1	1×10^0
HTLV-I *env*		
124 & 125	1×10^2	1×10^0
HTLV-I/II *pX*		
50 & 51	1×10^1	1×10^0
126 & 127	1×10^1	1×10^0

Figure 3.6: Sensitivity of PCR for detection of HTLV-I LTR sequences using primers 118 & 119.

PCR for HTLV-I LTR sequences using primers 118 & 119 was performed on serial dilutions of C8166 DNA in placental DNA (total quantity 1 μg DNA) and products were examined on an ethidium bromide-stained polyacrylamide gel following electrophoresis. The expected PCR product size is 183 bp. Additional bands at approximately 300 bp represent migration of single-stranded PCR product. M = ϕX174/*Hae*III molecular weight marker. bp = base pairs.

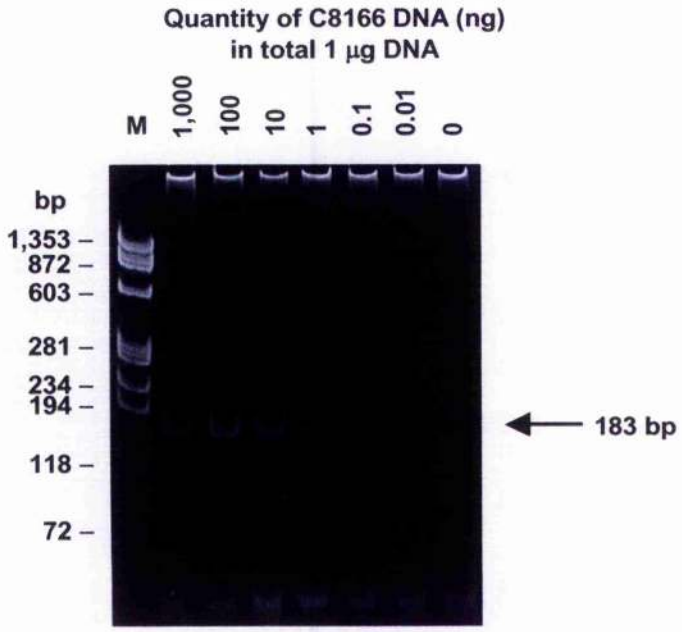


Figure 3.7: Hybridisation of probe pMT2$_i$ to HTLV-I LTR PCR products amplified using primers 118 & 119.

HTLV-I LTR PCR products from Figure 3.6 amplified using primers 118 & 119 were electroblotted onto nylon membrane and hybridised to probe pMT2$_i$ radiolabelled by random priming. PCR products of 183 bp were positive on hybridisation, as were PCR products that migrated as single-stranded bands at approximately 300 bp. M = Molecular weight marker lane. bp = base pairs.

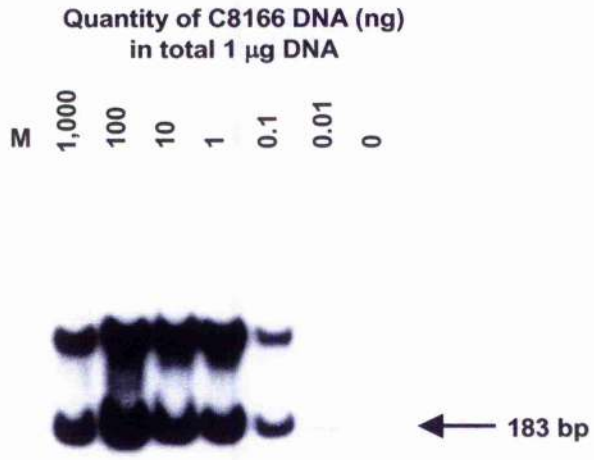


Figure 3.8: Sensitivity of PCR for detection of HTLV-I *gag* sequences using primers 120 & 121.

PCR for HTLV-I *gag* sequences using primers 120 & 121 was performed on serial dilutions of C8166 DNA in placental DNA (total quantity 1 μg DNA) and products were examined on an ethidium bromide-stained polyacrylamide gel following electrophoresis. The expected PCR product size is 273 bp. Additional larger bands at approximately 400 and 850 bp may represent migration of single-stranded PCR products. M = ϕX174/*Hae*III molecular weight marker. bp = base pairs.

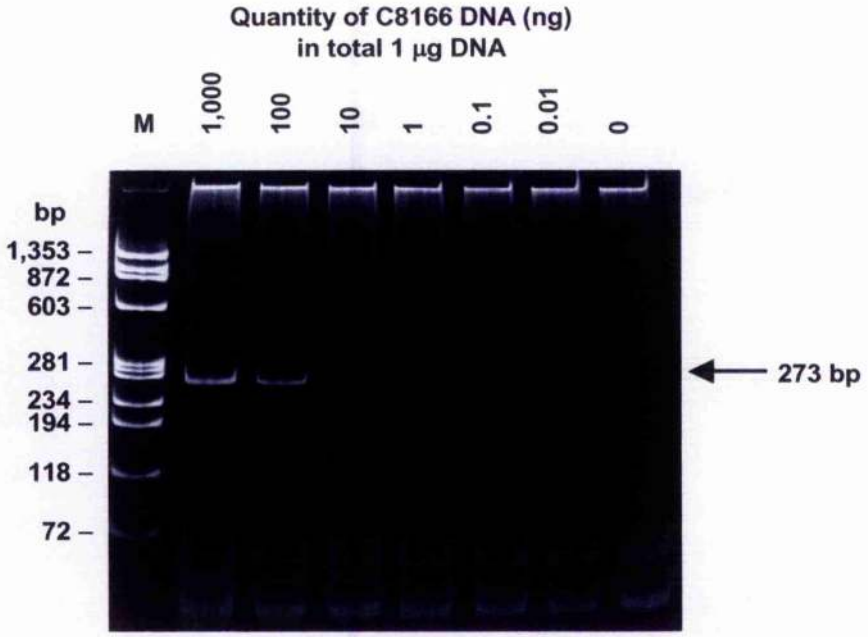


Figure 3.9: Hybridisation of probe pMT2$_i$ to HTLV-I *gag* PCR products amplified using primers 120 & 121.

HTLV-I *gag* PCR products from Figure 3.8 amplified using primers 120 & 121 were electroblotted onto nylon membrane and hybridised to probe pMT2$_i$ radiolabelled by random priming. PCR products of 273 bp, as well as larger bands including probable single-stranded PCR products, were positive on hybridisation. M = Molecular weight marker lane. bp = base pairs.

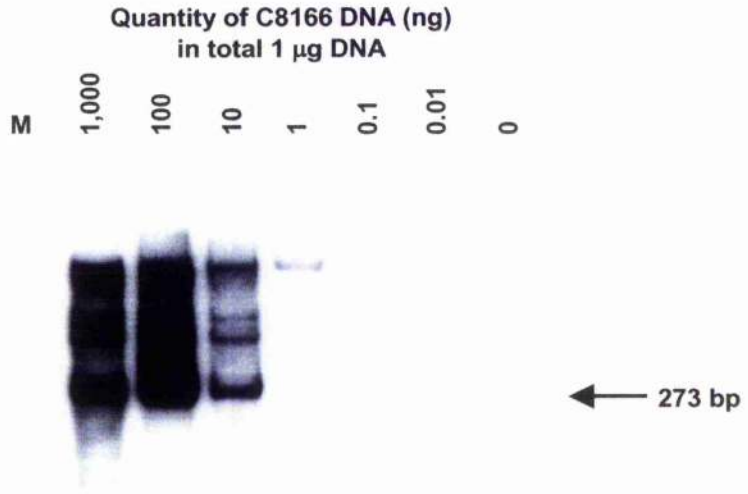


Figure 3.10: Sensitivity of PCR for detection of HTLV-I *gag* sequences using primers 122 & 123.

The sensitivity of PCR for detecting HTLV-I *gag* sequences using primers 122 & 123 was determined using serial dilutions of C8166 DNA in placental DNA (total quantity 1 μg DNA). In conjunction with the sensitivity assay, PCRs were performed on 1 μg skin DNA from three patients with cutaneous lymphoid infiltrates, along with water controls. PCR products were separated by polyacrylamide gel electrophoresis and stained with ethidium bromide. The expected PCR product size is 134 bp. M = ϕX174/*Hae*III molecular weight marker.
P = Patient number. CBCL = Cutaneous B cell lymphoma. MF = Mycosis fungoides. SS = Sèzary syndrome. bp = base pairs.

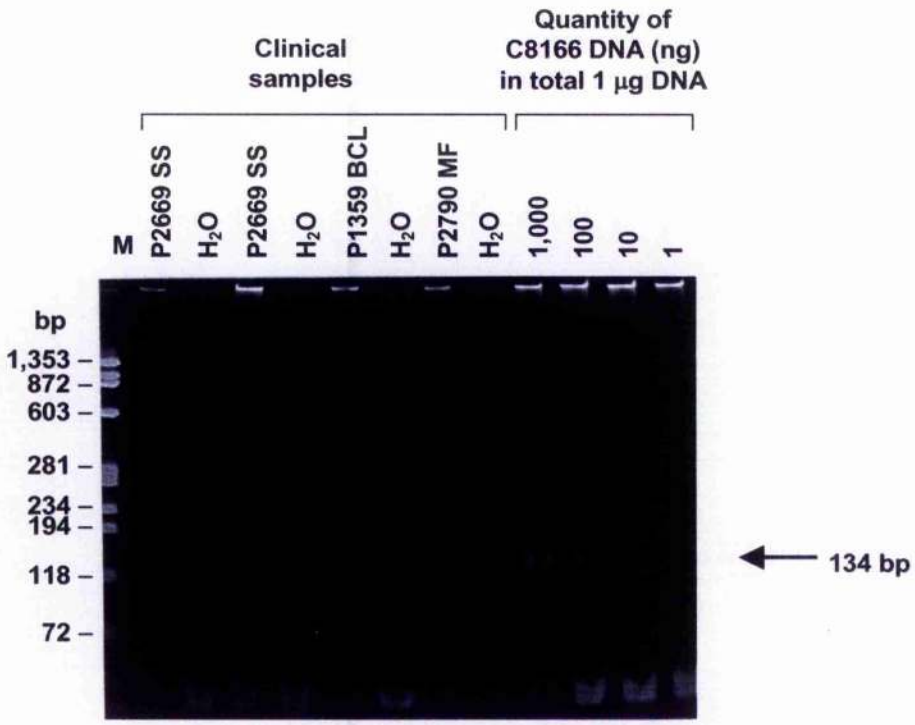


Figure 3.11: Hybridisation of probe pMT2$_i$ to HTLV-I *gag* PCR products amplified using primers 122 & 123.

HTLV-I *gag* PCR products from Figure 3.10 amplified using primers 122 & 123 were electroblotted onto nylon membrane and hybridised to probe pMT2$_i$ radiolabelled by random priming. PCR products of 134 bp were positive on hybridisation and there was hybridisation to products that migrated as single strands at higher molecular weight. M = Molecular weight marker lane. P = Patient number. CBCL = Cutaneous B cell lymphoma. MF = Mycosis fungoides. SS = Sèzary syndrome. bp = base pairs.

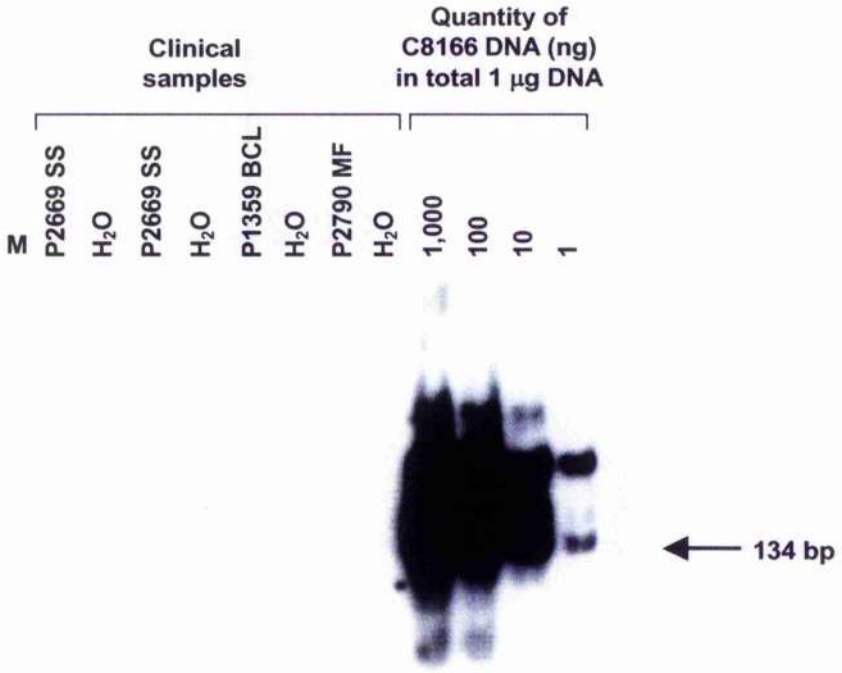


Figure 3.12: Sensitivity of PCR for detection of HTLV-I *pol* sequences using primers 1 & 2.

PCR for HTLV-I *pol* sequences using primers 1 & 2 was performed on serial dilutions of C8166 DNA in placental DNA (total quantity 1 μg DNA) and products were examined on an ethidium bromide-stained polyacrylamide gel following electrophoresis. The expected PCR product size is 119 bp. Additional bands at approximately 150 bp represent migration of single-stranded PCR product. M = ϕX174/*Hae*III molecular weight marker. bp = base pairs.

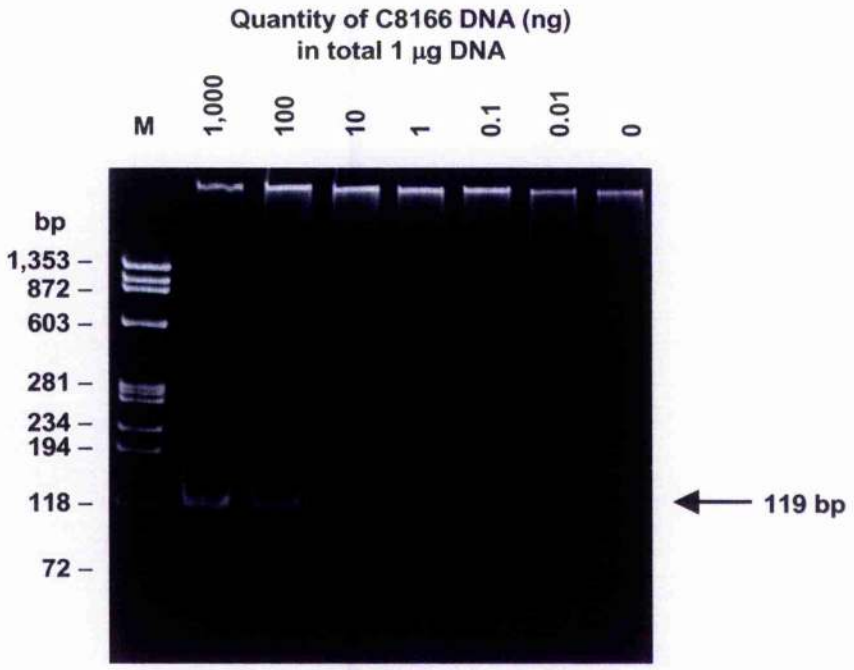


Figure 3.13: Hybridisation of probe pMT2$_i$ to HTLV-I *pol* PCR products amplified using primers 1 & 2.

HTLV-I *pol* PCR products from Figure 3.12 amplified using primers 1 & 2 were electroblotted onto nylon membrane and hybridised to probe pMT2$_i$ radiolabelled by random priming. PCR products of 119 bp were strongly positive on hybridisation. PCR product that migrated as single stranded bands at approximately 150 bp was also hybridisation positive and there was weak hybridisation to unincorporated PCR primers. . A faint band visible by eye in the lane with 0.1 ng C8166 DNA is not reproduced in the photograph. M = Molecular weight marker lane. bp = base pairs.

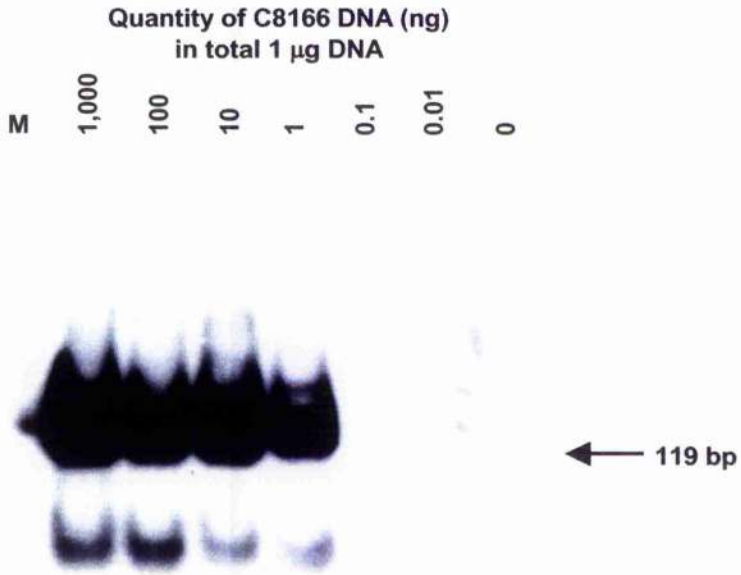


Figure 3.14: Sensitivity of PCR for detection of HTLV-I *pol* sequences using primers 67 & 68.

The sensitivity of PCR for detecting HTLV-I *pol* sequences using primers 67 & 68 was determined using serial dilutions of C8166 DNA in placental DNA (total quantity 1 μg DNA). In conjunction with the sensitivity assay, PCRs were performed on 1 μg skin DNA from three patients with cutaneous lymphoid infiltrates, along with water controls. PCR products were separated by polyacrylamide gel electrophoresis and stained with ethidium bromide. The expected PCR product size is 186 bp. Several bands migrating at larger sizes represent single-stranded PCR products. M = ϕX174/*Hae*III molecular weight marker. P = Patient number. CBCL = Cutaneous B cell lymphoma. MF = Mycosis fungoides. SS = Sèzary syndrome. bp = base pairs.

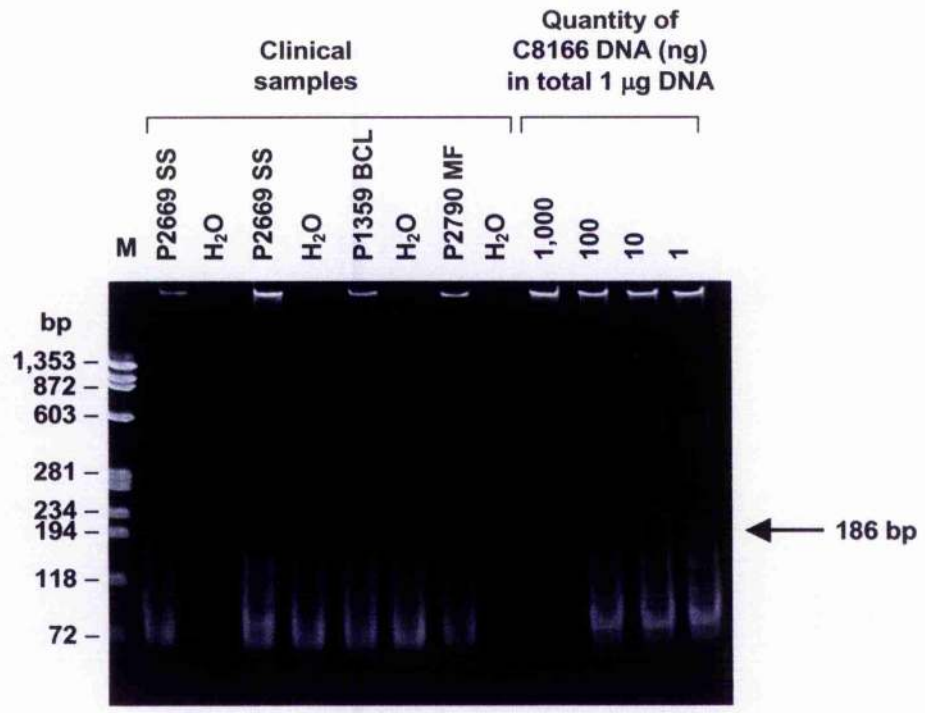


Figure 3.15: Hybridisation of probe pMT2$_i$ to HTLV-I *pol* PCR products amplified using primers 67 & 68.

HTLV-I *pol* PCR products from Figure 3.14 amplified using primers 67 & 68 were electroblotted onto nylon membrane and hybridised to probe pMT2$_i$ radiolabelled by random priming. PCR products of 186 bp were positive on hybridisation and there was hybridisation to products that migrated at higher molecular weight. Faint bands visible by eye in the lanes with 10 and 1 ng C8166 DNA are not reproduced in the photograph. M = Molecular weight marker lane. P = Patient number. CBCL = Cutaneous B cell lymphoma. MF = Mycosis fungoides. SS = Sèzary syndrome. bp = base pairs.

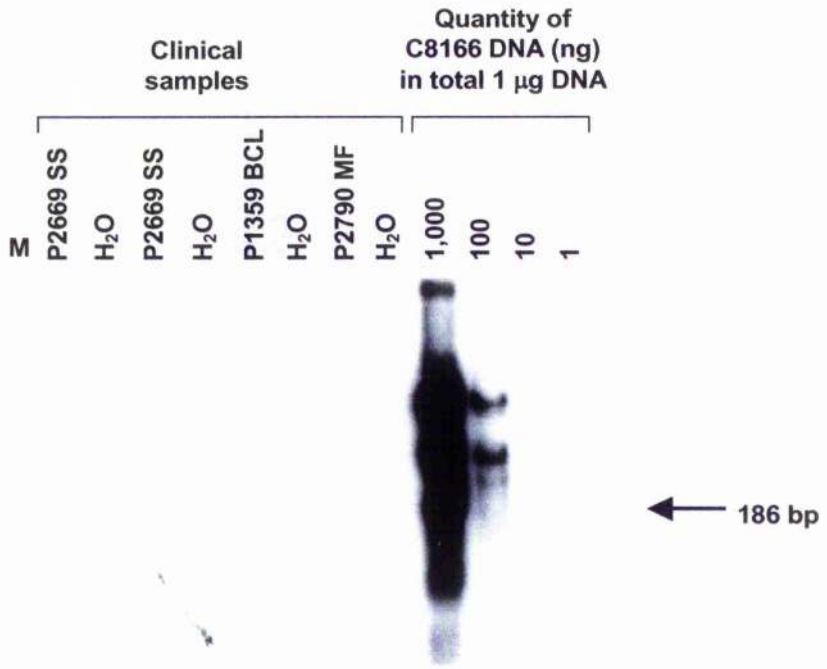


Figure 3.16: Sensitivity of PCR for detection of HTLV-I *env* sequences using primers 124 & 125.

PCR for HTLV-I *env* sequences using primers 124 & 125 was performed on serial dilutions of C8166 DNA in placental DNA (total quantity 1 μg DNA) and products were examined on an ethidium bromide-stained polyacrylamide gel following electrophoresis. The expected PCR product size is 327 bp. Specific PCR products from C8166 were detected as a doublet, probably reflecting amplification from different integrated proviruses in the DNA. Additional bands migrating at approximately 1,200 and 900 bp represent migration of corresponding single-stranded PCR product. M = ϕX174/*Hae*III molecular weight marker. bp = base pairs.

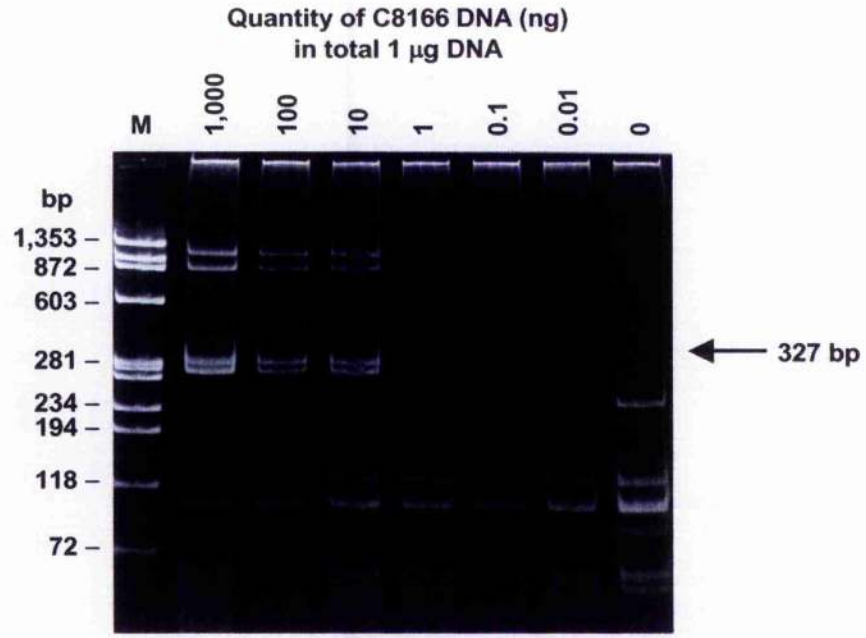


Figure 3.17: Hybridisation of probe pMT2$_i$ to HTLV-I *env* PCR products amplified using primers 124 & 125.

HTLV-I *env* PCR products from Figure 3.16 amplified using primers 124 & 125 were electroblotted onto nylon membrane and hybridised to probe pMT2$_i$ radiolabelled by random priming. PCR products of 327 bp were strongly positive on hybridisation, as were products of other sizes with decreasing intensity as the quantity of positive control template decreased. A faint band visible by eye in the lane with 0.01 ng C8166 DNA is not reproduced in the photograph. bp = base pairs.

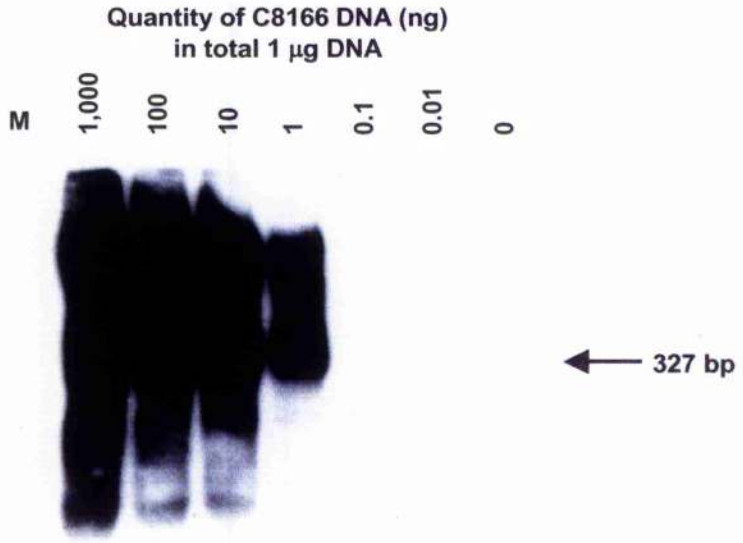


Figure 3.18: Sensitivity of PCR for detection of HTLV-I *pX* sequences using primers 50 & 51.

PCR for HTLV-I *pX* sequences using primers 50 & 51 was performed on serial dilutions of C8166 DNA in placental DNA (total quantity 1 μg DNA) and products were examined on an ethidium bromide-stained polyacrylamide gel following electrophoresis. The expected PCR product size is 159 bp. Bands at approximately 400 bp represent PCR product that has migrated as single-stranded DNA. Non-specific PCR products increase as the proportion of C8166 DNA decreases. M = ϕX174/*Hae*III molecular weight marker. bp = base pairs.

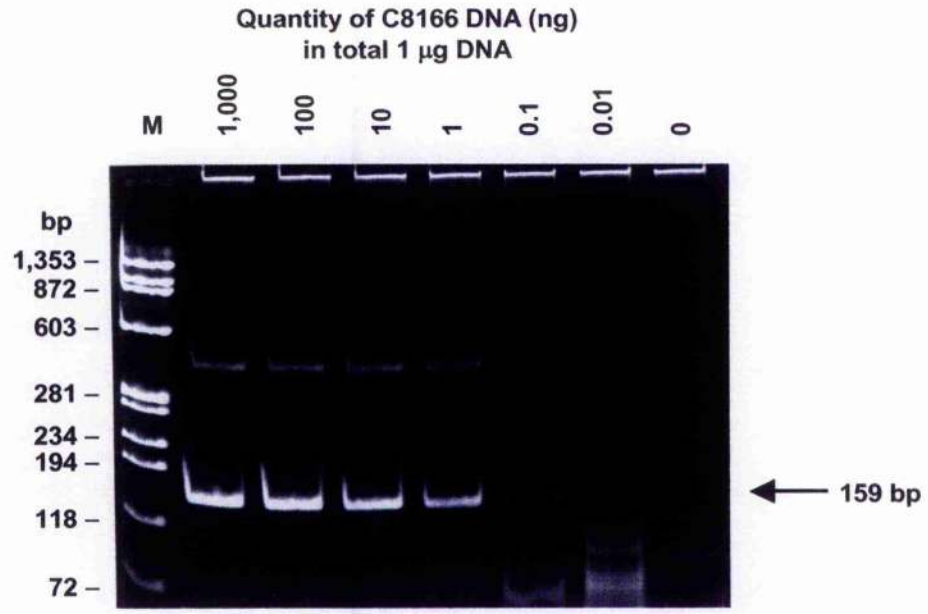


Figure 3.19: Hybridisation of probe pMT2$_i$ to HTLV-I *pX* PCR products amplified using primers 50 & 51.

HTLV-I *pX* PCR products from Figure 3.18 amplified using primers 50 & 51 were electroblotted onto nylon membrane and hybridised to probe pMT2$_i$ radiolabelled by random priming. PCR products of 159 bp were positive on hybridisation and there was hybridisation to bands that migrated at approximately 400 bp and represent single-stranded PCR product. Hybridisation to several non-specific bands is visible in the lane containing product from the PCR with no C8166 DNA template (1 μg placental DNA only). A faint band visible by eye in the lane with 0.01 ng C8166 DNA is not reproduced in the photograph. M = Molecular weight marker lane. bp = base pairs.

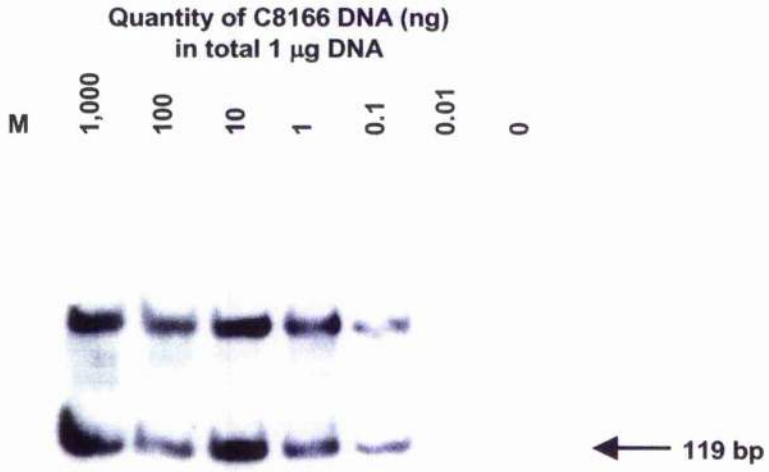


Figure 3.20: Sensitivity of PCR for detection of HTLV-I *pX* sequences using primers 126 & 127.

PCR for HTLV-I *pX* sequences using primers 126 & 127 was performed on serial dilutions of C8166 DNA in placental DNA (total quantity 1 μg DNA) and products were examined on an ethidium bromide-stained polyacrylamide gel following electrophoresis. The expected PCR product size is 127 bp. A slightly larger band at approximately 150 bp in some lanes probably represents an alternative product from one of three proviruses in C8166 DNA. Bands at approximately 275 bp are due to migration of single-stranded PCR products. M = ϕX174/*Hae*III molecular weight marker. bp = base pairs.

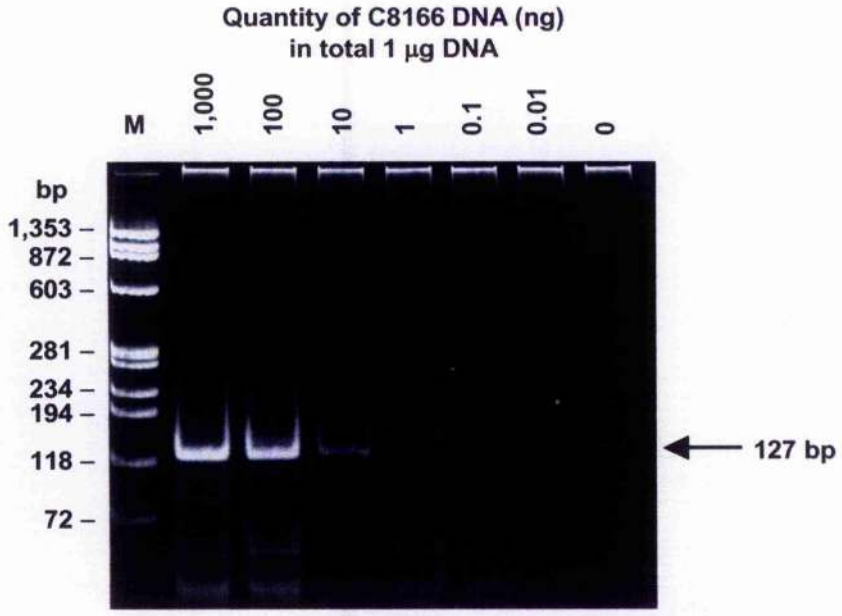


Figure 3.21: Hybridisation of probe pMT2$_i$ to HTLV-I *pX* PCR products amplified using primers 126 & 127.

HTLV-I *pX* PCR products from Figure 3.20 amplified using primers 126 & 127 were electroblotted onto nylon membrane and hybridised to probe pMT2$_i$ radiolabelled by random priming. PCR products of 127 bp, as well as larger single-stranded PCR products migrating at approximately 275 bp, were positive on hybridisation. A faint band visible by eye in the lane with 0.1 ng C8166 DNA is not reproduced in the photograph. M = Molecular weight marker lane. bp = base pairs.

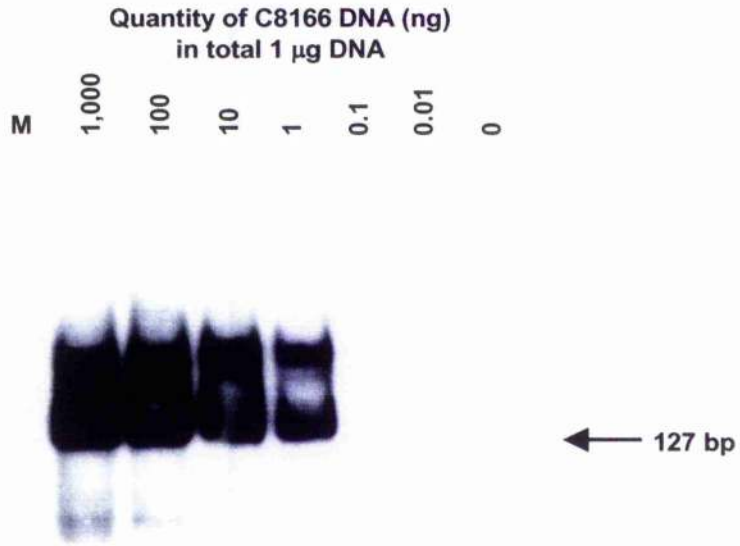


Figure 3.22: Sensitivity of PCR for detection of bovine leukaemia virus *gag* sequences using primers 194 & 195.

The sensitivity of PCR for detecting bovine leukaemia virus *gag* sequences with primers 194 & 195 was determined using serial dilutions of BLV-FLK DNA in FLK DNA (total quantity 1 μg DNA). In conjunction with the sensitivity assay, PCRs were performed on three samples of 1 x 10^5 peripheral blood mononuclear cells (PBMCs) from two patients with T cell acute lymphoblastic leukaemia (ALL) (patient 1130) and common ALL (patient 1131), along with water controls. PCR products were separated by polyacrylamide gel electrophoresis and stained with ethidium bromide. The expected PCR product size is 247 bp. M = ϕX174/*Hae*III molecular weight marker. P = Patient number. T-ALL = T cell acute lymphoblastic leukaemia. C-ALL = Common acute lymphoblastic leukaemia. BLV-FLK = Bovine leukaemia virus-infected foetal lamb kidney cells. bp = base pairs.

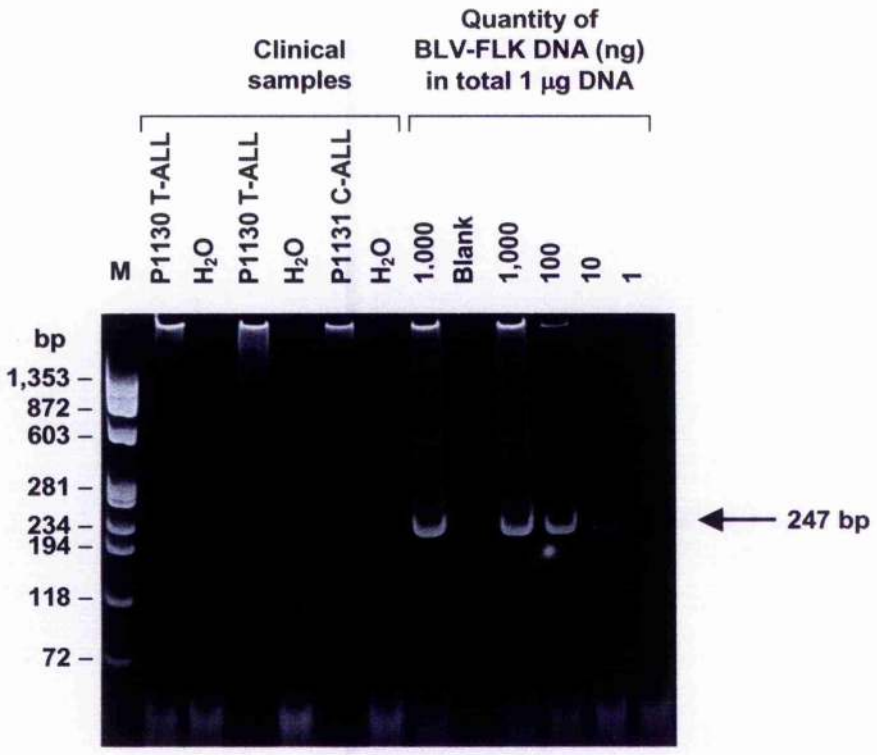


Figure 3.23: Hybridisation of a bovine leukaemia virus probe to *gag* PCR products amplified from BLV-FLK DNA using primers 194 & 195.

Bovine leukaemia virus (BLV) *gag* PCR products from Figure 3.22 amplified using primers 194 & 195 were electroblotted onto nylon membrane and hybridised to the BLV oligonucleotide probe 301 radiolabelled by 5' end-labelling. PCR products of 247 bp were positive on hybridisation and there was hybridisation to single-stranded PCR products that migrated at higher molecular weight. A faint band visible by eye in the lane with 1 ng C8166 DNA is not reproduced in the photograph. M = Molecular weight marker lane. P = Patient number. T-ALL = T cell acute lymphoblastic leukaemia. C-ALL = Common acute lymphoblastic leukaemia. BLV-FLK = Bovine leukaemia virus-infected foetal lamb kidney cells. bp = base pairs.

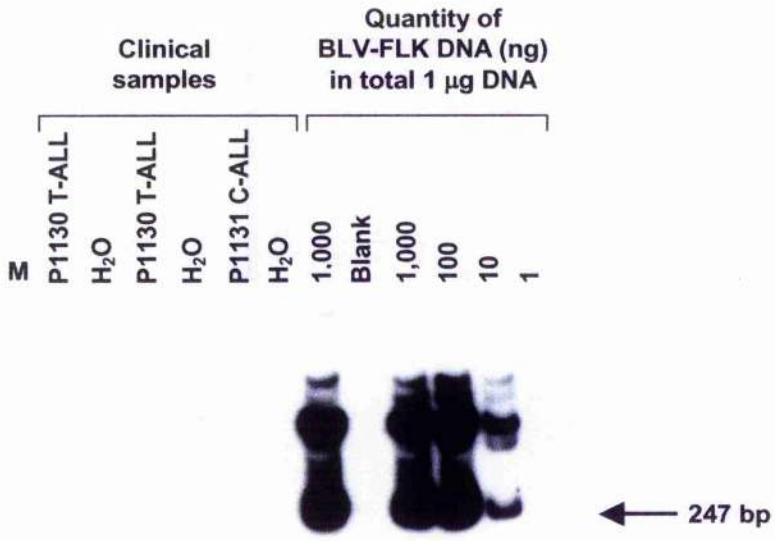
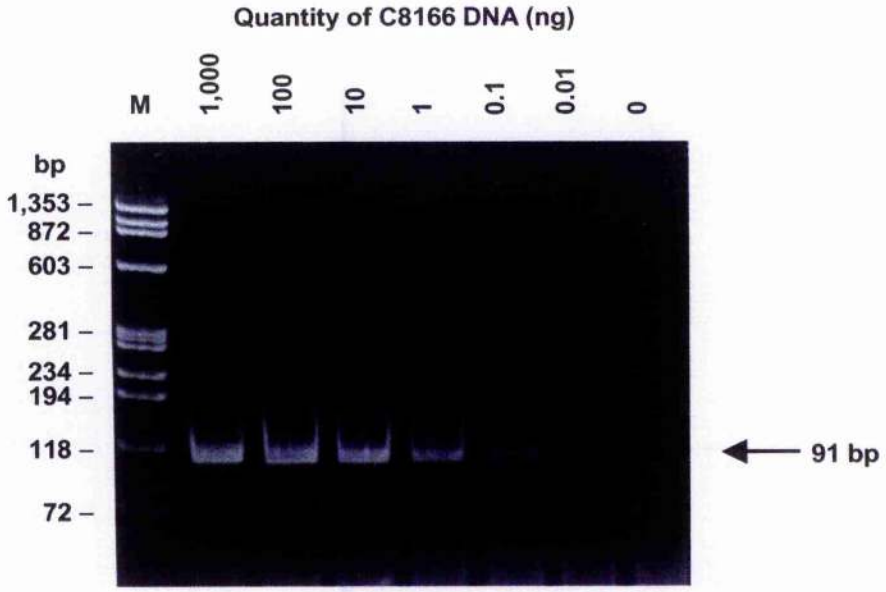


Figure 3.24: Sensitivity of PCR for detection of β-globin sequences in genomic DNA using primers 18 & 52.

PCR for β-globin sequences using primers 18 & 52 was performed on serial dilutions of C8166 DNA in water and products were examined on an ethidium bromide-stained polyacrylamide gel following electrophoresis. The expected PCR product size is 91 bp. M = ϕX174/*Hae*III molecular weight marker. bp = base pairs.



3.4.2 Cutaneous T cell lymphomas

Samples from 36 patients with cutaneous lymphoid infiltrates were examined by PCR for HTLV-I sequences. Clinical diagnoses in these patients were 21 cases of MF, five cases of SS, four cases of CBCL, three cases of angiocentric T cell lymphoma (ATCL) and one case each of CTCL (peripheral T cell lymphoma), small plaque parapsoriasis (SPP) and Langerhan's cell histiocytosis (LCH) (Table 3.1). Samples from all patients were positive with primers 18 & 52 for β globin by PCR, indicating the presence of amplifiable DNA (Tables 3.4, 3.5 and 3.6).

Three samples of PBMCs from two patients, one with SS (patient 1198) and one with CBCL (patient 1359), had faintly positive bands of approximately 159 bp on hybridisation for HTLV-I/II *tax* sequences amplified using primers 50 & 51 (Table 3.7) (Figures 3.25 and 3.26). Attempts were made to clone and sequence the 159 bp first round PCR products after gel purification, but the quantity of amplified material was too small to yield clonable DNA. The PCR products were re-amplified using nested primers that annealed to sequences internal to primers 50 & 51 (Section 3.3.7.5). Bands of 136 bp were obtained using the HTLV-I-specific primer set 282 & 283, whereas multiple non-specific bands, as well as bands of the approximate expected size, were obtained using the HTLV-II-specific primers 288 & 289. This suggested that HTLV-I-specific product was present.

The second round (nested) PCR products amplified using primers 282 & 283 from two submissions of PBMCs from patient 1198 (1198-1 and 1198-3) were successfully TA cloned and sequenced (Section 3.3.16.2), along with PCR products cloned from C8166 DNA following a single round amplification using the outer set of primers (50 & 51). The sequences amplified from these two samples from patient 1198 were identical and matched the sequence from C8166, but differed at four bases from the sequence of the same region of prototypical HTLV-I (Seiki *et al* 1983) (Figure 3.27). The first round PCR product from patient 1198 had been handled in the potentially contaminated molecular biology laboratory before the nested PCR was performed.

One sample of skin from a patient with MF (patient 436) had a faintly positive band of approximately 127 bp on hybridisation for HTLV-I *tax* sequences amplified using primers 126 & 127 (Table 3.7) (Figures 3.28 and 3.29). The first round PCR product from this sample was cloned after gel purification of the 127 bp band. The sequence of this PCR product was identical to that expected from the prototypical HTLV-I sequence, as well as the sequence amplified from positive control C8166 DNA using the same primers (Figure 3.30).

All other samples were negative by PCR using primers specific for HTLV-I LTR, *gag*, *pol*, *env* and *pX* regions.

3.4.3 Large granular lymphocytic leukaemia

Fresh and cultured PBMCs were tested from six patients with $CD3^+$ $CD8^+$ LGL leukaemia (Table 2.4). All samples were positive for β globin, indicating the presence of amplifiable DNA, although the signal from one patient was weak (Table 3.8).

Of six samples tested for HTLV-I/II *tax* using primers 50 & 51 and four samples tested for HTLV-I *pol* using primers 1 & 2, all were negative by PCR. Faint bands with sizes of approximately 119 and 180 bp were observed on ethidium bromide-stained gels in one patient following amplification with primers 1 & 2 but neither of these bands hybridised to the specific HTLV-I probe. No BLV *gag* PCR products were detected in two PBMC samples from two patients tested using primers 194 & 195.

3.4.4 Acute lymphoblastic leukaemia cases

PBMCs and bone marrow were tested from six patients with ALL of common, pre-B cell or T cell type (Table 2.5). All patients were male butchers from Cardiff. Seven samples of PBMCs and three of four bone marrow samples were positive for β globin, indicating the presence of amplifiable DNA (Table 3.8).

PBMCs and bone marrow samples from four patients tested for HTLV-I/II *tax* using primers 50 & 51 and HTLV-I *pol* using primers 1 & 2 were negative by PCR. Following amplification with primers 50 & 51, a faint band with a size of approximately 118 bp was detected in one sample, but this was negative on hybridisation. Using primers 1 & 2, faint bands with a size of approximately 120 bp were observed on ethidium bromide-stained gels in four samples from three patients. Additional bands also observed on ethidium bromide-stained gels were 90 and 230 bp in one sample, 350 bp in a second sample from the same patient and 170 bp in a sample from another patient. Faint bands of approximately 119 bp were detected in samples from two of these patients when amplification with primers 1 & 2 was repeated, whereas no PCR product was detected in another sample from one of these patients. These additional bands were negative on hybridisation to the specific HTLV-I probe.

No BLV *gag* PCR products were detected in six PBMC and four bone marrow samples from five patients tested using primers 194 & 195 (Table 3.9).

3.4.5 Polymerase chain reaction for detection of HTLV-I and HTLV-II in cultured cells

PCR was used to test for HTLV-I and HTLV-I/II sequences in four long-term T cell cultures established from skin samples of three patients with MF (patients 2531, 2600 and 2862) and one Epstein-Barr virus (EBV)-infected B-LCL established from a skin sample from a patient with CBCL (patient 2132) (Section 2.4.1) (Table 2.12). Established T cell lines grown in the laboratory were also tested for HTLV-I, HTLV-I/II and BLV sequences by PCR. These cell lines were J.JHAN, L-726A, HH, My-La and My-La MC, none of which is known to be infected with HTLV-I or HTLV-II (Sections 2.1.3.1 and 2.3.4). C8166 cells were used as positive controls. Pellets of 1 x 10^5 cells and 1 μg DNA extracted from cultured cells were tested by PCR with the complete panel of HTLV-I, HTLV-I/II and BLV primers using optimised conditions for each primer set. Parallel samples were also tested for β-globin sequences using primers 18 & 52. C8166 was positive with all HTLV-I and HTLV-I/II primer sets but was negative with the BLV primer sets. All other cultured cells tested by PCR were negative for HTLV-I, HTLV-I/II and BLV sequences.

Table 3.4: Polymerase chain reaction results from skin samples of patients with cutaneous lymphoid infiltrates.

Primer set	PCR product	Number tested	Number positive
18 & 52	β globin	37	37
118 & 119	HTLV-I LTR	26	0
120 & 121	HTLV-I *gag*	25	0
122 & 123	HTLV-I *gag*	24	0
1 & 2	HTLV-I *pol*	34	0
67 & 68	HTLV-I *pol*	29	0
124 & 125	HTLV-I *env*	28	0
3 & 4	HTLV-I *tax*	29	0
50 & 51	HTLV-I *tax*	34	0
126 & 127	HTLV-I *tax*	28	1

Table 3.5: Polymerase chain reaction results from peripheral blood mononuclear cells of patients with cutaneous lymphoid infiltrates.

Primer set	PCR product	Number tested	Number positive
18 & 52	β globin	16	16
118 & 119	HTLV-I LTR	14	0
120 & 121	HTLV-I *gag*	11	0
1 & 2	HTLV-I *pol*	15	0
124 & 125	HTLV-I *env*	12	0
50 & 51	HTLV-I *tax*	15	3

Table 3.6: Polymerase chain reaction results from lymph node samples of cutaneous T cell lymphoma patients.

Primer set	PCR product	Number tested	Number positive
18 & 52	β globin	5	5
118 & 119	HTLV-I LTR	5	0
120 & 121	HTLV-I *gag*	5	0
1 & 2	HTLV-I *pol*	5	0
124 & 125	HTLV-I *env*	5	0
50 & 51	HTLV-I *tax*	5	0
126 & 127	HTLV-I *tax*	4	0

Table 3.7: Details of polymerase chain reaction positive cutaneous T cell lymphoma cases.

Patient number	Age (years)	Sex	Clinical diagnosis	Sample number	Sample type	Primer set	PCR product
436	64	M	MF	15757	Skin	126 & 127	HTLV-I *tax*
1198	81	M	SS	13702	PBMCs	50 & 51	HTLV-I *tax*
				13944	PBMCs	50 & 51	HTLV-I *tax*
1359	NA	M	BCL	15644	PBMCs	50 & 51	HTLV-I *tax*

Figure 3.25: Amplification of HTLV-I *pX* sequences from clinical samples of patients 1198 and 1359 using primers 50 & 51.

Peripheral blood mononuclear cells (PBMCs) from a patient with Sèzary syndrome (patient 1198) and a patient with cutaneous B cell lymphoma (patient 1359) were positive for HTLV-I *pX* sequences when tested by PCR using primers 50 & 51. Samples are shown on a polyacrylamide gel stained with ethidium bromide. Water controls were run next to each sample. C8166 cells (1×10^5) were used as a positive control. Sensitivity was determined by electrophoresis of PCR products amplified from serial dilutions of C8166 cells in PBMCs on a different polyacrylamide gel (sensitivity 10 C8166 cells). Bands of the expected size (159 bp) could not be distinguished from non-specific bands on the polyacrylamide gel, but bands representing single-stranded DNA migrated at approximately 400 bp in lanes containing two samples from patient 1198 and one sample from patient 1359. These bands hybridised to a probe for HTLV-I (Figure 3.26). M = ϕX174/*Hae*III molecular weight marker. P = Patient number. SS = Sèzary syndrome. MF = Mycosis fungoides. CBCL = Cutaneous B cell lymphoma. bp = base pairs.

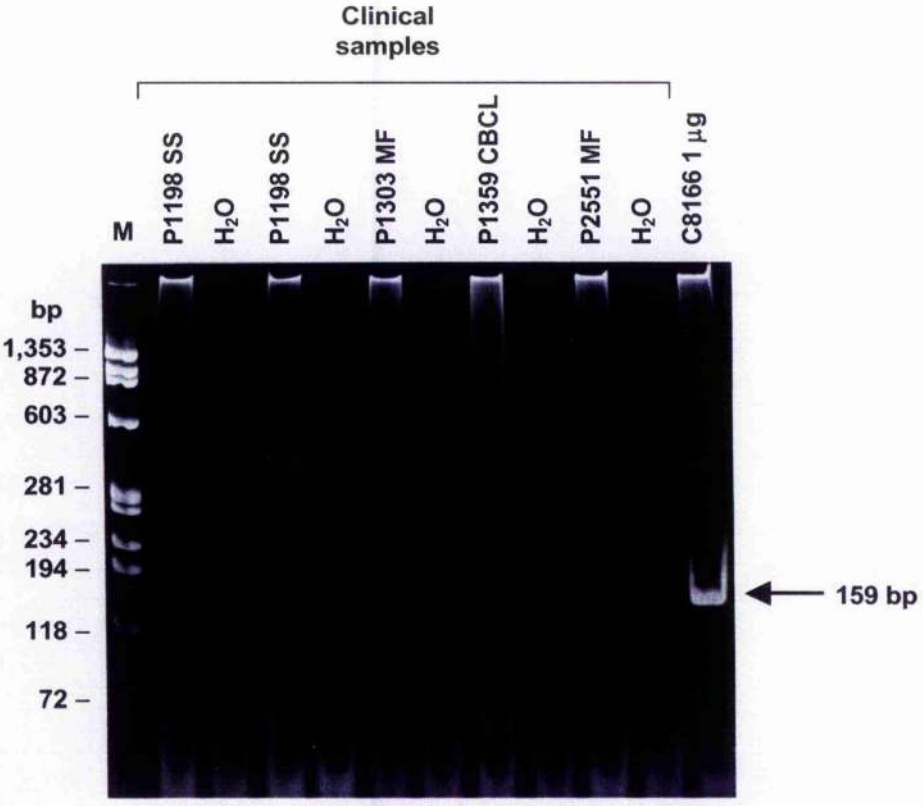


Figure 3.26: Hybridisation of probe pMT2$_i$ to HTLV-I *pX* PCR products amplified from clinical samples of patients 1198 and 1359 using primers 50 & 51.

HTLV-I *pX* PCR products from Figure 3.25 amplified using primers 50 & 51 were electroblotted onto nylon membrane and hybridised to probe pMT2$_i$ radiolabelled by random priming. PCR products of 159 bp from two samples of PBMCs from patient 1198 (Sèzary syndrome) and one sample of PBMCs from patient 1359 (cutaneous B cell lymphoma) were faintly positive on hybridisation. Positive control cells from the C8166 cell line were strongly positive on hybridisation. Bands that migrated at approximately 400 bp, representing single-stranded PCR products, were also positive on hybridisation. M = Molecular weight marker lane. P = Patient number. SS = Sèzary syndrome. MF = Mycosis fungoides. CBCL = Cutaneous B cell lymphoma. bp = base pairs.

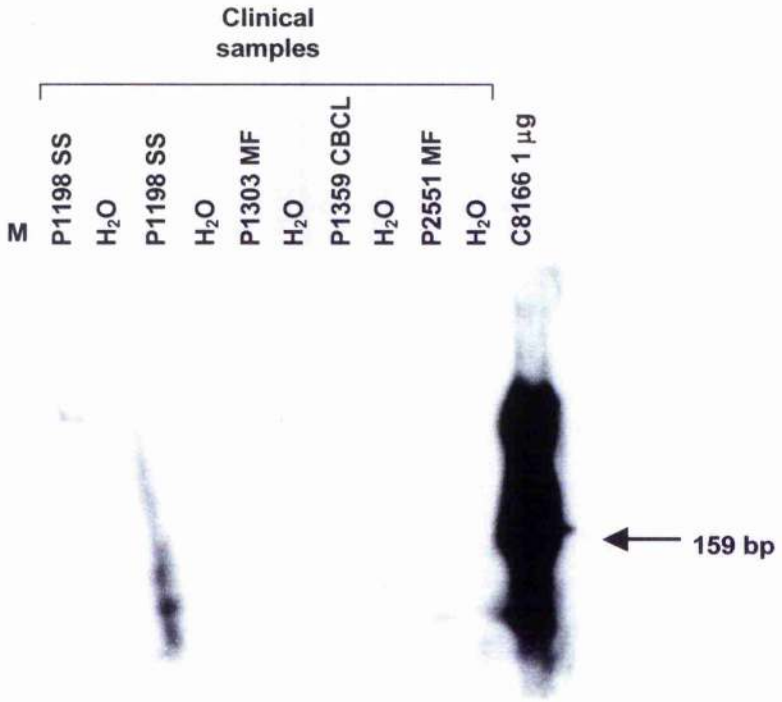


Figure 3.27: Sequences of nested HTLV-I *pX* PCR products amplified using primers 282 & 283 from patient 1198.

First round PCR products were amplified from peripheral blood mononuclear cells of patient 1198, who had SS, using primers 50 & 51. Second round (nested) PCR products were amplified using primers 282 & 283, then TA cloned and sequenced. The 136 bp sequence is aligned with prototypical HTLV-I (Seiki *et al* 1983) and sequences amplified from positive control DNA (C8166) using primers 50 & 51 in the same laboratory. Primers are underlined. The sequences from patient 1198 and C8166 are identical but differ at four bases from the prototypical HTLV-I sequence.

```
HTLV-I   AGTCTACGTGTTTGGAGACTGTGTACAAGGCGACTGGTGCCCCATCTCTGGGGGACTATGTTCGGCCC
 C8166   AGTCTACGTGTTTGGNGACTGTTTACAAGGCGACTGGTGCCCCACCTNTGGGGGACTATATCCGGCCC
1198-1   AGTCTACGTGTTTGGAGACTGTTTACAAGGCGACNGGTGCCCCACCTCTGGGGGACTATATCCGGCCC
1198-3   AGTCTACGTGTTTGGAGACTGTTTACAAGGCGACTGGTGCCCCACCTCTGGGGNACTATATCCGGCCC
         Primer 282

HTLV-I   GCCTACATCGTCACGCCCTACTGGCCACCTGTCCAGAGCATCAGATCACCTGGGACCCCATCGATGGA
 C8166   GCCTACATCGTCACGCCCTACTGGCCACCTGTCCAGAGCATCAGATCACCTGGGACCCCATCGATGGA
1198-1   GCCTACATCGTCNCGCCCTACTGGCCACCTGTCCAGAGCATCAGATCACCTGGGACCCCATCGATGGA
1198-3   GCCTACATCGTCACGCCCTACTGGCCACCTGTCCAGAGCATCAGATCACCTGGGACCCCATCGATGGA
                                                  Primer 283
```

HTLV-I	Prototypical HTLV-I sequence
C8166	Positive control HTLV-I-infected T cell line
1198-1	Second round PCR product from PBMCs of patient 1198, sample number 13702
1198-3	Second round PCR product from PBMCs of patient 1198, sample number 13944

Figure 3.28: Amplification of HTLV-I *pX* sequences from skin DNA of patient 436 using primers 126 & 127.

DNA samples (1 μg) from involved skin of five patients with mycosis fungoides (MF) and one patient with angiocentric T cell lymphoma were tested for HTLV-I *pX* sequences by PCR using primers 126 & 127. Water control PCRs were performed along with each sample. C8166 DNA (1 μg) was used as a positive control. Sensitivity was determined by electrophoresis of PCR products amplified from serial dilutions of C8166 DNA in placental DNA on a different polyacrylamide gel (sensitivity 1 ng positive control DNA). Bands of the expected size (127 bp) were detected in PCR products from lesional skin of patient 436, who had MF, as well as in positive control DNA. These bands hybridised to a probe for HTLV-I (Figure 3.29). Multiple non-specific bands were also amplified from clinical samples but did not hybridise to the HTLV-I probe. Polyacrylamide gel electrophoresis and ethidium bromide staining. M = ϕX174/*Hae*III molecular weight marker. P = Patient number. MF = Mycosis fungoides. ATCL = Angiocentric T cell lymphoma. bp = base pairs.

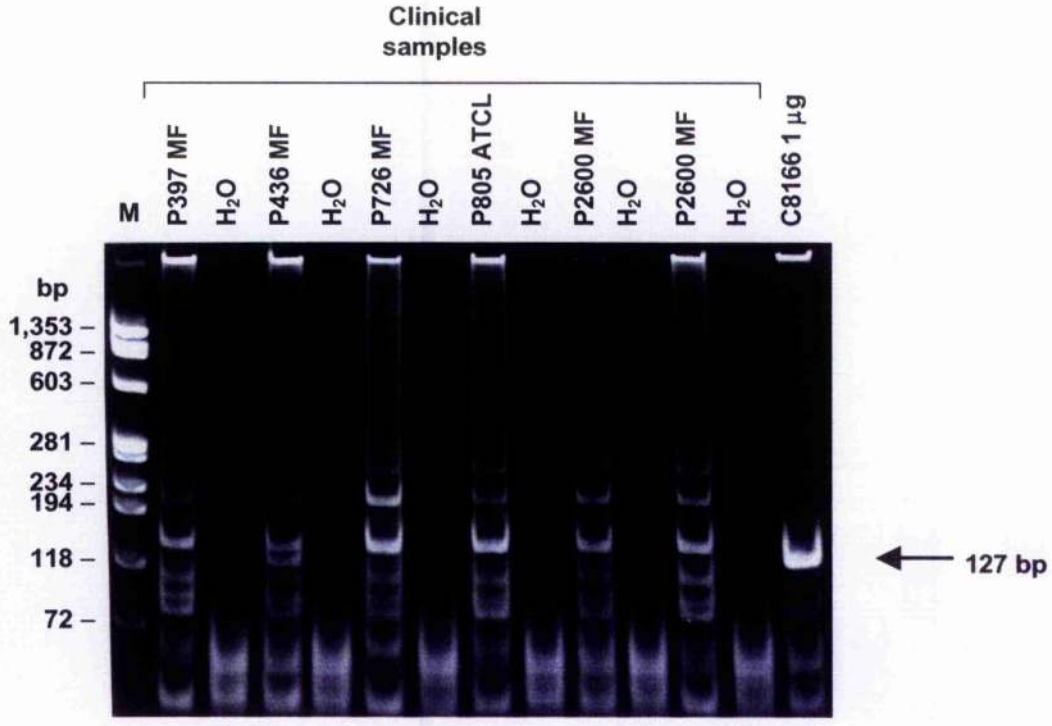


Figure 3.29: Hybridisation of probe pMT2$_i$ to HTLV-I *pX* PCR products amplified from skin DNA of patient 436 using primers 126 & 127.

HTLV-I *pX* PCR products from Figure 3.28 amplified using primers 126 & 127 were electroblotted onto nylon membrane and hybridised to probe pMT2$_i$ radiolabelled by random priming. PCR products of 127 bp from skin DNA of patient 436 and positive control DNA (C8166) were positive on hybridisation, whereas there was no hybridisation to non-specific PCR products. M = Molecular weight marker lane. P = Patient number. MF = Mycosis fungoides. ATCL = Angiocentric T cell lymphoma. bp = base pairs.

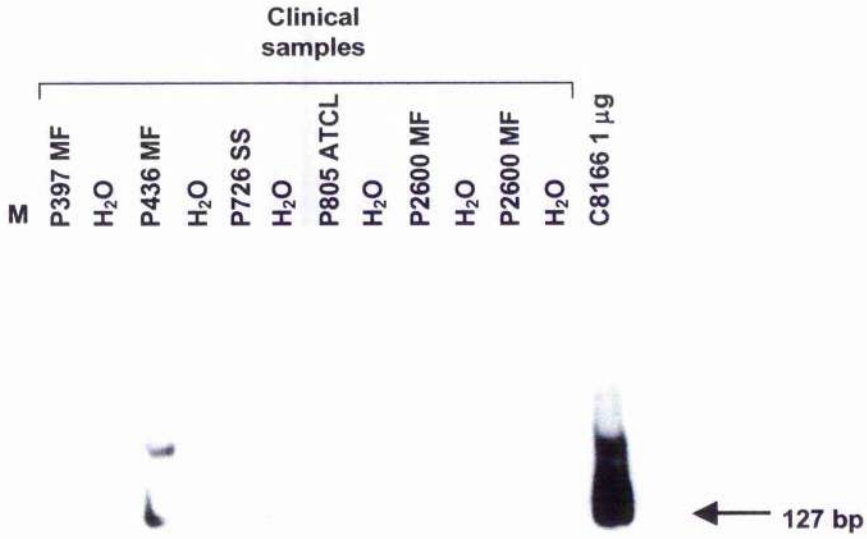


Figure 3.30: Sequences of polymerase chain reaction products amplified using primers 126 & 127 from patient 436.

PCR products were amplified by PCR using primers 126 & 127 from a skin sample of patient 436, who had mycosis fungoides, then cloned and sequenced. The 127 bp sequence is aligned with prototypical HTLV-I (Seiki *et al* 1983) and sequences amplified from positive control DNA (C8166) in the same laboratory. Primers are underlined.

```
 HTLV-I  CCAATCACTCATACAACCCCCAACATTCCACCCTCCTTCCTCCAGGCCATGCGCAAATACTCCC
   C8166 CCAATCACTCATACAACCCCCAACATTCCACCCTCCTTCCTCCAGGCCATGCGCAAATACTCCC
 305-1-2 CCAATCACTCATACAACCCCCAACATTCCACCCTCCTTCCTCCAGGCCATGCGCAAATACTCCC
 305-5-2 CCAATCACTCATACAACCCCCAACATTCCACCCTCCTTCCTCCAGGCCATGCGCAAATACTCCC
 305-6-3 CCAATCACTCATACAACCCCCAACATTCCACCCTCCTTCCTCCAGGCCATGCGCAAATACTCCC
          Primer 126

 HTLV-I  CCTTCCGAAATGGATACATGGAACCCACCCTTGGGCAGCACCTCCCAACCCTGTCTTTTCCAG
   C8166 CCTTCCGAAATGGATACATGGAACCCACCCTTGGGCAGCACCTCCCAACCCTGTCTTTTCCAG
 305-1-2 CCTTCCGAAATGGATACATGGAACCCACCCTTGGGCAGCACCTCCCAACCCTGTCTTTTCCAG
 305-5-2 CCTTCCGAAATGGATACATGGAACCCACCCTTGGGCAGCACCTCCCAACCCTGTCTTTTCCAG
 305-6-3 CCTTCCGAAATGGATACATGGAACCCACCCTTGGGCAGCACCTCCCAACCCTGTCTTTTCCAG
                                                  Primer 127
```

HTLV-I	Prototypical HTLV-I sequence
C8166	Positive control HTLV-I-infected T cell line
305-1-2	First round amplification product
305-5-2	Second round amplification product
305-6-3	Second round amplification product

Table 3.8: Polymerase chain reaction results from peripheral blood mononuclear cells of cases of large granular lymphocytic leukaemia.

Primer set	PCR product	Number tested	Number positive
18 & 52	β globin	6	6
1 & 2	HTLV-I *pol*	4	0
50 & 51	HTLV-I *tax*	6	0
194 & 195	BLV *gag*	2	0

Table 3.9: Polymerase chain reaction results from peripheral blood mononuclear cells of acute lymphoblastic leukaemia cases in butchers from Cardiff.

Primer set	PCR product	Number tested	Number positive
18 & 52	β globin	11	10
1 & 2	HTLV-I *pol*	6	0
50 & 51	HTLV-I *tax*	10	0
194 & 195	BLV *gag*	10	0

3.4.6 Consensus retroviral polymerase chain reaction

The expected size of HTLV-I PCR products amplified using the consensus HTLV/BLV LTR primers 174 & 175 was 506 bp (Figure 3.2). Products of approximately 550 bp that hybridised to the HTLV-I probe $pMT2_i$ were amplified from pMT2 when these primers were used in a standard PCR (annealing temperature 55°C) at concentrations of 1.0 μM with 1.5 mM $MgCl_2$ (Section 3.3.7.6). Bands of approximately 600 bp were amplified from C8166 DNA and human placental DNA under the same PCR conditions but did not hybridise to the HTLV-I probe. PCR products of a variety of different sizes were amplified from pMT2, C8166 and placental DNA at different annealing temperatures, but only the products from pMT2 hybridised to $pMT2_i$. Specific PCR products could be amplified from pMT2 using 1.0 to 2.5 mM $MgCl_2$. Touchdown PCR resulted in PCR products of approximately 400 bp from pMT2 with 1.5 and 2.5 mM $MgCl_2$ and 450 bp from C8166 with 2.5 mM $MgCl_2$ only. Although smaller than the expected product size, both bands hybridised to $pMT2_i$, suggesting that non-specific priming from plasmid and proviral sequences may have occurred.

PCR using 1.0 μM each of primers 174 & 176 and 1.5 mM $MgCl_2$ resulted in variable, weak amplification of a specific 1,858 bp LTR-*gag* product from pMT2, as well as smaller bands that hybridised to $pMT2_i$. Several bands of 100 to 250 bp amplified from C8166 also hybridised to $pMT2_i$, whereas similar bands amplified from placental DNA were negative. In a $MgCl_2$ titration using pMT2 as template, the number of non-specific PCR bands increased as the $MgCl_2$ concentration increased from 1.0 to 3.0 mM. The specific 1,858 bp product was amplified from pMT2 only using touchdown PCR. This band, as well as several smaller bands amplified from pMT2 and C8166, hybridised to $pMT2_i$.

The consensus retroviral primers 296 & 297 were used in an attempt to amplify HTLV-I *pol* sequences from pMT2, C8166 and BLV-FLK DNA using human placental DNA and FLK DNA as controls. Titrations of C8166 DNA in human placental DNA from 1 μg to 0.01 ng in a total of 1 μg DNA were tested, as were titrations of C8166 DNA in water. The primers were used at 1.0 to 4.0 μM each with $MgCl_2$ concentrations of 1.5, 2.5 and 3.5 mM in standard PCR reactions with annealing temperatures of 55, 60 and 65°C. A touchdown PCR protocol with the same compositions of PCR reaction mixes was also applied. A band of approximately 130 bp consistent with the expected product size of 133 bp was amplified from pMT2 using 1.0 μM of each primer and 3.5 mM $MgCl_2$. A band of moderately high intensity that migrated at approximately 180 bp was frequently amplified from both C8166 and human placental DNA using 3.5 or 2.5 and sometimes 1.5 mM $MgCl_2$. However, at 2.5 and 1.5 mM $MgCl_2$ there were many additional bands in all samples. Only PCR products from pMT2 hybridised to $pMT2_i$. The 180 bp PCR product from C8166 was gel-purified, TA cloned and sequenced. Excluding the primers, the sequence had 98% identity over 137 bases to a sequence on human chromosome 4 using the BLAST programme but had no significant matches with retroviral sequences. The specific PCR product amplified from pMT2 was also sequenced and shown to contain the expected 133 bp HTLV-I retroviral *pol* sequence. Bands of approximately 160 and 130 bp were amplified from both BLV-FLK and FLK DNA using 3.5, 2.5 and 1.5 mM $MgCl_2$.

3.5 Discussion

HTLV-I, HTLV-II and related sequences have previously been detected in HTLV-I/II seronegative or indeterminately seroreactive patients with MF and SS by PCR, SB hybridisation and sequencing (Kaplanski *et al* 1986, Hall *et al* 1991, Zucker-Franklin *et al* 1991, D'Incan *et al* 1992, Kiss *et al* 1992, Srivastava *et al* 1992, Chan *et al* 1993, Kiss *et al* 1993, Whittaker and Luzzato 1993, Bazarbachi *et al* 1994, Manca *et al* 1994) (Section 1.11.1.5). In a number of cases, molecular analysis has indicated clonal integration of incomplete or variant HTLV-I provirus in neoplastic cells, with preferential retention of HTLV-I/II *tax* sequences (Kaplanski *et al* 1986, Hall *et al* 1991, Zucker-Franklin *et al* 1992, Pancake and Zucker-Franklin 1993, Manca *et al* 1994). Other patients with CTCL have no evidence of HTLV-I infection (Capésius *et al* 1991, Lisby *et al* 1992, Bazarbachi *et al* 1994).

The amplification of HTLV-I/II *tax* sequences from two patients with CTCL (one SS, one MF) in this study would have been of significance if these results were verified as genuine evidence of infection with HTLV-I. To eliminate the possibility of contamination with PCR products, probes or positive control DNA, attempts were made to clone and sequence PCR products from the hybridisation-positive samples. The PCR products amplified from two different samples from a patient with SS were identical and matched the sequence from the positive control DNA used in the laboratory, but differed at four bases from the sequence of prototypical HTLV-I. It was concluded that these PCR products were the result of contamination from positive control (C8166) DNA. The first round PCR products from these samples had been handled in the molecular biology laboratory before the nested PCR was performed and therefore contamination could have resulted during either the first or second round PCRs.

The first round PCR product amplified from a patient with MF using a different set of primers also had an identical sequence to that amplified from positive control DNA, as well as prototypical HTLV-I. Therefore, it cannot be determined whether this PCR product was the result of contamination. It would be necessary to amplify HTLV-I sequences from these same clinical samples using other sets of *tax* primers and identify unique sequence signatures to confirm that the patients were infected with HTLV-I or a related virus. An alternative approach would be to have another sample of the same type collected from the same patient at the same time tested by PCR for HTLV-I *tax* sequences in a different laboratory. Sequence errors introduced by *Taq* polymerase could also be resolved by replicating the amplification, cloning and sequencing of PCR products from samples. However, in the cases in which false positive results were suspected in this study there was insufficient DNA available to retest all samples.

HTLV-I/II *tax* PCR products were also detected in one patient with CBCL, but it was not possible to re-amplify, clone and sequence these products. Therefore, this result cannot be verified. The detection of HTLV-I/II sequences in patients with cutaneous BCL has not previously been reported, but HTLV-I has been associated with B cell CLL (Mann *et al* 1987).

HTLV-II sequences have also been detected by PCR in patients with CTCL (Zucker-Franklin *et al* 1992) and LGL leukaemia (Loughran *et al* 1992, Martin *et al* 1993, Heneine *et al* 1994, Loughran *et al* 1994). In this study, no HTLV-I, HTLV-II or related sequences were detected in six patients with LGL leukaemia. Similarly, no HTLV-I, HTLV-II or BLV sequences were detected in six butchers from Cardiff with ALL.

Differences in sensitivities of primer sets used for amplification of HTLV-I and HTLV-II sequences were noted in this study, despite careful optimisation (Section 3.4.1) (Table 3.3). This means that it might be possible to detect sequences from one portion of a provirus by PCR using a highly sensitive set of primers, whereas other proviral regions are negative by PCR because the specific sets of primers for those regions are less sensitive. In addition, highly sensitive primer sets are more likely to yield false positive results if there is contamination from PCR products, plasmids or positive control DNA. The implications of these results are discussed further in Chapter 5.

The use of two sets of consensus oligonucleotide primers based on alignment of HTLV/BLV group virus LTR and *gag* sequences showed limited potential for a group-specific PCR assay. Specific PCR products of the appropriate sizes could be amplified from purified plasmid (pMT2) containing the full-length HTLV-I sequence, but not from the HTLV-I-infected cell line C8166. Similar non-specific bands were frequently amplified from C8166 and placental DNA.

While consensus primers can be designed for amplification of *pol* sequences of most known retroviruses, they lack specificity when used on complex (genomic) DNA, resulting in amplification of many endogenous sequences. Preferential amplification of endogenous viruses, retrotransposons or non-specific cellular sequences reduces the sensitivity of PCR for detection of accompanying proviral DNA using consensus primers. This can be overcome by reverse transcribing purified viral RNA to cDNA before amplification by PCR but for this approach it is necessary to isolate the uncharacterised virus to ensure adequate quantities of template (Donehawer *et al* 1990).

In this study most samples from patients with CTCL, LGL leukaemia and ALL were negative for HTLV-I, HTLV-II and BLV sequences where tested by PCR in combination with Southern blot hybridisation. Positive results could be accounted for by contamination. Therefore, no unequivocal evidence for infection with HTLV/BLV group retroviruses was demonstrated. Molecular approaches to the detection of HTLV-I-related retroviruses in patients with CTCL are discussed further in Chapter 5.

Chapter 4

The Role of Human T Lymphotropic Virus Type I *pX* Gene Products in Apoptosis

4.1 Introduction

4.1.1 Role of apoptosis in HTLV-I leukaemogenesis

4.1.1.1 Oncogenic properties of HTLV-I pX proteins

The Tax protein of human T lymphotropic virus type I (HTLV-I) is the main regulator of viral transcription and has transforming properties in a number of experimental systems (Section 1.8.3). Established rodent fibroblast cell lines can be transformed by Tax (Tanaka *et al* 1990, Smith and Greene 1991). Primary rat embryo fibroblasts (REFs) can be immortalised by Tax alone or transformed by Tax in cooperation with oncogenes such as Ras (Pozzatti *et al* 1990, Matsumoto *et al* 1994). Oncogenic features of the HTLV-I $p12^{I}$ protein have also been demonstrated (Section 1.8.4). $p12^{I}$ enhances transformation of C127 fibroblasts by the bovine papillomavirus type 1 (BPV-1) E5 protein (Schlegel *et al* 1986, Franchini *et al* 1993). The contribution of other HTLV-I *pX* gene products to leukaemogenesis is unknown. Rex acts as a post-transcriptional regulator of HTLV-I gene expression (Inoue *et al* 1987, Hidaka *et al* 1988, Nosaka *et al* 1989) (Section 1.3.5.2). The presence of a nuclear export signal (NES) in $p21^{III}$ and a nucleolar localisation signal (NLS) in $p30^{II}$ (Tof) suggest that these proteins could have a regulatory function. However, there is insufficient information available to attribute roles to $p21^{III}$, Tof, $p13^{II}$ and $p12^{I}$.

4.1.1.2 Induction of apoptosis by Tax

Rodent fibroblasts expressing Tax, including transformed Rat1 fibroblasts and non-transformed REFs and 3Y1 rat fibroblasts, undergo apoptosis when deprived of serum and this effect is inhibited by Bcl-2 (Sakurai *et al* 1992b, Yamada *et al* 1994, Fujita and Shiku 1995) (Section 1.8.7.4). Tax appears to promote cell cycle progression in the absence of external growth factors (Yamada *et al* 1994) and thus the response of cells expressing Tax to withdrawal of growth factors is similar to that of cells expressing c-Myc (Evan *et al* 1992) (Sections 1.8.6 and 1.8.7). This has led to the hypothesis that Tax activates pathways leading either to proliferation or cell death, depending on the availability of survival factors. Tax might exert this effect directly or indirectly through up-regulation of c-*myc* or *fos* (Fujita and Shiku 1993, Smeyne *et al* 1993). Since several oncogenic viruses produce proteins that induce proliferation and inhibit apoptosis (Section 4.1.3), it is possible that HTLV-I *pX* region gene products with currently unknown function could oppose the pro-apoptotic properties of Tax and thus cooperate with Tax in oncogenesis.

4.1.2 c-Myc-model of proliferation and apoptosis

4.1.2.1 Differential responses of rodent fibroblasts to c-Myc expression

The oncogene c-Myc appears to prime cells for either proliferation or apoptosis, depending on whether they are permissive for survival (Evan *et al* 1992) (Section 1.8.6). Rat1 cells constitutively expressing c-Myc undergo transformation and have a high rate of proliferation when grown in medium containing normal concentrations of serum (Small *et al* 1987). Eilers *et al* (1989) transfected Rat1 cells with a gene encoding a chimaeric c-Myc-murine oestrogen receptor (c-Myc-ER) protein, allowing translocation of functional c-Myc to the nucleus when the cells were exposed to oestrogen. Transient expression of c-Myc in the presence of oestrogen resulted in reversible proliferation (conditional transformation) in the absence of other mitogenic stimuli (Eilers *et al* 1989). Evan *et al* (1992) demonstrated that these Rat1 cells undergo apoptosis when c-Myc expression induced by oestrogen occurs under conditions of serum deprivation. Cells with deregulated expression of c-Myc are unable to withdraw from the cell cycle and arrest growth, but when grown in medium with a low concentration of serum the rate of population increase is slowed due to an increase in the rate of apoptosis. Induction of apoptosis by c-Myc has also been demonstrated in REFs, rat vascular smooth muscle cells and Swiss 3T3 cells (Harrington *et al* 1994).

4.1.2.2 Chimaeric c-Myc-modified oestrogen receptor system

The system of c-Myc induction by oestrogen in Rat1-c-*myc*-ER cells (Section 4.1.2.1) has been improved by substitution of the oestrogen receptor (ER) with a modified receptor (ER^{TM}) that responds to 4-hydroxytamoxifen (4-HT) (Littlewood *et al* 1995). This removes the need for charcoal-dextran stripping of natural steroid hormones from foetal bovine serum (FBS), previously necessary to prevent constitutive activation of the ER. Cells transfected with chimaeric ER proteins could not be grown in phenol red, whereas this indicator can be included in the medium used to grow cells with the ER^{TM}. Therefore, Rat1 cells stably transfected with a chimaeric c-*myc*-ER^{TM} gene (Rat1-c-*myc*-ER^{TM} cells) were used in this study (Littlewood *et al* 1995).

4.1.3 Inhibition of apoptosis by oncogenic DNA viruses

Several oncogenic viruses express proteins that inhibit apoptosis. These may cooperate with growth-promoting oncoproteins produced by the same viruses. This strategy allows cell proliferation to be switched on without activating pathways that lead to apoptosis. The E1B proteins of adenovirus type 5 block apoptosis induced by the viral oncoprotein E1A; $p55^{E1B}$ binds to and inactivates p53 by promoting degradation through the ubiquitin system, whereas $p19^{E1B}$ inhibits apoptosis by a mechanism similar to that of Bcl-2 (Scheffner *et al* 1990, Werness *et al* 1990, Rao *et al* 1992, White *et al* 1992, Lowe and Ruley 1993, Lowe *et al* 1994). The simian virus 40 (SV40) large tumour (large T) antigen and the E6 proteins of highly oncogenic human papillomaviruses (HPVs) also bind to and inactivate or promote the degradation of p53 (Lane and Crawford 1979, Sarnow *et al* 1982, Mietz *et al* 1992).

The Epstein-Barr virus (EBV) gene product BHRF1 is a Bcl-2 homologue that inhibits apoptosis and may cooperate with other EBV transforming genes (Henderson *et al* 1993, Marshall *et al* 1999). Other oncogenic herpesviruses, including human herpesvirus type 8 (HHV-8, Kaposi's sarcoma-associated virus) and *Herpesvirus saimiri*, also encode Bcl-2 homologues (Cheng *et al* 1997, Nava *et al* 1997). SV40 large T and EBV BHRF1 inhibit c-Myc-induced apoptosis (Hermeking *et al* 1994, Fanidi *et al* 1998). Expression of anti-apoptotic proteins thus appears to be a common strategy in infection by oncogenic DNA viruses. HTLV-I could use a similar mechanism to maintain the survival of infected T cells *in vivo*.

4.1.4 Investigation of the role of HTLV-I pX genes in proliferation and apoptosis

In this study the hypothesis was tested that the HTLV-I *pX* gene products Tax, Rex, $p21^{III}$, Tof, $p13^{II}$ and $p12^{I}$ contribute to leukaemogenesis by modulating apoptosis. One model used to test this hypothesis was the ability of *pX* gene products, in particular Tax, to induce apoptosis in Rat1 cells under conditions of serum deprivation, similar to c-Myc (Evan *et al* 1992). The other model was the ability of Tax or other *pX* gene products to prevent apoptosis induced by c-Myc in Rat1 cells under conditions of serum deprivation. At the start of the study it was not known that Tax induces apoptosis in rat fibroblasts deprived of serum. However, as this property became apparent, it was realised that Tax provided a parallel model in which to test for anti-apoptotic properties of other *pX* proteins. The *pX* genes *tax*, *rex*, $p21^{III}$, $p30^{II}$, $p13^{II}$ and $p12^{I}$ were cloned into the eucaryotic expression vector pcDNAI/Neo and introduced into Rat1-c-*myc*-ER^{TM} cells (Section 4.1.2.2). When these cells are exposed to 4-HT, the chimaeric protein translocates to the nucleus, allowing expression of c-Myc transcriptional activity. c-Myc activates the cell cycle, inducing proliferation, but at the same time activates the apoptosis pathway, leading to programmed death when cells are deprived of survival factors present in serum (Evan *et al* 1992). HTLV-I *pX* region gene products were tested for their ability to maintain the survival of cells deprived of serum that would otherwise die by apoptosis induced by c-Myc or Tax.

4.2 Methods

4.2.1 Cloning HTLV-I pX genes

4.2.1.1 Source of HTLV-I *tax* and *rex* plasmids

Plasmids containing $p40^{IV}$ (*tax*) and $p27^{III}$ (*rex*) were kindly provided by Dr Moyra Campbell, Leukaemia Research Fund (LRF) Virus Centre. Competent bacteria (*E coli* DH5α) were transformed with these plasmids and grown on LB agar containing 50 μg/ml ampicillin (Section 3.3.13.2). Large scale preparations of plasmids were purified by CsCl gradient centrifugation (Sections 3.3.14.2 and 3.3.14.3). The 6.6 kb plasmid LRF-MC-p27 contained a 1.6 kb *tax* insert cloned into pUC18 (2.7 kb) at *Bam*HI and *Xba*I sites (Appendix 1). An *Sph*I site within the insert had been deleted to remove the *rex* initiation codon. The *tax* insert was flanked by the 255 bp CD3ε promoter and 2.0 kb CD3ε enhancer (Hall *et al* 1998).

The 4.8 kb plasmid LRF-MC-p33 contained a 2.1 kb *rex* insert cloned into pIC20R (2.7 kb) at *Bam*HI and *Hin*dIII restriction sites (Appendix 1). The *rex* insert had been subcloned from *Hin*dIII sites in pKCR27x (Siomi *et al* 1988, Nosaka *et al* 1989). The insert expressed Rex but mutations prevented expression of $p21^{III}$ and allowed expression of an inactive, truncated protein comprising the N terminal 60 amino acids of Tax.

4.2.1.2 Amplification and cloning of $p21^{III}$

The HTLV-I $p21^{III}$ gene was amplified by PCR from pMT2, a full-length HTLV-I molecular clone in the vector pUC (Seiki *et al* 1982) (Section 3.3.11.2). Primers were designed with *Bam*HI and *Xba*I restriction sites (Figure 4.1) (Appendix 1) and the PCR was performed under standard conditions with an annealing temperature of 55°C (Section 3.3.7.1). The reaction mix contained dTTP, 1.0 μM each primer and 1.5 mM $MgCl_2$. The PCR product (356 bp) was ligated into the TA cloning vector pCRII (Invitrogen) (Section 3.3.16.2), grown in *E coli* DH5α and the plasmid construct was purified by CsCl gradient centrifugation.

4.2.1.3 Source of HTLV-I $p30^{II}$, $p13^{II}$ and $p12^{I}$ plasmid constructs

Plasmids containing the HTLV-I $p30^{II}$, $p13^{II}$ and $p12^{I}$ genes were kindly provided by Dr Genoveffa Franchini, Laboratory of Tumor Cell Biology, National Institutes of Health, Bethesda, Maryland, USA. These consisted of tagged *pX* inserts cloned into the 3.2 kb vector HCMV-HSpA: $p30^{II}$-HA1, $p13^{II}$-HA1 and $p12^{I}$-AU1. HCMV-HSpA contains the pUC19 polylinker cloned into *Hin*dIII/*Sma*I sites of HCMV-SEAP (Schwartz *et al* 1990) (Appendix 1). Competent *E coli* DH5α were transformed with the $p30^{II}$-HA1, $p13^{II}$-HA1 and $p12^{I}$-AU1-HCMV-HSpA constructs. Transformants were grown on LB agar containing 50 μg/ml ampicillin and the plasmids were purified by CsCl gradient centrifugation then quantified by spectrophotometry.

4.2.1.4 Subcloning HTLV-I *pX* genes into pcDNAI/Neo

The 7.0 kb (6,969 bp) vector pcDNAI/Neo (Invitrogen) was used for expression of HTLV-I *pX* gene products in eucaryotic cells. Genes cloned into this vector are constitutively expressed from the human cytomegalovirus (HCMV) promoter. pcDNAI/Neo has a neomycin resistance gene, permitting selection in eucaryotic cells with Geneticin (G418, Life Technologies).

The HTLV-I *pX* inserts *tax*, *rex*, $p21^{III}$, $p30^{II}$, $p13^{II}$ and $p12^{I}$ were subcloned from their source vectors into pcDNAI/Neo using RE sites that permitted directional cloning (Table 4.1). The *tax*, $p21^{III}$, $p30^{II}$, $p13^{II}$ and $p12^{I}$ inserts were excised from their respective vectors using *Bam*HI and *Xba*I and the *rex* insert was excised from LRF-MC-p27 by digestion with *Bam*HI and *Eco*RV (Section 3.3.15). Inserts were separated from the vector backbone by agarose gel electrophoresis and gel purified (Gelase, Cambio) (Section 3.3.10.3). *Bam*HI/*Xba*I and *Bam*HI/*Eco*RV restriction endonuclease (RE) digests of pcDNAI/Neo were prepared and gel-purified at the same time. Inserts were ligated into pcDNAI/Neo using T4 ligase (Section 3.3.14).

Figure 4.1: Specific oligonucleotide primers for amplification of the HTLV-I p21^{III} sequence by the polymerase chain reaction.

LRF oligonucleotide number		Primer sequence 5' to 3' with HTLV-I nucleotide position	Length (b)	T_m (°C)	Size of PCR product (bp)
HTLV-I p21^{III}					
		*Bam*HI			
190	Sense	7499 GCCGGGATCCATGGACGCGTTATCGGTCCA 7518	30	38	356
191	Antisense	7834 GCCGTCTAGATCACGTGGGGCAGGAGGGGC 7815	30	38	
		*Xba*I			

4.2.1.5 Transformation of MC1061/P3 with pcDNAI/Neo constructs

Competent bacteria of *E coli* strain MC1061/P3 (Ultracomp *E coli*, Invitrogen) were transformed with pcDNAI/Neo alone or with pcDNAI/Neo containing ligated inserts according to a modification of the protocol outlined in Section 3.3.13.2. Bacteria that had been stored at -80°C were thawed on ice and 100 µl transferred to a 5 ml polypropylene tube (2097, Falcon). A fresh solution of 2-mercaptoethanol (2-ME) was added to the bacteria to a final concentration of 25 mM. Ligation reaction products (5 µl) were mixed with the bacteria and held on ice for 30 min. The tubes were treated at 42°C in a water bath for 75 sec then placed on ice for 2 min. SOC medium (900 µl) (Appendix 5: A5.10.1) was added and the transformed bacteria were incubated at 37°C for 1 hour on an orbital incubator (225 rpm). After centrifuging at 400 *g* (1500 rpm) in a bench centrifuge (BR401, Denley), the supernatant was discarded and the bacteria were resuspended in 200 µl SOC medium. Equal volumes were spread on LB agar plates containing 25 µg/ml ampicillin or 10 µg/ml tetracycline (Appendix 5: A5.10). Growth on LB agar containing ampicillin, with no growth on medium containing tetracycline, was an indication of successful transformation without reversion of MC1061/P3 from type.

4.2.1.6 Selection and purification of pcDNAI/Neo *pX* constructs

Small scale preparations of plasmid DNA (Section 3.3.14.1) followed by RE digestion were used to select MC1061/P3 transformants that contained the desired pcDNAI/Neo constructs. A large scale preparation of each plasmid was made by alkaline lysis and CsCl density gradient centrifugation, then quantified by spectrophotometry (Sections 3.3.14.2 and 3.3.14.3). Inserts were sequenced in both directions at the University of Birmingham (Alta Bioscience) using T7 and SP6 sequencing primers (Appendix 5: A5.12.1). Each purified plasmid construct was diluted to 0.4 µg/µl in preparation for transfection of Rat1 cells.

4.2.2 Transfection of Rat1 cells

4.2.2.1 Culture of Rat1 and Rat1-c-*myc*-ER^{TM} cells

Rat1 and Rat1-c-*myc*-ER^{TM} cells were obtained from Dr Gerard Evan, Imperial Cancer Research Fund (ICRF), Lincoln's Inn Fields, London (Littlewood *et al* 1995). Rat1 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies) containing 10% heat-inactivated foetal bovine serum (hi-FBS), 400 mg/ml streptomycin, 400 U/ml penicillin and 2 mM L-glutamine (Appendix 4: A4.2.1.3). Rat1-c-*myc*-ER^{TM} cells were grown in the same medium containing 5 µg/ml puromycin to maintain selection for the transfected genotype. These cell lines formed adherent monolayers and were subcultured by trypsinisation with 1.5 to 3 ml 0.25% crude trypsin in 1 M EDTA (trypsin-EDTA) (Section 2.3.3.1). Cells cultured in sealed flasks were aerated with 5% CO_2 and cells grown in tissue culture dishes (Nunclon, Nunc) were grown in a 5% CO_2 incubator at 37°C.

Table 4.1 Plasmids used in studying the role of HTLV-I pX genes in apoptosis.

Plasmid	Vector	Size of vector (kb)	Insert	Size of insert (bp)	Insertion site	Tag
Tax-pcDNAI/Neo	pcDNAI/Neo	7.0	HTLV-I $p40^{IV}$	2600	*Bam*HI/*Xba*I	–
Rex-pcDNAI/Neo	pcDNAI/Neo	7.0	HTLV-I $p27^{III}$	2100	*Bam*HI/*Eco*RV	–
$p21^{III}$-pcDNAI/Neo	pcDNAI/Neo	7.0	HTLV-I $p21^{III}$	2100	*Bam*HI/*Xba*I	–
$p30^{II}$-TSP-pcDNAI/Neo	pcDNAI/Neo	3.2	HTLV-I $p30^{II}$	770	*Bam*HI/*Xba*I	TSP
$p13^{II}$-HA1-pcDNAI/Neo	pcDNAI/Neo	3.2	HTLV-I $p13^{II}$	360	*Bam*HI/*Xba*I	HA1
$p12^{I}$-AU1-pcDNAI/Neo	pcDNAI/Neo	3.2	HTLV-I $p12^{I}$	430	*Bam*HI/*Xba*I	AU1
pcDNAI/Neo	pcDNAI/Neo	7.0	–	–	–	–

AU1 Bovine papillomavirus type 1 AU1 epitope (DTYRYI) (~30 bp)
HA1 Influenza virus haemagglutinin epitope (YPYDVPDYASL) (~50 bp)
TSP Thrombospondin epitope (CSVTCG) (~30 bp)
– Not present

4.2.2.2 Transfection of Rat1 cells with plasmid constructs

Rat1-c-*myc*-ER^{TM} cells were transfected with 1 μg of each pcDNAI/Neo plasmid construct (Section 4.4.1). Rat1 cells without the c-*myc*-ER^{TM} gene were also transfected with selected plasmids. Some cultures were transfected with two active plasmids, for example p12^{I}-AU1-pcDNAI/Neo and Tax-pcDNAI/Neo, in which case 1 μg of each plasmid was co-transfected.

Cells were grown to 50% confluence in 10 cm diameter tissue culture dishes, then the medium was withdrawn and replaced with 10 ml serum-free DMEM. Plasmid DNA (1 μg) was diluted in 50 μl sterile ultrapure water in a 1.5 ml sterile polypropylene microcentrifuge tube. In a separate 1.5 ml tube 20 μl liposomal transfectant reagent (LipofectAMINE, Life Technologies) was mixed with 30 μl sterile ultrapure water. The DNA and liposomes were combined in a 15 ml screw-top polypropylene centrifuge tube and held at room temperature for 30 min to allow complexes to form. The 100 μl volume containing DNA-liposome complexes was then poured gently into the tissue culture dish containing cells in serum-free medium, mixed by gentle swirling and incubated at 37°C for 5 hours in a CO_2 incubator.

The serum-free medium was replaced with DMEM + 20% hi-FBS and the cells were grown in this medium with daily replacement for two days. The cells were trypsinised, serial two-fold dilutions prepared in DMEM + 10% hi-FBS and re-seeded into a series of six tissue culture dishes at 1 x 10^1 to 1 x 10^3 cells/ml. After one day in culture, the medium was replaced with medium containing 5 μg/ml puromycin and 1 mg/ml Geneticin to select for transfectants while maintaining the c-*myc*-ER^{TM} phenotype. This medium was replaced after 3 days.

4.2.2.3 Selection of transfected Rat1 clones

Clones were selected 7 days after cells had been reseeded at different densities on tissue culture dishes. Colonies derived from a single cell were identified, the medium was removed and plates were rinsed briefly with 1.5 ml trypsin-EDTA. Marked colonies were isolated using metal or plastic cloning rings affixed to the bottom of the tissue culture dishes with sterile Vaseline.

Trypsin-EDTA (200 μl) was pipetted into the well formed by each ring and the tissue culture dishes were incubated at 37°C for 10 min in 5% CO_2 to dissociate the cells. The solution in each well was pipetted up and down to dislodge remaining adherent cells and the trypsinised cells were transferred to a flask containing 5 ml DMEM + 10% hi-FBS with puromycin and Geneticin at standard concentrations. Each transfectant was subjected to a second round of clonal selection. These cell lines were maintained in medium containing puromycin and Geneticin at 37°C in 5% CO_2 and trypsinised for subculturing or to prepare cells for seeding of tissue culture dishes.

4.2.3 Detection of pX mRNA expression

4.2.3.1 Isolation of RNA from Rat1 cells

RNA was extracted using the acid guanidinium thiocyanate method with phenol-chloroform extraction (Chomczynski and Sacchi 1987). Rat1 cells and Rat1-c-*myc*-ER^{TM} cells transfected with HTLV-I *pX* plasmid constructs were grown to near-confluence in 75 cm^2 tissue culture flasks. The medium was removed and 4 ml RNAzol B (Life Technologies) was added to lyse the cells (Appendix 5: A5.13.1). The lysate was pipetted up and down several times to dislodge adherent cells, then transferred to a 15 ml polypropylene centrifuge tube. The mixture was shaken vigorously with 200 µl chloroform for 15 sec, placed on ice for 5 min and centrifuged at 1,500 *g* (2,700 rpm) in a GPR bench centrifuge (Beckman) with a GH 3.7 swinging bucket rotor for 15 min at 4°C.

The upper aqueous phase containing dissolved RNA was transferred to one or more 1.5 ml microcentrifuge tubes and the RNA was precipitated by mixing with an equal volume of isopropanol and holding at 4°C for 15 min. Tubes were centrifuged at 10,500 *g* (13,000 rpm) for 5 min in a bench microcentrifuge (MicroCentaur) at 4°C, then the RNA pellet was washed with 70% ethanol in ultrapure water containing 0.05% diethyl pyrocarbonate (DEPC) and re-centrifuged.

The tubes were desiccated under vacuum for 10 min then dissolved in ultrapure water containing 1 mM EDTA pH 7.0, 0.5% SDS and 0.05% DEPC. The concentration was determined by spectrophotometry with calculations made on the basis that 40 µg/ml of single-stranded RNA has an optical density at 260 nm (OD_{260}) of approximately 1.0.

4.2.3.2 Northern blot hybridisation

Northern blot hybridisation to detect expression of HTLV-I *pX* mRNA in transfected cells was performed by Dr Moyra Campbell in the LRF Virus Centre. A 10 µl volume containing 10 µg RNA was mixed with 20 µl formamide buffer (Appendix 5: A5.13.2), heated at 65°C for 15 min to denature the RNA, then placed on ice for 5 min. RNA loading buffer/dye (2 µl) was added and the samples were separated by gel electrophoresis (5 V/cm) on a 1% agarose gel in 3-(N-morpholino)-2-hydroxypropane sulphonic acid (MOPS) buffer 1x with 6% formaldehyde prepared using ultrapure water with 0.05% DEPC (Appendix 5: A5.13.2). λ*Hin*dIII and ϕX174/*Hae*III (Life Technologies) were used as size markers in combination with visualisation of 28S and 18S ribosomal RNA bands after staining with ethidium bromide.

The RNA was transferred to nylon membrane (Hybond-N, Amersham) by Northern blotting using SSC 20x as the transfer buffer, rinsed in SSC 3x, then cross-linked with ultraviolet (UV) light (Section 3.3.10) (Appendix 5: A5.7). Blots were hybridised to the HTLV-I probe pMT2$_i$ labelled with α ^{32}P dCTP by random priming (Sections 3.3.11.2 and 3.3.11.4). The following formula was used for calculation of the T_m for RNA:RNA hybridisations (Section 3.3.12.1):

Formula 4.1: $T_m = 67 + 16.6 \log_{10}[M^+] + 0.8(\%G+C) - 500/L - P - 0.5(\%Formamide)$ °C

Where: $[M^+]$ = Adjusted molar Monovalent cation concentration: $\frac{[Na^+]}{1 + 0.7\,[Na^+]}$

%G+C = Percentage of G and C nucleotides in the DNA probe

L = Length of the DNA:RNA duplex in bp

P = Percentage base mismatch

The calculated T_m for pMT2$_i$ labelled with α ^{32}P dCTP by random priming was 74°C. Hybridisation was performed overnight at 37°C in standard hybridisation solution containing SSC 3x with 50% formamide, as for Southern blot (SB) hybridisation (Section 3.3.12) (Appendix 5: A5.9). The high stringency washes were performed with SSC 0.5x, 0.1% SDS. Autoradiographic film was exposed for 3 days.

4.2.4 Induction of apoptosis

4.2.4.1 Serum starvation of Rat1 and Rat1-c-*myc*-ER^{TM} cells

Rat1 cells, Rat1-c-*myc*-ER^{TM} cells (selected with puromycin) and cells transfected with HTLV-I *pX* pcDNAI/Neo constructs (selected with puromycin and Geneticin) were trypsinised and resuspended in DMEM + 10% hi-FBS. Serial two-fold dilutions were prepared and 500 µl aliquots were pipetted into duplicate wells of an eight well chamber slide (ChamberSlide, Lab-Tek, Nunc). This resulted in a series of approximately 2 x 10^5, 1 x 10^5, 5 x 10^4 and 2.5 x 10^4 cells in each of two duplicate rows.

The chamber slides were incubated at 37°C in 5% CO_2 for 24 hours to allow cells to adhere to the substrate. The medium was aspirated from each well and replaced with 500 µl DMEM without serum. Cells were maintained in this medium for 48 hours to undergo cell cycle arrest.

4.2.4.2 Induction of c-Myc-ER^{TM} expression

Nuclear expression of the c-Myc-ER^{TM} protein was activated in Rat1-c-*myc*-ER^{TM} cells on day 2 of serum starvation by the addition of 4-HT to a final concentration of 0.1 µM. Cells in 500 µl medium in each well of one row of a chamber slide were induced by adding 5 µl of 10 µM 4-HT. Cells in the duplicate row remained uninduced as negative controls. Experiments were performed twice with duplicate cultures in each experiment.

4.2.4.3. Microscopic examination

Cells were examined microscopically using an inverted light microscope (Labovert, Leitz) 6, 24 and 48 hours following withdrawal of serum and 6 and 24 hours following induction of c-Myc-ER^{TM} expression with 4-HT.

Morphological changes occurring in cells undergoing apoptosis include loss of cell-cell contact, cell shrinkage, condensation of chromatin in the nucleus and disintegration of the nucleolus and nuclear membrane (Kerr *et al* 1972) (Section 1.8.7.1). As the dying cell disintegrates, the cell contents are fragmented to produce membrane-bound apoptotic bodies.

4.2.4.4 Staining with propidium iodide

Cells were stained with propidium iodide (PI) 24 or 48 hours following withdrawal of serum and 6 and 24 hours following induction with 4-HT. PI is a fluorescent vital dye that does not cross the plasma membrane of viable cells. In dying cells in the late stages of apoptosis, PI enters the cell and intercalates DNA. It also stains DNA within apoptotic bodies. The dye fluoresces in the orange range of the spectrum.

Medium was removed from the wells of chamber slides to be stained with PI and the cells were fixed with 200 µl acetic acid-alcohol fixative for 30 min at room temperature (Appendix 4: A4.6). The fixative was aspirated and replaced with 200 µl 4 M HCl for 30 min at room temperature to denature the DNA. This solution was replaced with 0.1 M sodium borate for 5 min to neutralise the cells. After aspiration, the chamber slides were air dried for 5 min. The cells were then incubated at 37°C for 30 min in 200 µl of a solution containing 5 µg/ml PI and 100 µg/ml RNase A in PBS 1x. This solution was removed and the walls of the chambers were removed from the glass slide along with the gasket.

Approximately 100 µl of mounting solution was pipetted onto each slide, overlaid with a glass coverslip and sealed at the edges with clear nail varnish. The mounting solution (Vectashield, Vector Laboratories) contained glycerol and PBS 1x at a ratio of 9:1 with 1,4-diazobicylo(2,2,2)-octane (DABCO) as an antiquenching agent (Johnson *et al* 1982). The slide was wrapped in aluminium foil to protect the cells from light and held at 4°C for 5 to 10 min for the mounting solution to set. The slides were examined under oil immersion using a fluorescent microscope (Laborlux K, Leitz) with a 562 to 588 nm band pass (green) filter.

4.3 Results

4.3.1 Detection of HTLV-I pX *expression in Rat1 cells*

The HTLV-I *pX* genes *tax*, *rex*, $p21^{III}$, $p30^{II}$, $p13^{II}$ and $p12^{I}$ were cloned into pcDNAI/Neo and transfected into Rat1 and Rat1-c-*myc*-ER^{TM} cells. Each plasmid construct was transfected alone and the Tax-pcDNAI/Neo construct was also co-transfected with each of the other *pX*-pcDNAI/Neo plasmids. Cells transfected with pcDNAI/Neo without an insert were used as negative controls. Transfectants were selected by cultivation in medium containing Geneticin and puromycin. RNA extracted from transfected cell cultures was hybridised to the HTLV-I probe $pMT2_I$ labelled with α ^{32}P dCTP by random priming. Positive hybridisation to bands of the appropriate size was detected in all transfected cell lines (Table 4.1), whereas non-transfected Rat1 cells were negative. Cells co-transfected with *pX*-pcDNAI/Neo and one other *pX* plasmid had two bands corresponding to the respective inserts. The assay for *pX* mRNA expression was not quantitative but the strongest bands were detected with *tax*, *rex* and $p21^{III}$.

4.3.2 Induction of apoptosis by expression of c-Myc

Rat1 cells and Rat1-c-*myc*-ER^{TM} cells grew to confluence when cultivated in DMEM + 10% hi-FBS, forming adherent monolayers in tissue culture flasks and dishes. When serum was withdrawn, there was cessation of proliferation and spreading in non-confluent cultures. Confluent cultures, which had stopped proliferating due to contact inhibition in the presence of serum, exhibited no obvious morphological changes by light microscopy when serum was withdrawn (Figure 4.2). Addition of 4-HT to Rat1-c-*myc*-ER^{TM} cultures grown in the absence of serum resulted in cell death, with rounding up and detachment of fibroblasts, while cells that remained adherent had attenuated cytoplasmic extensions (Figure 4.3). Cell death was visible initially at 6 hours and more than 50% of cells had died and detached by 24 hours. These changes were also observed consistently in Rat1-c-*myc*-ER^{TM} cells transfected with the vector pcDNAI/Neo that did not contain an insert and acted as a negative control. Addition of 4-HT to confluent Rat1-c-*myc*-ER^{TM} cells in the presence of serum had no visible effect within the time frame of observations. Addition of 4-HT had no effect on Rat1 cells cultured with or without serum.

4.3.3 Induction of apoptosis by expression of Tax

Transfection of cells with Tax-pcDNAI/Neo would have resulted in constitutive expression of Tax from the HCMV promoter. Rat1 and Rat1-c-*myc*-ER^{TM} cells expressing Tax exhibited mild piling up (focus formation), suggestive of transformation, with occasional large multinucleate cells scattered through cultures (Figure 4.4). When serum was withdrawn from these cultures, cell death was visible initially at 24 hours and by 48 hours 50 to 90% of cells had died (Table 4.2) (Figure 4.5). It was not possible to perform experiments with induction of c-Myc expression by 4-HT in these cells according to the selected protocol. However, the addition of 4-HT at 24 or 48 hours following withdrawal of serum did not alter the fate of Rat1 and Rat1-c-*myc*-ER^{TM} cells transfected with Tax-pcDNAI/Neo (Table 4.2) (Figure 4.6).

4.3.4 Effects of Rex, p21^{III}, p30^{II}, p13^{II} and p12^{I} on c-Myc and Tax-induced apoptosis

Constitutive expression of Rex, p21^{III}, Tof, p13^{II} and p12^{I} from the pcDNAI/Neo HCMV promoter had no effect on Rat1 or Rat1-c-*myc*-ER^{TM} cells transfected with single plasmids containing any one of these genes (Table 4.2). In the presence of serum Rat1-c-*myc*-ER^{TM} cells transfected with these genes continued to proliferate and spread until confluent, whereas withdrawal of serum resulted in cessation of growth, similar to non-transfected cells. When Rat1-c-*myc*-ER^{TM} cells expressing these genes were exposed to 4-HT under conditions of serum deprivation, the cells died in a temporal pattern that was similar to non-transfected cells (Figure 4.7). Thus, there was no inhibition of c-Myc-induced apoptosis by any HTLV-I *pX* genes.

Rat1-c-*myc*-ER^{TM} cells were co-transfected with Tax-pcDNAI/Neo and pcDNAI/Neo expressing Rex, p21^{III}, p30^{II} (Tof) p13^{II} or p12^{I}. No morphological changes were evident in these cultures that were different from cells transfected with Tax-pcDNAI/Neo alone. When serum was withdrawn, the cells died with a time course and morphological pattern similar to that of Rat1 and Rat1-c-*myc*-ER^{TM} cells expressing Tax alone (Figure 4.8). Thus, Rex, p21^{III}, Tof, p13^{II} and p12^{I} did not oppose the apoptotic effects of Tax (Table 4.2).

4.3.5 Staining with propidium iodide

Staining of nuclei with propidium iodide (PI) in detaching and degenerating cells was visible by fluorescence microscopy in only small numbers of Rat1-c-*myc*-ER^{TM} cells from confluent monolayers grown in the presence of serum or 24 and 48 hours following withdrawal of serum (Figure 4.9). There was increased intranuclear staining in serum-deprived Rat1-c-*myc*-ER^{TM} cells stained 6 hours after treatment with 4-HT, as well as staining of DNA in fragments of degenerating cells (apoptotic bodies) (Figure 4.10). Serum-deprived Rat1-c-*myc*-ER^{TM} cells stained with PI 48 hours after treatment with 4-HT exhibited extensive degeneration and detachment. PI-stained DNA was visible in adherent debris and in many cells that remained attached. However, most degenerating cells were washed away during the staining procedure.

Rat1-c-*myc*-ER^{TM} cells transfected with Tax-pcDNAI/Neo had mild piling up and occasional multinucleate cells when grown in the presence of serum (Figure 4.11). Only a few degenerating cells with condensed nuclei or apoptotic bodies were visible. Rat1-c-*myc*-ER^{TM} cells transfected with Tax-pcDNAI/Neo exhibited extensive degeneration and detachment of cells when PI staining was performed 24 and 48 hours after withdrawal of serum. PI staining of DNA was visible in fragmented cells, as well as attenuated cells that remained adherent (Figure 4.12). Induced expression of c-Myc in serum-deprived cells resulted in extensive degeneration and detachment, with numerous apoptotic bodies visible in PI-stained slides. c-Myc-induced apoptosis was not inhibited by Rex, p21^{III}, p30^{II} (Tof) p13^{II} or p12^{I} (Figure 4.13). Similarly, apoptosis induced by Tax in serum-deprived Rat1-c-*myc*-ER^{TM} cells was not inhibited by Rex, p21^{III}, p30^{II} (Tof) p13^{II} or p12^{I} (Figure 4.14).

Table 4.2 Effect of induced expression of c-Myc following withdrawal of serum on survival of Rat1-c-*myc*-ER^{TM} cells constitutively expressing HTLV-I *pX* region genes.

HTLV-I *pX* gene expression	Normal serum	Low serum	
	c-Myc–	c-Myc–	c-Myc+
pcDNAI/Neo	+	+	–
p12^{I}	+	+	–
p13^{II}	+	+	–
p30^{II}	+	+	–
p21^{III}	+	+	–
Rex	+	+	–
Tax	+	–	–*
Tax + p12^{I}	+	–	ND
Tax + p13^{II}	+	–	ND
Tax + p30^{II}	+	–	ND
Tax + p21^{III}	+	–	ND
Tax + Rex	+	–	ND

c-Myc–	No c-Myc expression
c-Myc+	c-Myc expression induced by 4-HT
+	Survival
–	Death by apoptosis
*	Many cells already dead at time of addition of 4-HT
ND	Not done

Figure 4.2: Rat1-c-*myc*-ER^{TM} cells following withdrawal of serum.

Rat1-c-*myc*-ER^{TM} cells grown in the presence of serum had multiplied and spread across the bottom of the tissue culture flask to form a confluent monolayer that had ceased proliferating due to contact inhibition. These cells remained stable following withdrawal of serum. A few rounded, detached cells are visible (arrow). Original 200x magnification.

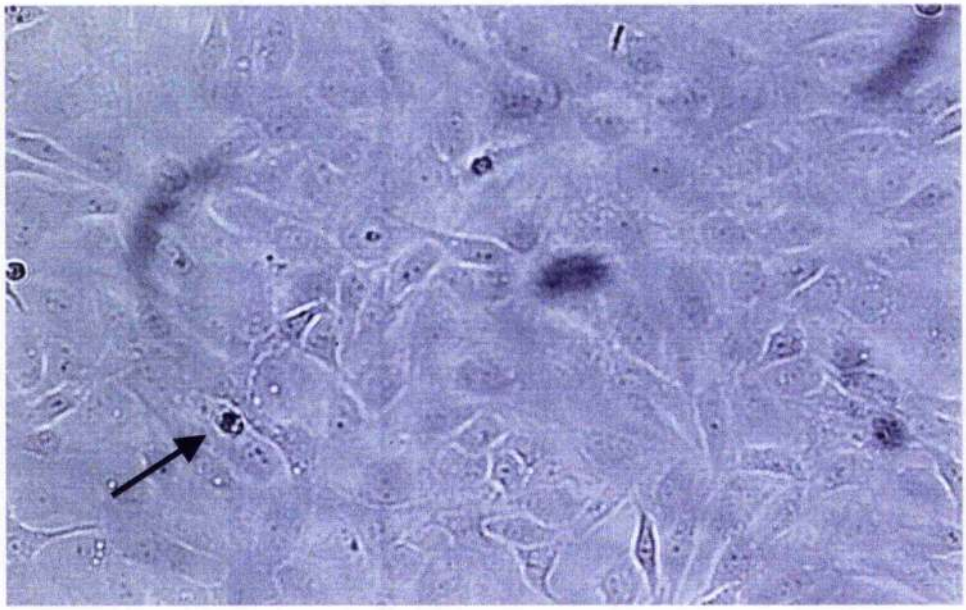


Figure 4.3: Apoptosis in serum-starved Rat1-c-*myc*-ER^{TM} cells following induction of c-Myc expression.

c-Myc expression was induced in Rat1-c-*myc*-ER^{TM} cells by addition of 4-hydroxytamoxifen 48 hours following withdrawal of serum. At 6 hours following c-Myc induction there is attenuation (arrows), rounding up (arrow heads) and detachment of fibroblasts dying by apoptosis. Original 200x magnification.

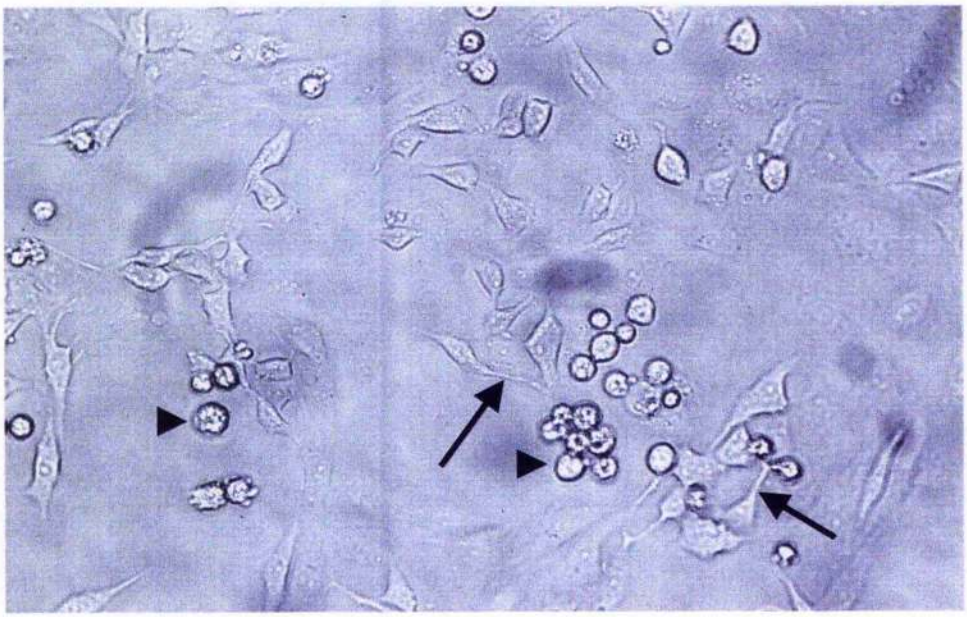


Figure 4.4: Rat1-c-*myc*-ER^{TM} cells expressing Tax grown in serum.

Rat1-c-*myc*-ER^{TM} cells transfected with Tax-pcDNAI/Neo were grown in the presence of serum. These cells proliferated to form a confluent monolayers with mild piling up (focus formation) (arrow) and occasional multinucleate cells (arrow head). A few rounded, detached cells are visible. Original 200x magnification.

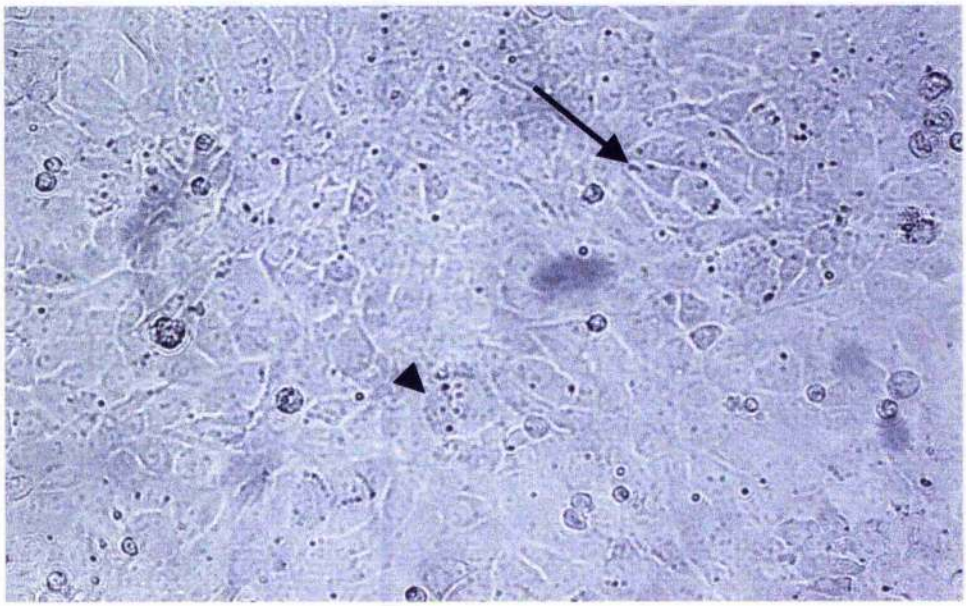


Figure 4.5: Effect of withdrawal of serum on Rat1-c-*myc*-ER^{TM} cells expressing Tax.

Rat1-c-*myc*-ER^{TM} cells transfected with Tax-pcDNAI/Neo formed confluent monolayers when grown in the presence of serum. At 24 hours following withdrawal of serum there is extensive cell death characterised by attenuation (arrows), rounding up (arrow heads) and detachment. Original 200x magnification.

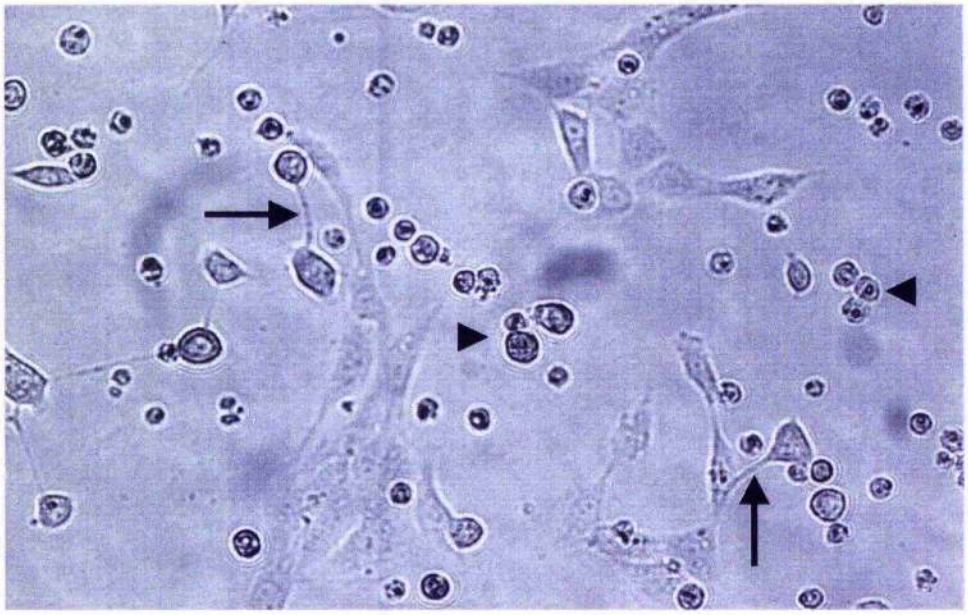


Figure 4.6: Effect of induction of c-Myc expression in serum-starved Rat1-c-*myc*-ER^{TM} cells expressing Tax.

Rat1-c-*myc*-ER^{TM} cells transfected with Tax-pcDNAI/Neo experienced apoptosis following withdrawal of serum (Figure 4.5). When c-Myc was induced in these cells by addition of 4-hydroxytamoxifen 24 hours following withdrawal of serum, there was no alteration in the pattern of cell death. Cells exhibit attenuation (arrow), rounding up (arrow heads) and detachment. Original 200x magnification.

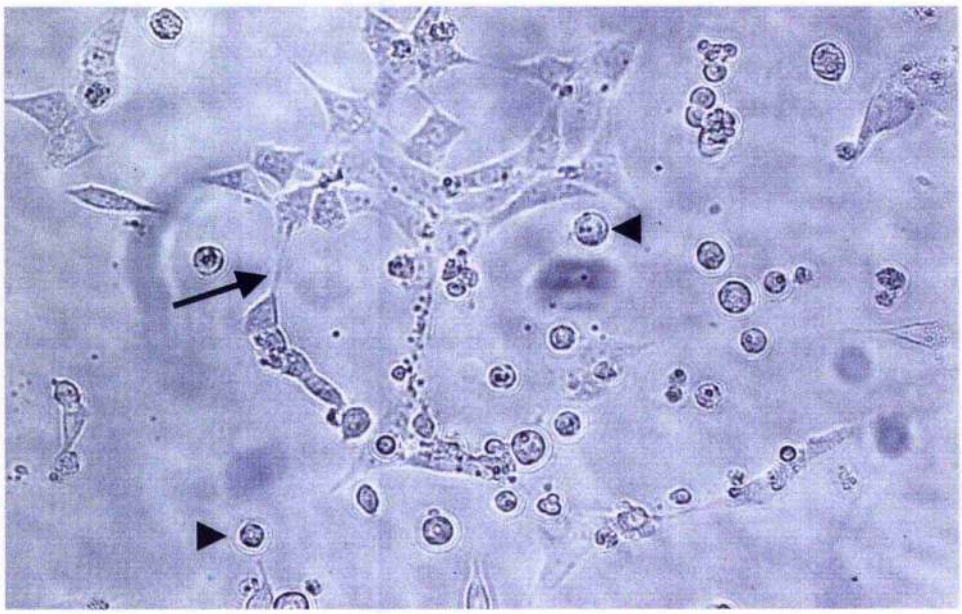


Figure 4.7: Effect of induction of c-Myc expression on serum-starved Rat1-c-*myc*-ER^{TM} cells expressing p12^{I}.

c-Myc expression was induced in Rat1-c-*myc*-ER^{TM} cells transfected with p12^{I}-pcDNAI/Neo by addition of 4-hydroxytamoxifen 48 hours following withdrawal of serum. At 24 hours after induction of c-Myc dying cells have rounded up and detached from the substrate. Original 200x magnification.

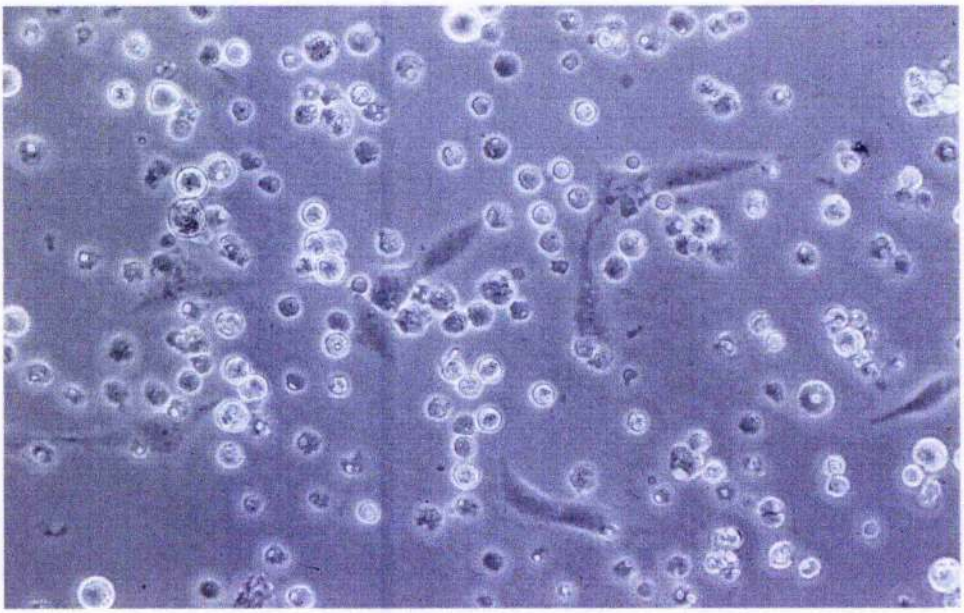


Figure 4.8: Effect of serum starvation on Rat1-c-*myc*-ER^{TM} cells expressing HTLV-I Tax and p12^{I}.

Rat1-c-*myc*-ER^{TM} cells co-transfected with Tax-pcDNAI/Neo and p12^{I}-pcDNAI/Neo were allowed to grow to near-confluence in the presence of serum. At 24 hours following withdrawal of serum, a substantial proportion of cells are dying, characterised by rounding up and detachment. Expression of p12^{I} has not inhibited cell death induced by Tax in serum-starved cells. Original 200x magnification.

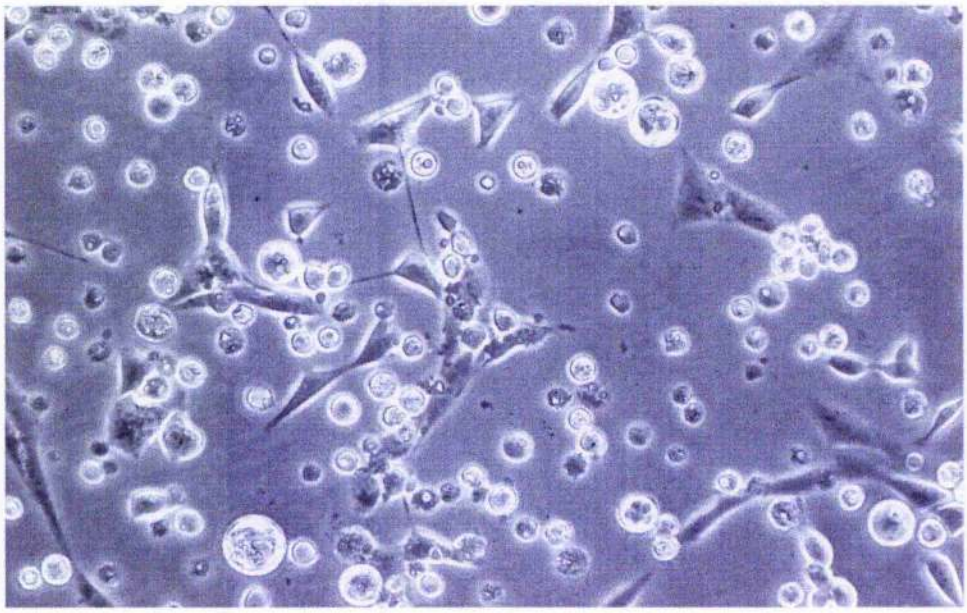


Figure 4.9: Propidium iodide staining of Rat1-c-*myc*-ER^{TM} cells following withdrawal of serum.

Near-confluent monolayer of Rat1-c-*myc*-ER^{TM} cells grown in the presence of serum shows survival of most cells 48 hours after withdrawal of serum. Cells detached from two sites in the field have been washed away during the staining procedure (arrows). Propidium iodide staining. Fluorescence microscopy. Original 400x magnification.

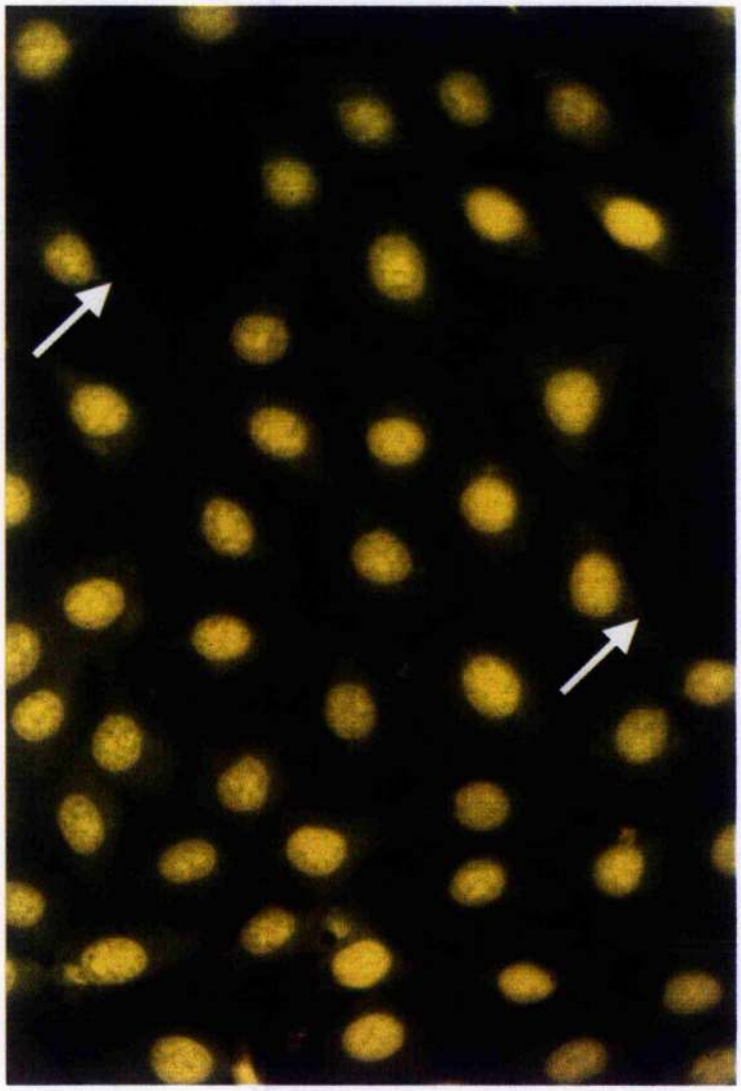


Figure 4.10: Propidium iodide staining of Rat1-c-*myc*-ER^{TM} cells following serum deprivation and induction of c-Myc expression.

c-Myc expression was induced in Rat1-c-*myc*-ER^{TM} cells by addition of 4-hydroxytamoxifen 48 hours following withdrawal of serum. At 6 hours following c-Myc induction there are fragmented nuclear bodies containing condensed DNA that have arisen from fibroblasts dying by apoptosis (arrows). Propidium iodide staining. Fluorescence microscopy. Original 400x magnification.

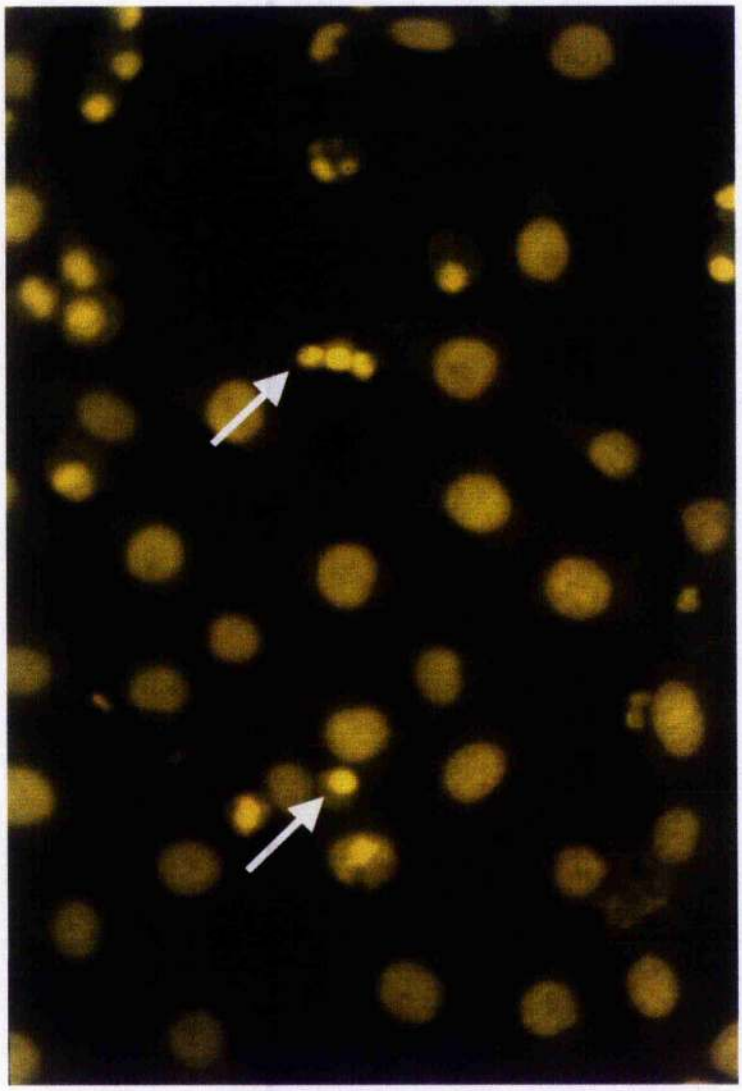


Figure 4.11: Propidium iodide staining of Rat1-c-*myc*-ER^{TM} cells expressing Tax grown in serum.

Rat1-c-*myc*-ER^{TM} cells transfected with Tax-pcDNAI/Neo and grown in the presence of serum form a near-confluent monolayer with mild piling up and some multinucleate cells. A single rounded, condensed nucleus from a dead cell can be seen (arrow). A mitotic figure is visible in a dividing cell (arrow head). Propidium iodide staining. Fluorescence microscopy. Original 400x magnification.

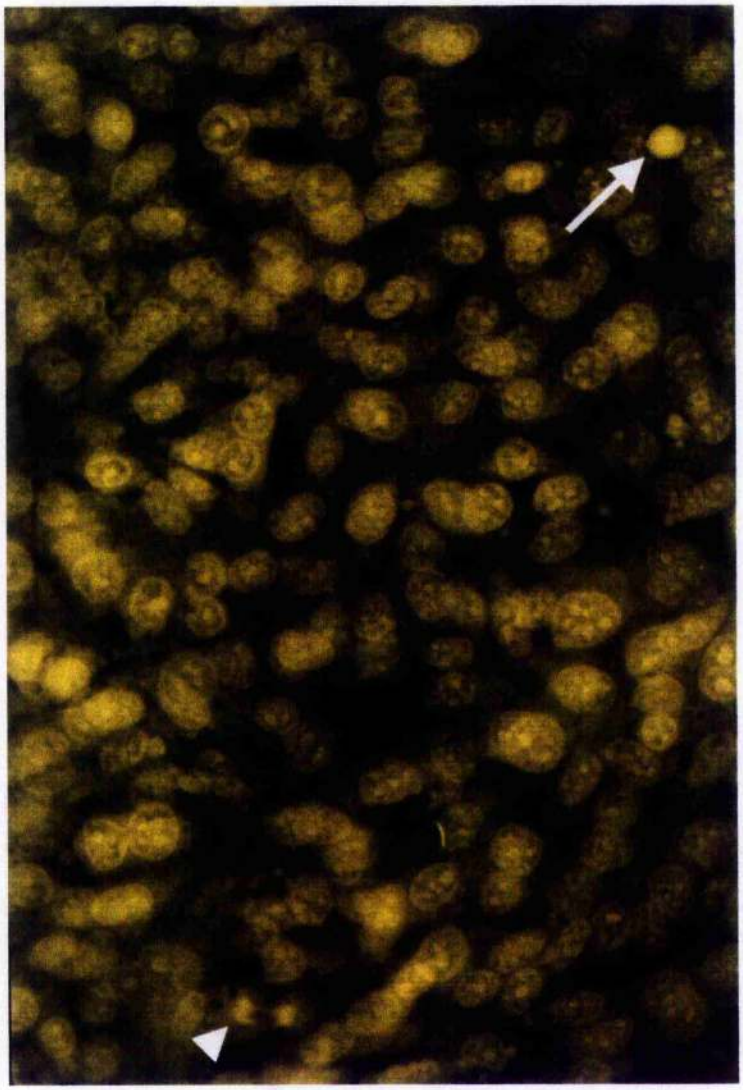


Figure 4.12: Propidium iodide staining of Rat1-c-*myc*-ER^{TM} cells expressing Tax following withdrawal of serum.

Rat1-c-*myc*-ER^{TM} cells transfected with Tax-pcDNAI/Neo photographed 24 hours following withdrawal of serum exhibit attenuation (arrows) and formation of apoptotic bodies in degenerating nuclei of dying cells (arrow heads). Detached cells have been washed away during the staining procedure. Propidium iodide staining. Fluorescence microscopy. Original 400x magnification.

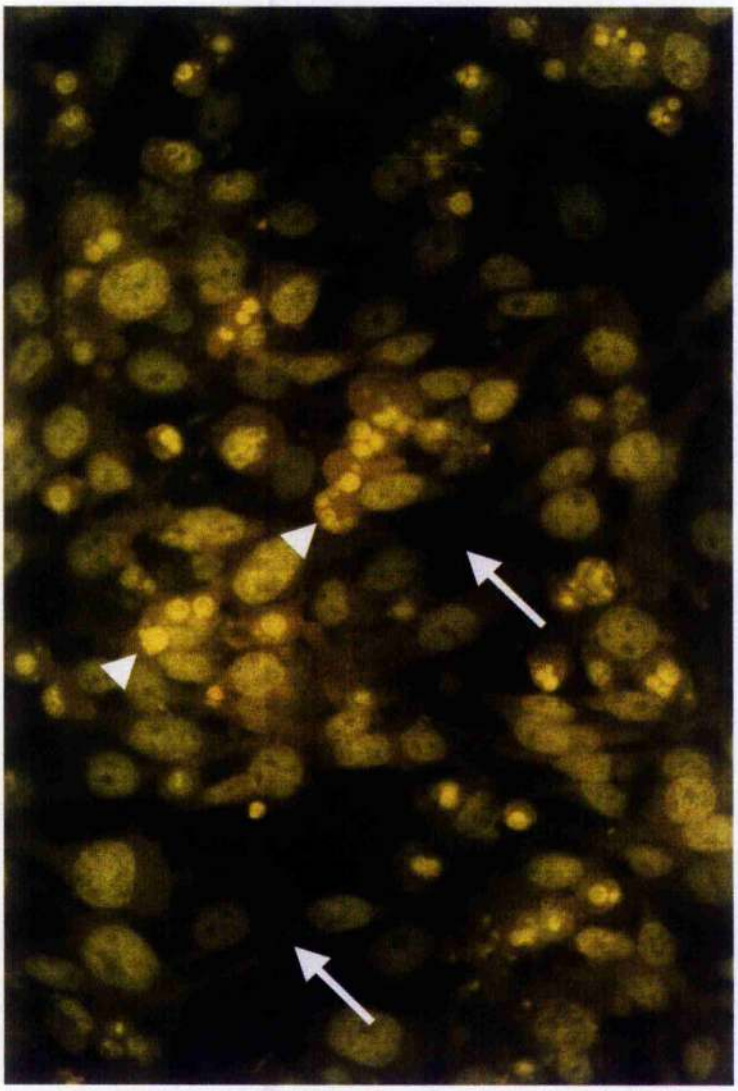


Figure 4.13: Propidium iodide staining of Rat1-c-*myc*-ER^{TM} cells expressing p12^{I} with induced expression of c-Myc expression following deprivation of serum

c-Myc expression was induced in Rat1-c-*myc*-ER^{TM} cells transfected with p12^{I}-pcDNAI/Neo by addition of 4-hydroxytamoxifen 48 hours following withdrawal of serum. At 24 hours after induction of c-Myc there are multiple foci of cell loss with numerous degenerating cells and apoptotic bodies (arrows). Propidium iodide staining. Fluorescence microscopy. Original 400x magnification.

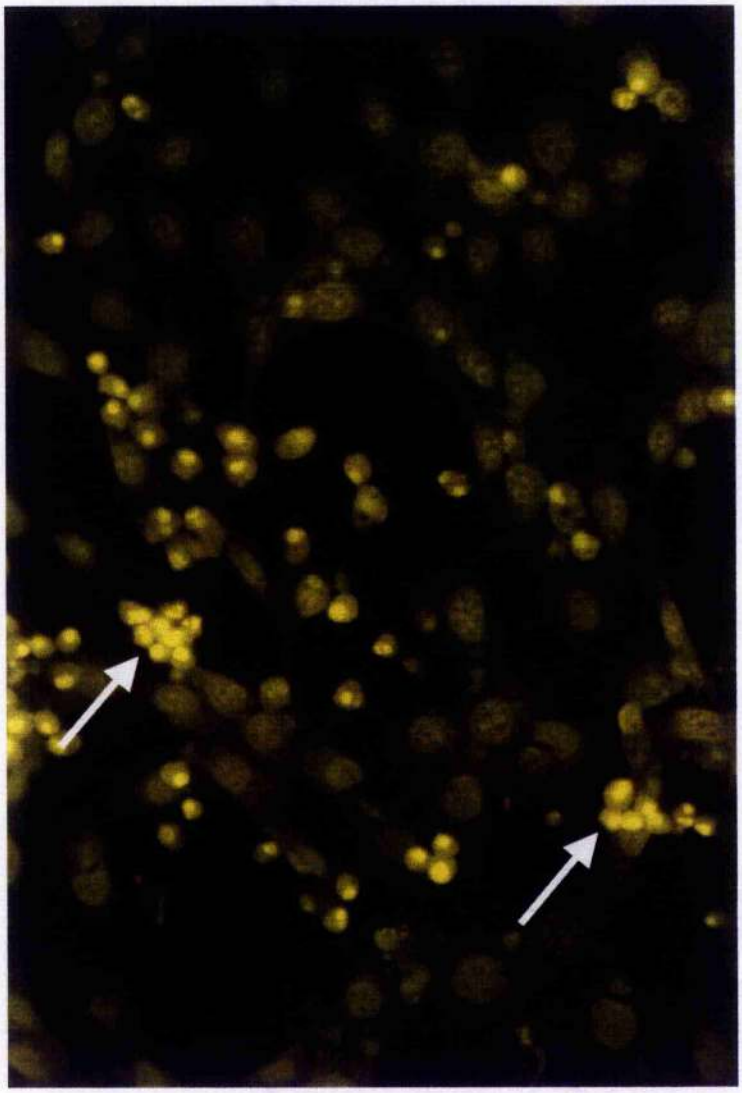
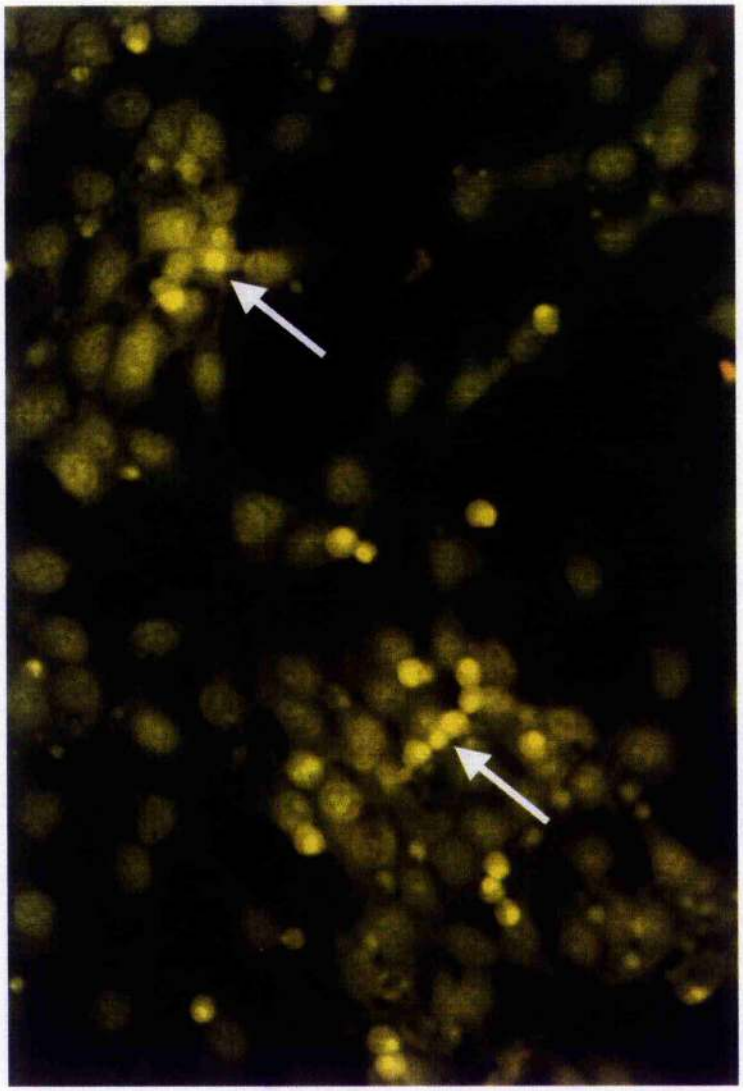


Figure 4.14: Propidium iodide staining of Rat1-c-*myc*-ER^{TM} cells expressing HTLV-I Tax and p12^{I} following serum starvation.

Serum was withdrawn from near-confluent Rat1-c-*myc*-ER^{TM} cells co-transfected with Tax-pcDNAI/Neo and p12^{I}-pcDNAI/Neo. At 24 hours following serum deprivation there are multiple foci of cell loss and formation of apoptotic bodies in degenerating cells (arrows). Expression of p12^{I} has not inhibited cell death induced by Tax in serum-starved cells. Propidium iodide staining. Fluorescence microscopy. Original 400x magnification.



4.4 Discussion

In this study combinations of HTLV-I *pX* genes were transfected into Rat1-c-*myc*-ER^{TM} cells and tested for their ability to protect these cells from apoptosis following activation of c-Myc by 4-HT under conditions of serum deprivation. Rat1 and Rat1-c-*myc*-ER^{TM} cells survived but ceased growing when serum was withdrawn. Activation of c-Myc in these cells under conditions of serum deprivation resulted in cell death due to apoptosis (Evan *et al* 1992). Constitutive expression of Tax induced cell death in Rat1 and Rat1-c-*myc*-ER^{TM} cells following withdrawal of serum, consistent with the observations of other groups using rodent fibroblasts in different experimental systems (Sakurai *et al* 1992b, Yamada *et al* 1994, Fujita and Shiku 1995). Within the constraints of the temporal pattern of cell death in serum-starved Rat1-c-*myc*-ER^{TM} cells expressing Tax, the fate of these cells was unaffected by induced expression of c-Myc.

Constitutive expression of Rex, p21^{III}, Tof, p13^{II} and p12^{I} in Rat1-c-*myc*-ER^{TM} cells did not have any detectable effect on proliferation or survival when cells were cultivated in normal or reduced concentrations of serum. Co-expression of these gene products with Tax did not prevent Tax-induced apoptosis of serum-starved Rat1-c-*myc*-ER^{TM} cells. Similarly, constitutive expression of Rex, p21^{III}, Tof, p13^{II} or p12^{I} did not prevent apoptosis induced in serum-starved Rat1-c-*myc*-ER^{TM} cells by activation of c-Myc. Therefore, no role in induction or inhibition of apoptosis by Rex, p21^{III}, Tof, p13^{II} or p12^{I} was demonstrated in this experimental system and these gene products did not appear to cooperate with Tax or c-Myc. It is uncertain whether prevention of apoptosis is one of the oncogenic mechanisms used by HTLV-I.

In this study cell death due to apoptosis was demonstrated by visible changes in cells by standard light microscopy and by examination of PI-stained cells by fluorescence light microscopy. These changes are largely qualitative. Quantification of apoptosis could be achieved by a variety of techniques, including the use of video time-lapse photography to follow the fate of individual cells (Evan *et al* 1992), flow cytometry analysis of DNA strand breaks labelled with bromo-dUTP (Li and Darzynkiewicz 1995), detection of phosphatidylserine expression on apoptotic cells by flow cytometry using annexin V (Koopman *et al* 1994) and staining of DNA strand breaks in cells on slides stained by the terminal deoxynucleotidyl transfer-mediated dUTP-biotin nick end labelling (TUNEL) (Kasagi *et al* 1994). Use of these techniques could allow more subtle effects resulting from interactions between HTLV-I *pX* gene products to be detected.

HTLV-I *pX* mRNA expression was detected in transfected Rat1-c-*myc*-ER^{TM} cells in this study by Northern blot hybridisation. Expression of mRNA does not necessarily imply expression of a functional protein. The observed effects in Tax-pcDNAI/Neo-transfected cells deprived of serum indicate that functional Tax was expressed in these cells. However, it would be desirable to demonstrate expression of Rex, p21^{III}, Tof, p13^{II} and p12^{I} proteins by functional or immunological assays to be confident that the apparent absence of effects due to these gene products was not due to lack of expression of active proteins. Monoclonal antibodies (MAbs) could be used to stain specific HTLV-I *pX* proteins or MAbs against amino acid tags such as AU1, HA1 and TSP in Tof, p13^{II} and p12^{I} (Table 4.1) could be used as

markers for protein expression. In 2003 an attempt was made to revive 35 transfected cell lines that had been stored in liquid nitrogen to test for expression of HTLV-I *pX* proteins but none of the cell lines were viable.

This study demonstrated that Tax induces apoptosis in Rat1 cells deprived of serum and that other HTLV-I *pX* gene products did not prevent apoptosis induced by either Tax or c-Myc under these conditions. The implications of these results and potential future studies are discussed further in Chapter 5.

Chapter 5

General Discussion

5.1 Investigation of the role of retroviruses in human T cell leukaemias and lymphomas

The main aim of this project was to determine if human T lymphotropic virus type I (HTLV-I) or related retroviruses are associated with cutaneous T cell lymphomas (CTCLs), particularly mycosis fungoides (MF) and Sézary syndrome (SS). Additional aims were to determine if HTLV-I-related viruses are involved in large granular lymphocytic (LGL) leukaemia and if infection with bovine leukaemia virus (BLV) or other members of the HTLV/BLV group of viruses is present in selected cases of adult acute lymphoblastic leukaemia (ALL). The possibility that undiscovered retroviruses might be involved in human T cell leukaemias and lymphomas was also considered. Identifying an association between a virus and a neoplasm is a first step in confirming the aetiology of virus-induced neoplasia. A final aim of the project was to determine if HTLV-I *pX* region gene products are able to prevent apoptosis. Identifying anti-apoptotic properties in *pX* proteins with currently unknown function would contribute to an understanding of the mechanisms of HTLV-I leukaemogenesis.

MF is an epidermotropic $CD4^+$ CTCL and SS is considered to be the leukaemic form of the same disease (Edelson 1980). These neoplasms were targeted for investigation because they have clinical and pathological similarities to some forms of adult T cell leukaemia/lymphoma (ATL), an aggressive $CD4^+$ T cell neoplasm that is caused by HTLV-I (Blattner *et al* 1982, Hinuma *et al* 1982b, Nagatani *et al* 1990). Infections with HTLV-I and HTLV-II have been demonstrated in some patients with LGL leukaemia and therefore samples from cases of this lymphoproliferative disorder were included in the study (Loughran *et al* 1992, Martin *et al* 1993). Cases of acute ALL in butchers from the Cardiff area were examined for evidence of infection with BLV because of their occupational exposure to cattle (Whittaker 1991). A combination of cell culture and molecular biology techniques was used in an attempt to identify HTLV-I and related viruses in these neoplasms.

Many other groups have attempted to establish associations between HTLV-I, HTLV-II or related retroviruses and CTCLs (Hall 1994, Lessin *et al* 1994). Most cases of MF/SS can be distinguished from ATL on the basis of clinicopathological features and the presence or absence of HTLV-I infection (Yamaguchi *et al* 1984, Nagatani *et al* 1990, Levine *et al* 1994a). Many cases initially diagnosed as MF/SS were reclassified as forms of ATL following the discovery of HTLV-I (Poiesz *et al* 1980a, 1981, Blattner *et al* 1982, Blayney *et al* 1983b). However, infection with HTLV-I or HTLV-II has been reported in a number of cases that conform to the case definition of MF/SS (Kaplanski *et al* 1986, Zucker-Franklin *et al* 1991, 1992, Bazarbachi *et al* 1994, Manca *et al* 1994). These results are part of a spectrum of evidence implicating HTLV-I-related retroviruses in T cell neoplasia distinct from ATL in HTLV-I non-endemic regions (Capésius *et al* 1991, D'Incan *et al* 1992, Lapis *et al* 1992,

Lisby *et al* 1992, Bazarbachi *et al* 1993, Chan *et al* 1993, Whittaker and Luzatto 1993, Pancake *et al* 1995). However, the conflicting results of many of these studies indicate the need for stringent criteria to confirm that positive findings are genuine and have aetiological significance. Such criteria include establishing unequivocal case definitions for MF/SS and other CTCLs to enable them to be distinguished from ATL. The concept of HTLV-I positive MF/SS distinct from ATL remains controversial and may depend on strict pathological definitions.

Classification systems for T cell neoplasia are continually being updated and caution should be exercised in the interpretation of studies that have used different criteria for definition of cases (Willemze *et al* 1997, 1999, Fink-Puches *et al* 2002). The classification of LGL leukaemia, which has T cell and natural killer (NK) cell forms, is still developing (Semenzato *et al* 1997). The relationship between clonal LGL leukaemia and lymphoproliferative disease of LGLs (LDLGLs) in which clonality cannot be demonstrated remains to be elucidated (Scott *et al* 1993, Richards *et al* 1995). Adult ALL is well-defined, but childhood ALL has received more attention in the search for an infectious aetiology because of temporal and spatial clustering (Alexander 1993, Harris *et al* 1994, Birch *et al* 2000).

Establishing an association between a virus and a defined clinicopathological entity depends on the acceptability of evidence that the virus is present. Gold standards for diagnosis of HTLV-I infection by serology in patients with ATL may not be applicable to MF/SS or LGL leukaemia if the serological response is altered in patients with these diseases. Variations in patterns of seroreactivity are difficult to interpret in this context, particularly as variant HTLVs have not so far been detected in seroindeterminate patients (Maruyama *et al* 1989, Ranki *et al* 1990, Srivastava *et al* 1990, Nerurkar *et al* 1992, Madeleine *et al* 1993, Re *et al* 1993, Busch *et al* 2000). Therefore, serological testing was not performed in this study. Molecular techniques are increasingly being used for detection of viral sequences in blood and tissues but the most definitive evidence of retroviral infection remains isolation of infectious virus. In studies testing the hypothesis of a viral aetiology of MF/SS, a goal would be to isolate a new species of exogenous retrovirus or a distinct strain of HTLV-I or HTLV-II.

If the presence of infectious virus or viral sequences is confirmed in patients with T cell neoplasia, then further investigation is required to determine if the virus has an aetiological role in the disease (Evans 1976, Fredricks and Relman 1996). The presence of a retrovirus in patients with CTCL, even if integrated in neoplastic cells, is not proof of a causal role in oncogenesis. Retroviruses may be carried as passengers in neoplastic cells, with infection representing tropism for proliferating T cells rather than an aetiological role. Early isolates of HTLV-II were obtained from T cell variants of hairy cell leukaemia (HCL) and $CD8^+$ T cell lymphocytosis in patients with B cell HCL, but HTLV-II does not appear to be aetiologically involved in HCL (Rosenblatt *et al* 1987, Hjelle *et al* 1991a). Similarly, cases of LGL leukaemia in which HTLV-I and HTLV-II have been demonstrated may reflect coincidental infection in patients from populations harbouring these viruses (Heneine *et al* 1994, Loughran *et al* 1994b).

5.2 Detection of retroviruses in human T cell leukaemias and lymphomas by cell culture

In the first part of this study attempts were made to isolate HTLV-I or other retroviruses in cell culture from patients with cutaneous lymphoid infiltrates. HTLV-I was first detected in a $CD4^+$ T cell line established from a patient with ATL (Poiesz *et al* 1980a, b) and HTLV-II was first identified in a $CD8^+$ T cell line derived from a patient with a T cell variant of HCL (Kalyanaraman *et al* 1982b). HTLV-I-infected T cell lines can be readily established from ATL patients using mitogens, interleukin (IL) 2, conditioned medium (CM) and by cocultivation of peripheral blood mononuclear cells (PBMCs) with umbilical cord blood mononuclear cells (UCMCs) (Gazdar *et al* 1980, Miyoshi *et al* 1981a, b, Markham *et al* 1983). A number of $CD4^+$ T cell lines have been established from patients with MF and SS using IL2, IL4, IL7 and CM as growth stimulants (Kaltoft *et al* 1984, 1987, Abrams *et al* 1991a, b, Crae *et al* 1991, Starkebaum *et al* 1991, Kaltoft *et al* 1992). Although retrovirus-like particles have sometimes been demonstrated by electron microscopy (EM) and reverse transcriptase (RT) activity sometimes detected in cell culture supernatants in these cell lines, there is no definitive evidence of infection with exogenous retroviruses. Two groups have identified HTLV-I or HTLV-II sequences by the polymerase chain reaction (PCR) in lymphocytes cultivated from patients with MF/SS using IL2 and granulocyte-macrophage colony stimulating factor (GMCSF) as growth stimulants but infectious retroviruses have not been purified or propagated (Zucker-Franklin *et al* 1991, 1992, Ghosh *et al* 1994). Similar cell culture techniques were adopted in this study in an attempt to propagate T cell lines that might harbour T lymphotropic viruses such as HTLV-I and HTLV-II. Cultivated cells were examined by EM for evidence of retrovirus infection and culture supernatants were assayed for RT activity. Cultivated cells were also tested for HTLV-I and HTLV-II by PCR.

PBMCs and lymphocytes derived from skin and lymph nodes were cultivated from 18 patients with cutaneous lymphoid infiltrates. These were 11 patients with MF, three patients with SS, one patient with CTCL (peripheral T cell lymphoma) and three patients with cutaneous B cell lymphoma (CBCL). Four $CD8^+$ T cell lines were cultivated for more than 6 months from the skin of three patients with MF. Since these patients had $CD4^+$ T cell neoplasia, the cultivated cells probably arose from non-neoplastic $CD8^+$ tumour infiltrating lymphocytes (TILs). It has been shown that most T cell clones propagated from MF lesions are not derived from neoplastic MF/Sézary cells (Ho *et al* 1990, Harwix *et al* 2001). One Epstein-Barr virus (EBV)-infected B-lymphoblastoid cell line (B-LCL) was established from a patient with CBCL, consistent with the known B cell immortalising properties of this ubiquitous virus (Pattengale *et al* 1973). No evidence of retrovirus infection was found in cultured lymphocytes by EM or RT assay and no HTLV-I or HTLV-II sequences were demonstrated by PCR.

Reports of detection of retrovirus-like particles by EM and RT activity in cultured lymphocytes from patients with MF/SS (Kaltoft *et al* 1984, 1987, Saal *et al* 1989, Zucker-Franklin *et al* 1991, Bazarbachi *et al* 1994) need to be verified by purification and characterisation of the putative virus, demonstration of infectivity and sequencing. None of the studies so far reported have been able to meet these criteria. It is necessary to exclude the possibility that putative virus isolates are the result of expression of endogenous retroviruses in cultured

cells (Löwer *et al* 1993, Herbst *et al* 1996). Virus-like particles may be produced following activation of endogenous retrovirus-like elements in the altered environment of neoplastic tissue or in cell cultures. It is also necessary to ensure that propagated viruses have not been derived from laboratory contamination with exogenous or endogenous retroviruses, especially those of non-human primate origin (Reitz *et al* 1976, Smith *et al* 1979, Popovic *et al* 1982). Contamination of cell cultures with non-human primate and murine retroviruses has been a frequent distraction during attempts to isolate retroviruses from human patients with neoplasia. Cell lines established from patients with CTCL should also be characterised by immunophenotyping, karyotyping and molecular analysis as necessary to determine if they are derived from the neoplastic clone. In many studies reporting HTLV-I-related viruses in cell cultures from patients with CTCL the propagated cells have not been confirmed to be of T cell origin (Manzari *et al* 1987, Hall *et al* 1991, Zucker-Franklin *et al* 1991, 1992).

Human T cells infected with HTLV-I and HTLV-II can be immortalised (remaining IL2-dependent) or transformed (growing independently of IL2) *in vitro* (Miyoshi *et al* 1981c, Chen *et al* 1983). Normal human T cells are able to survive in standard cell cultures for 20 to 30 days if unstimulated or 30 to 60 days if activated (Perillo *et al* 1989). Use of a variety of stimulants and cocultivation techniques can increase the longevity of T cells in culture and result in the selective outgrowth of specific T cell clones, such as the $CD8^+$ T cell lines established in this study. Success in establishing T cell lines could also be due to infection with immortalising viruses but extending the lifespan of propagated cells per se does not necessarily increase the chances of isolating new retroviruses. The cell culture approach to isolation of new viruses should therefore be directed at maintaining an environment in which the *in vitro* growth of cells is optimised for virus isolation without merely perpetuating the survival of normal T cells. This could be achieved by selective induction of specific T cell types and subtypes and stimulation of virion production from latent proviruses.

5.3 Use of molecular techniques to detect HTLV-related retroviruses in human T cell leukaemias and lymphomas

There is a wide range in reported frequencies of detection of HTLV-I and HTLV-II sequences by PCR in patients with MF/SS (Capésius *et al* 1991, Hall *et al* 1991, D'Incan *et al* 1992, Lapis *et al* 1992, Lisby *et al* 1992, Bazarbachi *et al* 1993, Chan *et al* 1993, Manca *et al* 1994, Pancake *et al* 1995). A number of groups have provided molecular evidence that incomplete or defective genomes of HTLV-I, or possibly variant viruses, are present in some patients (Hall *et al* 1991, Zucker-Franklin *et al* 1991, Srivastava *et al* 1992, Ghosh *et al* 1994, Pancake *et al* 1995). HTLV-I *pX* sequences, especially *tax* sequences, are detected more frequently than other regions of the genome in these cases. Defective HTLV-I proviruses with preferential retention of the *pX* region have been detected in a small proportion of patients with ATL (Konishi *et al* 1984, Korber *et al* 1991, Ohshima *et al* 1991, Kubota *et al* 1995). Similarly, the *pX* region is retained in defective BLV proviruses integrated in leukaemic B cells of cattle with enzootic bovine leucosis (EBL) (Kettmann *et al* 1982). Therefore, the hypothesis was tested that incomplete or defective HTLV-I or HTLV-II proviruses are integrated in neoplastic cells of patients with MF/SS and that *tax* sequences are detected more frequently by PCR than other regions of the viral genome.

In the second part of this study samples from 36 patients with cutaneous lymphoid infiltrates, six patients with LGL leukaemia and six patients with adult ALL were tested for the presence of HTLV-I and HTLV-I/II using PCR primer sets that amplified sequences from different regions of the HTLV-I proviral genome. Patients with LGL leukaemia and adult ALL were also tested for BLV *gag* sequences by PCR. An important feature of this study was the careful optimisation of PCR conditions for each primer set and the use of serial dilutions of positive control DNA and cells to quantify the sensitivity of each assay. Sensitivity was monitored in PCRs conducted on each batch of clinical samples to ensure that an adequate limit of detection was achieved. Precautions were taken to minimise PCR contamination. PCR products were amplified from three patients (one MF, one SS and one CBCL) using HTLV-I or HTLV-I/II *tax* primers. However, the sequences of cloned PCR products were identical to those amplified from C8166 cell line DNA used as a positive control. It was concluded that these PCR products were the result of contamination, despite the care taken in preparing PCR reagents and handling clinical samples.

False positive results due to PCR contamination have been a major problem in attempts to associate HTLV-I or HTLV-II with a number of diseases. HTLV-I sequences detected by PCR were reported in patients with multiple sclerosis (Koprowski *et al* 1985, Greenberg *et al* 1989a, Reddy *et al* 1989) but extensive follow-up studies have been negative (Ehrlich *et al* 1991). The use of PCR to detect viruses must be conducted with stringent precautions to eliminate contamination from PCR products, plasmids containing cloned viral sequences, positive control DNA samples and infected clinical samples. When there is controversy regarding the veracity of findings, for example when disparate results are obtained by different groups, positive findings must be subjected to high levels of scrutiny. Criteria for accepting a positive result as genuine need to take account of the precautions to prevent contamination at all stages of sample and reagent preparation and analysis.

In molecular studies of the involvement of HTLV-I-related viruses in T cell neoplasia, an acceptable level of proof in some circumstances would be to accept results only when sequences of PCR products differ significantly from reference strains of HTLV-I and HTLV-II, particularly those used as positive controls. It may be not be possible to resolve the issue of whether prototypical strains of HTLV-I are involved in the aetiology of CTCLs, since cases of MF/SS and T cell lymphomas (TCLs) that are HTLV-I-associated could be classified as variants, particularly cutaneous forms, of ATL. It may only be possible to identify a retrovirus as the cause of MF/SS by isolation of a distinct sequence variant of virus that can be classified as a new strain or new virus species. It would then be necessary to demonstrate that this virus is consistently associated with MF/SS.

Since this molecular investigation was conducted, several other PCR-based studies have been published that report no evidence of HTLV-I or HTLV-II infection in MF/SS patients (Böni *et al* 1996, Wood *et* al 1996b, Bazarbachi *et al* 1997, Fujihara *et al* 1997, Wood *et al* 1997, Daliani *et al* 1998, Kim *et al* 1998). The carefully controlled study of Wood *et al* (1996a, b) provides a good model for future molecular studies attempting to identify viral sequences in lesions of patients with a variety of diseases. Similar to the molecular investigations described in this thesis, a PCR system for detection of HTLV-I in paraffin-embedded histological sections was carefully optimised, controlling for specificity by

Southern blot (SB) hybridisation using an oligonucleotide probe and nucleotide sequencing (Wood *et al* 1996a). The integrity of total DNA was assessed by PCR for the nucleophosmin gene and the integrity of T cell DNA was assessed by PCR for the T cell receptor (TCR). Using PCR and SB hybridisation, HTLV-I *pol* sequences were initially detected in three (7%) of 42, *env* sequences in 2 (5%) of 37 and *pX* sequences in 7 (17%) of 42 patients from the USA with MF/SS (Wood *et al* 1996b). However, after treatment of samples with ultraviolet (UV) light, only three of 12 previously positive cases remained positive, suggesting that the earlier positive results were due to trace contamination with PCR reagents or sample DNA. Furthermore, *pX* and *pol* sequences cloned from one PCR-positive sample were identical to that of the positive control; the same sample was negative for HTLV-I by dot blot hybridisation of genomic DNA and new samples from the two remaining positive cases were negative by PCR. When material from 28 additional cases of MF/SS from the USA was subjected to PCR with the use of UV light to reduce contamination, all were negative for *pX* sequences. The conclusion from this study was that positive PCR results for HTLV-I in MF/SS patients were due to trace contamination with PCR products (Wood *et al* 1996b).

Measures to prevent contamination in many studies may not have been sufficiently stringent to prevent false positive results. Associations between diseases and viruses should be viewed with scepticism if they are based on PCR results without other supporting evidence. If a retrovirus is involved in CTCL, it may not be related to HTLV-I or HTLV-II, it might be present in the skin in quantities below the detection limit of PCR or it might have been eliminated from the skin before clinical lesions develop (Lisby *et al* 1992). The best evidence to date, including the results described in this thesis, thus indicate that HTLV-I and HTLV-II are not involved in CTCL (Lessin *et al* 1996, Wood *et al* 1996b)

Supporting these observations, no HTLV-I sequences were detected by PCR in PBMCs or skin lesions of 127 patients with CTCL (108 with MF/SS) from Europe and the USA using HTLV-I and HTLV-II *gag*, *pol*, *env*, *pX* and LTR specific primers (Bazarbachi *et al* 1997). No HTLV-I sequences were detected by PCR in 50 Swiss and German patients with CTCL (Böni *et al* 1996) or 16 HTLV-I seronegative patients with MF/SS from north eastern USA (Fujihara *et al* 1997). Similarly, PCR analyses conducted since this study have not shown any evidence of HTLV-I or HTLV-II in cases of LGL leukaemia and T prolymphocytic leukaemia in non-endemic regions (Pawson *et al* 1997).

However, some groups have continued to report detection of HTLV-I sequences by PCR in patients with CTCL (Zucker-Franklin 2001). Pancake and Zucker-Franklin (1996) detected HTLV-I *tax* sequences by *in situ* PCR in infiltrating lymphocytes, as well as keratinocyte-like cells, in the skin of 11 of 12 patients with MF. The skin PCR negative patient had HTLV-I sequences demonstrable by PCR and Southern blot (SB) hybridisation in PBMCs (Khan *et al* 1996). HTLV-II *tax* and *pol* sequences were detected by PCR in tissues from one (5%) of 22 patients with CTCL in the USA, although clonal HTLV-II integration could not be detected by SB hybridisation in PBMCs of this patient; all patients were negative for HTLV-I *tax* or *pol* sequences (Li *et al* 1996). Kikuchi *et al* (1997a) reported that no HTLV-I sequences were detected in 50 HTLV-I seronegative Japanese patients with CTCL, then later reported that HTLV-I *gag*, *pol*, *env* and/or *pX* sequences were detected by PCR in two HTLV-I seronegative patients with CTCL, although monoclonal integration of HTLV-I provirus could

not be confirmed in these patients by SB hybridisation (Kikuchi *et al* 1997b). Similarly, HTLV-I *gag*, *pol*, *env*, *pX* and LTR sequences were detected by PCR in PBMCs of an HTLV-I seronegative Japanese patient with SS, but no monoclonal integration of HTLV-I provirus could be detected by SB hybridisation (Miyoshi *et al* 1998). Twelve of 28 patients with CTCL (27 MF, one SS) had antibodies against HTLV-I by WB ELISA and HTLV-I sequences were amplified by PCR from PBMCs of six patients (Shohat *et al* 1999). An HTLV-II associated CTCL has been identified in an HIV-1-infected AIDS patient (Poiesz *et al* 2000).

Differences between studies in associating HTLV-I with MF/SS have been attributed to differences in the methods of processing samples for detection of HTLV-I sequences (Pancake and Zucker-Franklin 1996, Zucker-Franklin and Pancake 1998b). Zucker-Franklin and Pancake (1998b) showed that the use of whole-cell lysates instead of DNA extracts and the use of fresh instead of cultured cells for PCR increased the ability to detect HTLV-I *tax* sequences in samples from MF patients. They also observed that positive PCR products from clinical samples are more likely when *tax* primers are used than primers from other regions of the HTLV-I genome, when there are more than 30 cycles of amplification and when SB hybridisation is applied to PCR products (Ghosh *et al* 1994, Pancake *et al* 1995b, Pancake and Zucker-Franklin 1996). However, contamination is also more likely with extended PCR cycles using highly sensitive primer sets. Since it is difficult to assess precautions taken by other groups to prevent PCR contamination, independent studies are necessary to verify positive findings. The only study to have used a double blind method for confirmation of PCR results from the same patients at different times of collection reported positive results for HTLV-I *pol* and *tax* sequences in 10 (34%) of 29 patients with MF from Italy (Manca *et al* 1994). The need for carefully controlled independent studies will continue while ever there are claims of positive associations between HTLV-I or related viruses and CTCL.

In 50 MF patients positive for HTLV-I *tax* DNA and mRNA by PCR, 83% had antibodies against HTLV-I Tax, whereas only 5% had reactivity to structural proteins in standard HTLV-I serological assays (Pancake *et al* 1996a). Although anti-Tax antibodies and *tax* sequences detected by PCR have been reported by some groups in 8 to 11% of normal blood donors and 4 to 33% of intravenous drug users (IVDUs) negative for HTLV-I in standard serological assays (Ehrlich *et al* 1989a, Pancake *et al* 1996a, b, Zucker-Franklin and Gorman 1997, Zucker-Franklin *et al* 1997, Zucker-Franklin and Pancake 1998a) these findings have not been supported in an independent, blind, multicentre study (Cowan *et al* 1999).

DNA extracted from skin samples of patients with MF usually contains only a small percentage of DNA from neoplastic T cells and therefore it is important to determine the sensitivity of detection of HTLV-I and HTLV-II sequences by PCR. The use of PCR to indicate the presence of incomplete HTLV-I or HTLV-II proviruses needs to be critically evaluated, since most studies have not adequately monitored the sensitivity of each primer set. A negative PCR result for some but not other regions of the HTLV genome could be due to poor sensitivity of particular primer sets in a panel. Alternatively, positive results could be due to contamination. It is notable that many *tax* primer sets appear to be more sensitive than primer sets used for PCR from other regions of the HTLV-I genome. The presence of HTLV-I or HTLV-II with variant sequences could also result in the failure to amplify products

by PCR. The possibility of variant HTLV-I sequences was taken into account in this study by using primers covering multiple regions of prototypical HTLV-I and by use of consensus primers. Also, the *pX* regions of HTLV-I and HTLV-II have sufficient similarity to cross-hybridise in SB hybridisation analyses, so the HTLV-I probe ($pMT2_I$) used in these experiments would be expected to hybridise to PCR products amplified using HTLV-I/II primers (Shaw *et al* 1984).

To verify that a patient with CTCL is infected with HTLV-I or a related virus, it will be necessary to obtain a substantial length of sequence, preferably of the entire provirus, for comparative analysis. Sequencing of a fragment derived from a PCR reaction is inadequate unless the sequence can be shown to be substantially different from sequences of any known HTLV-I or HTLV-II strain and different from any positive control DNA used for the same study. Small differences in the sequences of PCR products could be due to errors introduced by *Taq* polymerase during amplification. This possibility could be excluded by sequencing PCR products from multiple independent reactions or by the use of proof reading enzymes with higher fidelity. Otherwise, even with the use of positive and negative controls, contamination cannot be excluded with the stringency required for declaration of a new virus or variant given the level of controversy in this field. The lack of adequate sequence data is the major flaw of all studies so far reported that have attempted to associate HTLV-I, HTLV-II or related viruses with CTCL.

Regions of sequence similarity in the LTR and *gag* regions of HTLV-I, HTLV-II and BLV were used to design consensus PCR primers for the HTLV/BLV (*Deltaretrovirus*) group of viruses. However, these primers were shown to produce non-specific PCR products under a variety of conditions using HTLV-I and BLV DNA as templates. Further optimisation may improve the specificity of these primer sets or they may have specific applications in customised PCR systems. It would also be necessary to determine the sensitivity of these primer sets. Consensus retroviral PCR primers have been based on alignments of retroviral Pol region amino acid sequences and reverse translation to identify minimum degenerate codons (Mack and Sninsky *et al* 1988, Donehawer *et al* 1990). A major problem with these primers is that they will amplify homologous *pol* sequences from endogenous retroviral elements and therefore are only likely to be useful in PCR systems based on cDNA (Nelson *et al* 1999). Another approach is to use a modification of representational difference analysis (RDA) to preferentially amplify tester sequences that will not be present in driver DNA, thus limiting amplification of endogenous sequences (Lisitsyn *et al* 1993). This allows selective amplification of exogenous retroviruses from the tester DNA, which will not have partners in the driver DNA. RDA could also be applied to cDNA to search for retroviral RNA genomes (Hubank and Schatz 1994, Geng *et al* 1998).

A third HTLV-related retrovirus lineage, known as primate T lymphotropic virus type L (PTLV-L or PTLV-3), has been identified in baboons (*Papio hamadryas*) (Goubau *et al* 1994, Van Brussel *et al* 1997). There is sufficient similarity between *tax* sequences of PTLV-L and HTLV-I/II for this divergent group to be identified using generic *tax* primers (Busch *et al* 2000). As discussed above, no variant PTLV-L *tax* sequences were detected by PCR in 269 HTLV-I/II seroindeterminate blood donors in the USA (Busch *et al* 2000).

Although there is little evidence for the involvement of EBV in most human primary CTCLs, including MF/SS (Kanavaros *et al* 1994, Anagnostopoulos *et al* 1996, Angel *et al* 1996), an EBV-related virus, HV_{MNE}, has been associated with MF in a pig-tailed macaque (*Macaca nemestrina*) (Rivadeneira *et al* 1999). PCR using specific, degenerate and consensus primers for detection of herpesviruses could be applied to CTCLs (MacKenzie *et al* 2001, Gallagher *et al* 2002).

This project did not produce evidence that HTLV-I, HTLV-II or BLV are associated with MF/SS, LGL leukaemia or selected cases of adult ALL in the United Kingdom. The available reports attempting to implicate retroviruses in CTCLs similarly have failed to provide conclusive evidence for a primary aetiological role of HTLV-I or HTLV-II in MF/SS (Lessin *et al* 1994, Woods *et al* 1996b). Therefore, it is concluded that HTLV-I and HTLV-II are not the cause of CTCLs in HTLV non-endemic regions.

5.4 The role of HTLV-I *pX* gene products in apoptosis

The third part of this project examined the ability of HTLV-I *pX* gene products to inhibit apoptosis induced by c-Myc in Rat1 fibroblasts. When cells proliferate in response to the activation of oncogenes such as c-Myc, the apoptosis pathway is switched on concurrently and these cells die when survival factors such as those present in serum are withdrawn (Evan *et al* 1992). In models of oncogenesis there is a requirement for apoptosis to be blocked to enable proliferating cells to survive. Several oncogenic viruses produce proteins that inhibit apoptosis (Rao *et al* 1992, White *et al* 1992, Henderson *et al* 1993).

In this study it was hypothesised that HTLV-I *pX* region gene products with currently unknown functions might inhibit apoptosis. The system of c-Myc induction by 4-hydroxytamoxifen (4-HT) in Rat1 cells stably transfected with a chimaeric c-*myc*-modified receptor (ER^{TM}) gene provided an elegant model to test the anti-apoptotic properties of HTLV-I *pX* proteins (Evan *et al* 1992, Littlewood *et al* 1995). However, expression of Rex, $p21^{III}$, $p30^{II}$ (Tof), $p13^{II}$ or $p12^{I}$ did not inhibit c-Myc-induced apoptosis in Rat1 cells under conditions of serum deprivation. Furthermore, Rat1 cells expressing Tax underwent apoptosis when serum was withdrawn, indicating that Tax might trigger apoptotic pathways at the same time as it activates pathways leading to cell proliferation. Other workers similarly have demonstrated that Tax induces apoptosis in cells following withdrawal of cell survival signals (Sakurai *et al* 1992b, Yamada *et al* 1994, Fujita and Shiku 1995). Expression of Rex, $p21^{III}$, Tof, $p13^{II}$ or $p12^{I}$ alone had no effect on Rat1 cells in the absence of serum. Co-expression of these *pX* proteins with Tax did not inhibit Tax-induced apoptosis compared to expression of Tax alone and there was no evidence of a cooperative effect.

Although no inhibition of c-Myc or Tax-induced apoptosis by other HTLV-I *pX* proteins was observed in this experimental system, further studies should be undertaken to explore the functions of HTLV-I *pX* gene products in other systems for detecting anti-apoptotic effects. It would be highly desirable to develop an inducible HTLV-I *tax* gene expression system such as a cell line stably transfected with a *tax*-ER^{TM} plasmid construct. This would allow interactions between *tax* and other HTLV-I *pX* proteins to be explored in a more tightly controlled system.

5.5 Conclusions

The studies reported in this thesis have demonstrated no evidence for infection with HTLV-I or related viruses in patients with MF/SS or other CTCLs in Scotland using PCR and cell culture. PCR studies also showed no indication of HTLV-I, HTLV-II or BLV infection in cases of LGL leukaemia in the UK or in a cluster of ALL in butchers from the Cardiff region. The HTLV-I Tax protein was shown to induce apoptosis in rodent fibroblasts upon withdrawal of serum, whereas other HTLV-I *pX* gene products failed to inhibit Tax or c-Myc-induced apoptosis.

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Appendices

Appendix 1: Nucleotide and peptide sequences of HTLV-I *pX* region gene products.

Tax

```
   1  ATGGCCCACTTCCCAGGGTTTGGACAGAGTCTTCTTTTCGGATACCCAGT
        M  A  H  F  P  G  F  G  Q  S  L  L  F  G  Y  P  V

  51  CTACGTGTTTGGAGACTGTGTACAAGGCGACTGGTGCCCCATCTCTGGGG
        Y  V  F  G  D  C  V  Q  G  D  W  C  P  I  S  G

 101  GACTATGTTCGGCCCGCCTACATCGTCACGCCCTACTGGCCACCTGTCCA
        G  L  C  S  A  R  L  H  R  H  A  L  L  A  T  C  P

 151  GAGCATCAGATCACCTGGGACCCCATCGATGGACGCGTTATCGGCTCAGC
        E  H  Q  I  T  W  D  P  I  D  G  R  V  I  G  S  A

 201  TCTACAGTTCCTTATCCCTCGACTCCCCTCCTTCCCCACCCAGAGAACCT
        L  Q  F  L  I  P  R  L  P  S  F  P  T  Q  R  T

 251  CTAAGACCCTCAAGGTCCTTACCCCGCCAATCACTCATACAACCCCCAAC
        S  K  T  L  K  V  L  T  P  P  I  T  H  T  T  P  N

 301  ATTCCACCCTCCTTCCTCCAGGCCATGCGCAAATACTCCCCCTTCCGAAA
        I  P  P  S  F  L  Q  A  M  R  K  Y  S  P  F  R  N

 351  TGGATACATGGAACCCACCCTTGGGCAGCACCTCCCAACCCTGTCTTTTC
        G  Y  M  E  P  T  L  G  Q  H  L  P  T  L  S  F

 401  CAGACCCCGGACTCCGGCCCCAAAACCTGTACACCCTCTGGGGAGGCTCC
        P  D  P  G  L  R  P  Q  N  L  Y  T  L  W  G  G  S

 451  GTTGTCTGCATGTACCTCTACCAGCTTTCCCCCCCCATCACCTGGCCCCT
        V  V  C  M  Y  L  Y  Q  L  S  P  P  I  T  W  P  L

 501  CCTGCCCCACGTGATTTTTTGCCACCCCGGCCAGCTCGGGGCCTTCCTCA
        L  P  H  V  I  F  C  H  P  G  Q  L  G  A  F  L

 551  CCAATGTTCCCTACAAGCGAATAGAAGAACTCCTCTATAAAATTTCCCTC
        T  N  V  P  Y  K  R  I  E  E  L  L  Y  K  I  S  L

 601  ACCACAGGGGCCCTAATAATTCTACCCGAAGACTGTTTGCCCACCACCCT
        T  T  G  A  L  I  I  L  P  E  D  C  L  P  T  T  L

 651  TTTCCAGCCTGCTAGGGCACCCGTCACGCTAACAGCCTGGCAAAACGGCC
        F  Q  P  A  R  A  P  V  T  L  T  A  W  Q  N  G

 701  TCCTTCCGTTCCACTCAACCCTCACCACTCCAGGCCTTATTTGGACATTT
        L  L  P  F  H  S  T  L  T  T  P  G  L  I  W  T  F

 751  ACCGATGGCACGCCTATGATTTCCGGGCCCTGCCCTAAAGATGGCCAGCC
        T  D  G  T  P  M  I  S  G  P  C  P  K  D  G  Q  P

 801  ATCTTTAGTACTACAGTCCTCCTCCTTTATATTTCACAAATTTCAAACCA
        S  L  V  L  Q  S  S  S  F  I  F  H  K  F  Q  T

 851  AGGCCTACCACCCCTCATTTCTACTCTCACACGGCCTCATACAGTACTCT
        K  A  Y  H  P  S  F  L  L  S  H  G  L  I  Q  Y  S

 901  TCCTTTCATAGTTTACATCTCCTGTTTGAAGAATACACCAACATCCCCAT
        S  F  H  S  L  H  L  L  F  E  E  Y  T  N  I  P  I

 951  TTCTCTACTTTTTTAACGAAAAAGAGGCAGATGACAATGACCATGAGCCCC
        S  L  L  F  N  E  K  E  A  D  D  N  D  H  E  P

1001  AAATATCCCCCGGGGGCTTAGAGCCTCCCAGTGAAAAACATTTCCGAGAA
        Q  I  S  P  G  G  L  E  P  P  S  E  K  H  F  R  E

1051  ACAGAAGTCTGA
        T  E  V  *
```

Appendix 1: (continued).

Rex

```
1   ATGCCCAAGACCCGTCGGAGCCCCCGCCGATCCCAAAGAAAAAGACCTCC
      M  P  K  T  R  R  R  P  R  R  S  Q  R  K  R  P  P

51  AACACCATGGCCCACTTCCCAGGGTTTGCACAGAGTCTTCTTTTCGGATA
      T  P  W  P  T  S  Q  G  L  D  R  V  F  F  S  D

101 CCCAGTCTACGTGTTTGGAGACTGTGTACAAGGCGACTGGTGCCCCATCT
      T  Q  S  T  C  L  E  T  V  Y  K  A  T  G  A  P  S

151 CTGGGGGACTATGTTCGGCCCGCCTACATCGTCACGCCCTACTGGCCACC
      L  G  D  Y  V  R  P  A  Y  I  V  T  P  Y  W  P  P

201 TGTCCAGAGCATCAGATCACCTGGGACCCCATCGATGGACGCGTTATCGG
      V  Q  S  I  R  S  P  G  T  P  S  M  D  A  L  S

251 CTCAGCTCTACAGTTCCTTATCCCTCGACTCCCCTCCTTCCCCACCCAGA
      A  Q  L  Y  S  S  L  S  L  D  S  P  P  S  P  P  R

301 GAACCTCTAAGACCCTCAAGGTCCTTACCCCGCCAATCACTCATACAACC
      E  P  L  R  P  S  R  S  L  P  R  Q  S  L  I  Q  P

351 CCCAACATTCCACCCTCCTTCCTCCAGGCCATGCGCAAATACTCCCCCTT
      P  T  F  H  P  P  S  S  R  P  C  A  N  T  P  P

401 CCGAAATGGATACATGGAACCCACCCTTGGGCAGCACCTCCCAACCCTGT
      S  E  M  D  T  W  N  P  P  L  G  S  T  S  Q  P  C

451 CTTTTCCAGACCCCGGACTCCGGCCCCAAAACCTGTACACCCTCTGGGGA
      L  F  Q  T  P  D  S  G  P  K  T  C  T  P  S  G  E

501 GGCTCCGTTGTCTGCATGTACCTCTACCAGCTTTCCCCCCCCATCACCTG
      A  P  L  S  A  C  I  S  T  S  F  P  P  P  S  P

551 GCCCCTCCTGCCCCACGTGA
      G  P  S  C  P  T  *
```

Appendix 1: (continued).

$p21^{III}$

```
1   ATGGACGCGTTATCGGCTCAGCTCTACAGTTCCTTATCCCTCGACTCCCC
     M  D  A  L  S  A  Q  L  Y  S  S  L  S  L  D  S  P

51  TCCTTCCCCACCCAGAGAACCTCTAAGACCCTCAAGGTCCTTACCCCGCC
      P  S  P  P  R  E  P  L  R  P  S  R  S  L  P  R

101 AATCACTCATACAACCCCCAACATTCCACCCTCCTTCCTCCAGGCCATGC
     Q  S  L  I  Q  P  P  T  F  H  P  P  S  S  R  P  C

151 GCAAATACTCCCCCTTCCGAAATGGATACATGGAACCCACCCTTGGGCAG
     A  N  T  P  P  S  E  M  D  T  W  N  P  P  L  G  S

201 CACCTCCCAACCCTGTCTTTTCCAGACCCCGGACTCCGGCCCCAAAACCT
      T  S  Q  P  C  L  F  Q  T  P  D  S  G  P  K  T

251 GTACACCCTCTGGGGAGGCTCCGTTGTCTGCATGTACCTCTACCAGCTTT
     C  T  P  S  G  E  A  P  L  S  A  C  T  S  T  S  F

301 CCCCCCCCATCACCTGGCCCCTCCTGCCCCACGTGA
     P  P  P  S  P  G  P  S  C  P  T  *
```

Appendix 1: (continued).

p30^{II} (Tof)

```
   1  ATGGCACTATGCTGTTTCGCCTTCTCAGCCCCTTGTCTCCACTTGCGCTC
        M  A  L  C  C  F  A  F  S  A  P  C  L  H  L  R  S

  51  ACGGCGCTCCTGCTCTTCCTGCTTTCTCCGGGCGACGTCAGCGGCCTTCT
        R  R  S  C  S  S  C  F  L  R  A  T  S  A  A  F

 101  TCTCCGCCCGCCTCCTGCGCCGTGCCTTCTCCTCTCCTTCCTTTTCAAA
        F  S  A  R  L  L  R  R  A  F  S  S  S  F  L  F  K

 151  TACTCAGCAATCTGCTTTTCCTCCTCTTTCTCCCGCTCTTTTTTTCGCTT
        Y  S  A  I  C  F  S  S  S  F  S  R  S  F  F  R  F

 201  CCTCTTCTCCTCAGCCCGTCGCTGCCGCTCACGATGCGTTTCCCCGCGAG
        L  F  S  S  A  R  R  C  R  S  R  C  V  S  P  R

 251  GTGGCGCTTTCTCCCCTGGAAGGCCCCGTCGCAGCCGGCCGCGGCTTTCC
        G  G  A  F  S  P  G  R  P  R  R  S  R  P  R  L  S

 301  TCTTCTAAGGATAGCAAACCGTCAAGCACAGCTTCCTCCTCCTCCTTGTC
        S  S  K  D  S  K  P  S  S  T  A  S  S  S  S  L  S

 351  CTTTAACTCTTCCTCCAAGGATAATAGCCCGTCCACCAATTCCTCCACCA
        F  N  S  S  S  K  D  N  S  P  S  T  N  S  S  T

 401  GCAGGTCCTCCGGGCATGGCACAGGCAAGCATCGAAACAGCCCTACAGAT
        S  R  S  S  G  H  G  T  G  K  H  R  N  S  P  T  D

 451  ACAAAGTTAACCATGCTTATTATCAGCCCACTTCCCAGGGTTTGGACAGA
        T  K  L  T  M  L  I  I  S  P  L  P  R  V  W  T  E

 501  GTCTTCTTTTCGGATACCCAGTCTACGTGTTTGGAGACTGTGTACAAGGC
        S  S  F  R  I  P  S  L  R  V  W  R  L  C  T  R

 551  GACTGGTGCCCCATCTCTGGGGGACTATGTTCGGCCCGCCTACATCGTCA
        R  L  V  P  H  L  W  G  T  M  F  G  P  P  T  S  S

 601  CGCCCTACTGGCCACCTGTCCAGAGCATCAGATCACCTGGGACCCCATCG
        R  P  T  G  H  L  S  R  A  S  D  H  L  G  P  H  R

 651  ATGGACGCGTTATCGGCTCAGCTCTACAGTTCCTTATCCCTCGACTCCCC
        W  T  R  Y  R  L  S  S  T  V  P  Y  P  S  T  P

 701  TCCTTCCCCACCCAGAGAACCTCTAA
        L  L  P  H  P  E  N  L  *
```

Appendix 1: (continued).

p13^{II}

```
  1  ATGCTTATTATCAGCCCACTTCCCAGGGTTTGGACAGAGTCTTCTTTTCG
       M  L  I  I  S  P  L  P  R  V  W  T  E  S  S  F  R

 51  GATACCCAGTCTACGTGTTTGGAGACTGTGTACAAGGCGACTGGTGCCCC
       I  P  S  L  R  V  W  R  L  C  T  R  R  L  V  P

101  ATCTCTGGGGGACTATGTTCGGCCCGCCTACATCGTCACGCCCTACTGGC
       H  L  W  G  T  M  F  G  P  P  T  S  S  R  P  T  G

151  CACCTGTCCAGAGCATCAGATCACCTGGGACCCCATCGATGGACGCGTTA
       H  L  S  R  A  S  D  H  L  G  P  H  R  W  T  R  Y

201  TCGGCTCAGCCCTACAGTTCCTTATCCCTCGACTCCCCTCCTTCCCCACC
       R  L  S  S  T  V  P  Y  P  S  T  P  L  L  P  H

251  CAGAGAACCTCTAA
       P  E  N  L  *
```

Appendix 1: (continued).

p12^{I}

```
1   ATGCTGTTTCGCCTTCTCAGCCCCTTGTCTCCACTTGCGCTCACGGCGCT
     M  L  F  R  L  L  S  P  L  S  P  L  A  L  T  A  L

51  CCTGCTCTTCCTGCTTTCTCCGGGCGACGTCAGCGGCCTTCTTCTCCGCC
     L  L  F  L  L  S  P  G  D  V  S  G  L  L  L  R

101 CGCCTCCTGCGCCGTGCCTTCTCCTCTTCCTTCCTTTCAAATACTCAGC
    P  P  P  A  P  C  L  L  L  F  L  P  F  Q  I  L  S

151 AATCTGCTTTTCCTCCTCTTCTCCCGCTCTTTTTTCGCTTCCTCTTCT
     N  L  L  F  L  L  F  L  P  L  F  F  S  L  P  L  L

201 CCTCAGCCCGTCGCTGCCGCTCACGATGCGTTCCCCGCGAGGTGGCGCT
     L  S  P  S  L  P  L  T  M  R  F  P  A  R  W  R

251 CTCTCCCCTGGAAGGCCCCGTCGCAGCCGGCCGCGGCTTTCCTCTTCTAA
    F  L  P  W  K  A  P  S  Q  P  A  A  A  F  L  F  *
```

Appendix 2: International Union of Pure and Applied Chemistry (IUPAC) International Union of Biochemistry (IUB)/Genetics Computer Group (GCG) letter codes for nucleotide and amino acid sequences.

The nomenclature for incompletely specified bases in nucleic acid sequences is also depicted. (Nomenclature Committee of the International Union of Biochemistry 1985).

Nucleotides

Purines	A	Adenine
	G	Guanine
Pyrimidines	C	Cytosine
	T/U	Tnymine/Uracil

IUB/GCG	Meaning	Complement
A	A	T
C	C	G
G	G	C
T/U	T	A
M	A or C	K
R	A or G	Y
W	A or T	W
S	C or G	S
Y	C or T	R
K	G or T	M
V	A or C or G	B
H	A or C or T	D
D	A or G or T	H
B	C or G or T	V
X/N	G or A or T or C	X
.	not G or A or T or C	.

Appendix 2: (continued).

Amino acids

Symbol	3-letter	Meaning	Codons	IUB Depiction
A	Ala	Alanine	GCT,GCC,GCA,GCG	!GCX
B	Asp,Asn	Aspartic, Asparagine	GAT,GAC,AAT,AAC	!RAY
C	Cys	Cysteine	TGT,TGC	!TGY
D	Asp	Aspartic	GAT,GAC	!GAY
E	Glu	Glutamic	GAA,GAG	!GAR
F	Phe	Phenylalanine	TTT,TTC	!TTY
G	Gly	Glycine	GGT,GGC,GGA,GGG	!GGX
H	His	Histidine	CAT,CAC	!CAY
I	Ile	Isoleucine	ATT,ATC,ATA	!ATH
K	Lys	Lysine	AAA,AAG	!AAR
L	Leu	Leucine	TTG,TTA,CTT,CTC,CTA,CTG	!TTR,CTX,YTR;YTX
M	Met	Methionine	ATG	!ATG
N	Asn	Asparagine	AAT,AAC	!AAY
P	Pro	Proline	CCT,CCC,CCA,CCG	!CCX
Q	Gln	Glutamine	CAA,CAG	!CAR
R	Arg	Arginine	CGT,CGC,CGA,CGG,AGA,AGG	!CGX,AGR,MGR;MGX
S	Ser	Serine	TCT,TCC,TCA,TCG,AGT,AGC	!TCX,AGY;WSX
T	Thr	Threonine	ACT,ACC,ACA,ACG	!ACX
V	Val	Valine	GTT,GTC,GTA,GTG	!GTX
W	Trp	Tryptophan	TGG	!TGG
X	Xxx	Unknown		!XXX
Y	Tyr	Tyrosine	TAT, TAC	!TAY
Z	Glu,Gln	Glutamic, Glutamine	GAA,GAG,CAA,CAG	!SAR
*	End	Terminator	TAA, TAG, TGA	!TAR,TRA;TRR

Appendix 3: Sources of frequently used materials.

A & J Beveridge
Alpha Labs, UK
Alta Bioscience, University of Birmingham
Amersham, Little Chalfont
Applied Biosystems, Warrington
AT Biochem, Malvern, Pennsylvania
BDH (Merck), Poole, Dorset
Beckman Instruments, High Wycombe
Becton Dickinson, Cowley
J Bibby, Stone
Bio-Rad, Hemel Hempstead
Boehringer Manheim, Lewes
British Biotechnology, Abingdon
BRL, Bethesda, Maryland
Cambio, Cambridge
Costar, High Wycombe
Coulter, Luton
Dako, Glostrup, Denmark
Difco, East Molesey
Fisons Scientific Equipment, Loughborough
Fotodyne, New Berlin, Wisconsin
Genetic Research Instrumentation, Dunmow
Genosys, Cambridge
Greiner Labortechnik, Dursley
Hybaid, Teddington
ICN Biomedicals, Thame
International Laboratory Services, UK
Invitrogen, San Diego, California
J.Bio, Les Ulis Cedex, France
Leo Labs, UK
Life Technologies, Gibco BRL, Paisley
Millipore, Watford
MSE, supplied by Philip Harris, Paisley
Bando Chemical Industries, Nippon Shoji Kaisha, Japan
Nunc, supplied by Philip Harris, Paisley
Unipath, Basingstoke
Perkin-Elmer, Beaconsfield
Pharmacia LKB Biotechnology, St Albans
Philip Harris Scientific, Clydebank
Polaroid, St Albans
Promega, Southampton
Qiagen, Chatsworth, California

Appendix 3: (continued).

Rathburn Chemical Company, UK
Sarstedt, Beaumont Leys Schleicher & Schuell, West Germany
Scotlab Bioscience, Coatbridge
Sigma Chemical Company, Poole
Stratagene, Cambridge
Surgikos, Johnson & Johnson, Livingston
Techne, Cambridge
UVP, San Gabriel, California
Vector Laboratories, Burlingame, California
Whatman, Maidstone

Appendix 4: List of commonly used reagents, stock solutions and buffers for cell culture, immunophenotyping and reverse transcriptase assay.

A4.1 Tissue culture disposable plastic-ware

50 cm^2, 75 cm^2 and 250 cm^2 screw-top tissue culture flasks (Costar)
96-well microtitre tissue culture plate (Costar)
12-well tissue culture plate (Costar)
1, 5, 10 and 25 ml disposable filter-plugged polycarbonate pipettes (Costar)
5, 15 and 50 ml screw-top polypropylene centrifuge tubes (Falcon and Greiner)
0.6 and 1.5 ml flip-top and screw-top polypropylene microcentrifuge tubes (Elkay, Treff and Eppendorf, supplied by Scotlab)
20 ml screw-top polycarbonate Universal tube (Scotlab)
5 ml screw-top polycarbonate bijou (Scotlab)
1, 2.5, 5, 10 and 20 ml syringes (Scotlab)
0.2 and 0.45 μm sterile filters (Acrodisc, Gelman Sciences, supplied by Scotlab)
250 and 500 ml bottle filter systems with 0.2 or 0.45 μm cellulose acetate filter (Costar, Nalgene)
Cell scraper (Costar)
Sterile Petri dish, non-tissue culture grade (Scotlab)
Sterile Petri dish, tissue culture grade (Nunclon, Nunc)
Sterile plastic bulb pastettes (Scotlab)
Cryotubes (Nunc)
30 ml Corex glass centrifuge tubes (Sarstedt)

A4.2 Media for handling and culturing cells

A4.2.1 Standard tissue culture medium

A4.2.1.1 RPMI 1640 + 10% hi-FBS + 2ME

500 ml RPMI 1640 (Life Technologies)
50 ml heat-inactivated FBS (hi-FBS) (final concentration: nominal 10%; actual 8.7% FBS)
5 ml 200 mM (29.2 g/L) L-glutamine (final concentration: nominal 600 mg/L, 4 mM; actual 296 mg/L, 2 mM)
20 ml 1 x 10^4 U/ml penicillin (final concentration: nominal 400 U/ml; actual 348 U/ml) and 1 x 10^4 μg/ml streptomycin (final concentration: nominal 400 mg/ml; actual 348 μg/ml)
500 μL 5 x 10^{-2} M 2 (β) mercaptoethanol (2ME) (final concentration: nominal: 5 x 10^{-5} M; actual 4 x 10^{-5} M)
Stored at 4°C

Appendix 4: (continued).

A4.2.1.2 RPMI 1640 + 20% hi-FBS + 2ME

500 ml RPMI 1640
100 ml hi-FBS (final concentration: nominal 20%; actual 16% FBS)
5 ml 200 mM L-glutamine (final concentration: nominal 4 mM; actual 3.2 mM)
20 ml 1 x 10^4 U/ml penicillin (final concentration: nominal 400 U/ml; actual 320 U/ml) and 1x 10^4 µg/ml streptomycin (final concentration: nominal 400 mg/ml; actual 320 µg/ml)
500 µL 5 x 10^{-2} M 2 2ME (final concentration: nominal: 5 x 10^{-5} M; actual 4 x 10^{-5} M)
Stored at 4°C

A4.2.1.3 Dulbecco's Modified Eagle's Medium (DMEM) + 10% hi-FBS + 2ME

Dulbecco's Modified Eagle Medium with sodium pyruvate and 1 x 10^3 mg/L glucose (Life Technologies)
50 ml charcoal-dextran-stripped (cds)-hi-FBS (final concentration: nominal 10%; actual 8.7% FBS)
5 ml 200 mM L-glutamine (final concentration: nominal 4 mM; actual 2 mM)
20 ml 1 x 10^4 U/ml penicillin (final concentration: nominal 400 U/ml; actual 348 U/ml) and 1 x 10^4 µg/ml streptomycin (final concentration: nominal 400 mg/ml; actual 348 µg/ml)
500 µL 5 x 10^{-2} M 2ME (final concentration: nominal: 5 x 10^{-5} M; actual 4 x 10^{-5} M)
Stored at 4°C

A4.2.2 Transport medium

500 ml RPMI 1640
100 ml hi-FBS (final concentration: nominal 20%; actual 15% FBS)
5 ml 200 mM L-glutamine (final concentration: nominal 4 mM; actual 1.5 mM)
20 ml 1 x 10^4 U/ml penicillin (final concentration: nominal 400 U/ml; actual 300 U/ml) and 1 x 10^4 µg/ml streptomycin (final concentration: nominal 400 mg/ml; actual 300 µg/ml)
5 ml 250 µg/ml amphotericin B (final concentration: 2 µg/ml)
7.5 ml 10 mg/ml gentamicin (final concentration: 100 µg/ml)
12.5 ml 1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer (final concentration: 20 mM)
500 µL 5 x 10^{-2} M 2ME (final concentration: nominal: 5 x 10^{-5} M; actual 4 x 10^{-5} M)
300 µl 1 x 10^3 U/ml heparin (final concentration: 0.45 U/ml)
Stored at 4°C

A4.2.3 Additives for tissue culture medium

Heat-inactivated foetal bovine serum (hi-FBS) (Foetal calf serum, Life Technologies)
Heat-inactivated at 56°C for 30 min
Stored at -20°C

Appendix 4: (continued).

200 mM L-glutamine 200 (Life Technologies)
292 mg in 10 ml ultrapure water
Stored at -20°C

1 x 10^4 U/ml penicillin (Life Technologies)
Stored at -20°C

1 x 10^4 µg/ml streptomycin (Life Technologies)
Stored at -20°C

10 mg/ml gentamicin (Life Technologies)
Stored at 4°C

250 µg/ml amphotericin B (Fungizone, Life Technologies)
2.5 g in 50 ml deionised water
Filter sterilised
Stored at -20°C

50 mM 2-(β)-mercaptoethanol (2-ME) (Sigma)
39 mg in 10 ml HBSS
Stored at room temperature

1 x 10^3 U/ml preservative-free heparin (Leo Labs)
Stored at 4°C

1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer (Life Technologies)
Stored at room temperature

100 mg/ml α methylmannoside (methyl α-D-mannopyranoside) (Sigma)
1 g in 10 ml RPMI 1640 + 20% hi-FBS + 2ME
Filter sterilised
Stored at -20°C

Dimethyl sulphoxide (DMSO) (Sigma)
Stored at 4°C

0.2% w/v trypan blue (Sigma)
20 mg in 10 ml PBS 1x with 3 mM NaN_3
Stored at room temperature

Appendix 4: (continued).

Tissue culture grade distilled water (Life Technologies)
Stored at room temperature

A4.2.4 Solutions for suspending viable cells

Phosphate-buffered saline (PBS) 1x (Dulbecco's Phosphate-Buffered Saline without Mg^{2+} or Ca^{2+}, Life Technologies)
Stored at room temperature

Phosphate-buffered saline (PBS) 10x (Dulbecco's Phosphate-Buffered Saline pH 7.4 without Mg^{2+} or Ca^{2+}, Life Technologies)
Stored at room temperature

Hanks' balanced saline solution (HBSS) + 2% hi-FBS
500 ml Hanks' balanced saline solution (Life Technologies)
10 ml hi-FBS (final concentration: 2%)
Stored at 4°C

Tris-NaCl-EDTA (TNE) 1x
0.1 M NaCl
10 mM Tris.Cl
1 mM ethylenediaminetetraacetic acid (EDTA)
Adjusted to pH 8.0
Stored at room temperature

A4.2.5 Solutions for dissociating cells and tissues

Trypsin-EDTA
0.25% crude trypsin (Sigma)
1 M EDTA
Stored at –20°C

100 U/ml dispase II (Boehringer Manheim)
0.5 g (250 U) in 10 ml PBS 1x without Ca^{2+} or Mg^{2+}
Filter sterilised
Stored at -20°C

A4.2.6 Mitogens and cytokines

0.5 mg/ml phytohaemagglutinin-P (PHA-P, Sigma)
Filter sterilised
Stored at -20°C

Appendix 4: (continued).

0.5 mg/ml concanavalin A (ConA, Sigma or Boehringer Mannheim)
5 mg in 10 ml RPMI 1640 + 20% hi-FBS + 2ME
Filter sterilised
Stored at -20°C

5 U/μl interleukin 2 (IL2, Interleukin-2, human recombinant, Amersham)
50 μg (1.5×10^5 U) dissolved in 30 ml RPMI 1640 + 20% hi-FBS + 2ME
Stored at -20°C

5 μg/ml interleukin 4 (IL4, hIL-4, recombinant interleukin 4, human sequence, R & D Systems, British Biotechnology)
5 μg (2×10^3 to 5×10^3 U) dissolved in 1 ml PBS 1x containing 0.1% BSA
Stored at -20°C

1 μg/ml interleukin 7 (IL7) (hIL-7, recombinant interleukin 7, human sequence, R & D Systems, British Biotechnology)
5 μg (2×10^3 to 5×10^3 U) dissolved in 5 ml PBS 1x containing 0.1% BSA
Stored at -20°C

1×10^4 U/ml granulocyte-macrophage colony stimulating factor (GMCSF, GM-CSF, human recombinant, Boehringer Mannheim) in PBS 1x containing 0.1% BSA
Stored at -20°C

A4.3 Reagents for immunophenotyping cultured lymphocytes by flow cytometry

A4.3.1 Stock solutions

10% sodium azide (Sigma)
10 g in 100 ml sterile ultrapure water
Filter sterilised
Stored at 4°C

PAB
100 ml PBS 10x (final concentration: PBS 1x)
10 ml 10% sodium azide (final concentration: 0.1%)
2 g bovine serum albumin (final concentration: 2% w/v)
Made up to 1 L with deionised water
Filter sterilised
Stored at 4°C

Appendix 4: (continued).

20% rabbit serum in PAB
100 µl rabbit serum (Dako). Heat inactivated at 56° for 30 min. Filter sterilised. Stored at -20°C
400 µl PAB

1% paraformaldehyde in PBS 1x
10 g paraformaldehyde (BDH) in 450 ml deionised water (heated to 70°C)
Adjusted to pH 7.4 with 1 M NaOH
50 ml PBS 10x
Filter sterilised

20% paraformaldehyde in PBS 1x
10 g paraformaldehyde (BDH) in 20 ml deionised water (heated to 70°C)
Adjusted to pH 7.4 with 1 M NaOH
5 ml PBS 10x
Filter sterilised

A4.3.2 Monoclonal antibodies

Mouse anti-*Aspergillus niger* glucose oxidase IgG1-FITC κ chain (X927, Dako)
100 µg/ml in 0.05 M Tris.HCl pH7.2, 15 mM NaN_3 and 1% BSA (negative control)

Mouse anti-*A niger* glucose oxidase IgG1-FITC + IgG1-RPE κ chain (X932, Dako) in 0.05 M Tris.HCl pH7.2, 15 mM NaN_3 and 1% BSA (negative control)

Mouse anti-CD2-FITC + anti-CD19-RPE IgG1 κ chain (FR894, Dako) in 0.05 M Tris.HCl pH7.2, 15 mM NaN_3 and 1% BSA

Mouse anti-CD8-FITC + anti-CD4-RPE IgG1 κ chain (FR885, Dako) in 0.05 M Tris.HCl pH7.2, 15 mM NaN_3 and 1% BSA

Mouse anti-CD15-FITC IgM κ chain (F830, Dako)
75 µg/ml in 0.05 M Tris.HCl pH7.2, 15 mM NaN_3 and 1% BSA

Mouse anti-CD30-FITC IgG1 κ chain (F849, Dako)
100 µg/ml in 0.05 M Tris.HCl pH7.2, 15 mM NaN_3 and 1% BSA

Mouse anti-CD33-FITC IgG1 κ chain (F832, Dako)
100 µg/ml in 0.05 M Tris.HCl pH7.2, 15 mM NaN_3 and 1% BSA

Mouse anti-CD45 (LCA)-FITC IgG1 κ chain (F796, Dako)
100 µg/ml in 0.05 M Tris.HCl pH7.2, 15 mM NaN_3 and 1% BSA

Appendix 4: (continued).

A4.4 Reagents for conventional reverse transcriptase assay

A4.4.1 Stock solutions

0.1 M Dithiothreitol (DTT) (Sigma)
1.54 g in 100 ml ultrapure water
Stored at room temperature

2% Nonidet P-40 (NP-40) (Sigma)
2 ml plus 98 ml ultrapure water
Stored at room temperature

0.8 M Tris pH 8.1 (Trizma-8.1, Sigma)
5.59 g in 50 ml ultrapure water
Stored at room temperature

3 M NaCl (Sigma)
17.53 g in 100 ml ultrapure water
Stored at room temperature

Tris pH 8.1, 0.15 M NaCl
2.80 g Trizma-8.1
1.25 ml 3 M NaCl
Made up to 25 ml with ultrapure water
Stored at room temperature

1 M KCl (Sigma)
7.46 g in 100 ml ultrapure water
Stored at room temperature

20 mM $MnCl_2$ (Sigma)
396 mg in 100 ml ultrapure water
Stored at room temperature

0.1 M $MgCl_2$ (Sigma)
952 mg in 100 ml ultrapure water
Stored at room temperature

100% trichloroacetic acid (TCA) (BDH)
500 g in 500 ml ultrapure water
Stored at room temperature

Appendix 4: (continued).

10% TCA, 1% w/v sodium pyrophosphate ($NaPP_i$)
50 ml 100% TCA
5 g $NaPP_i$ (Fisons)
Made up to 500 ml with ultrapure water
Stored at room temperature

5% TCA, 1% w/v tetrasodium pyrophosphate ($Na_2P_20_7$, $NaPP_i$)
25 ml 100% TCA
5 g $NaPP_i$ (Fisons)
Made up to 500 ml with ultrapure water
Stored at room temperature

5% TCA
50 ml 100% TCA
Made up to 1 L with ultrapure water on day of use
Cooled to 4°C before use

5 U/ml Poly(rA).p$(dT)_{12-18}$ (Pharmacia)
5 U precipitate (15.7 A_{260} units/mg) in 1 ml ultrapure water
Stored at -20°C

5 U/ml Poly(dA).p$(dT)_{12-18}$ (Pharmacia)
5 U precipitate (16.0 A_{260} units/mg) in 1 ml ultrapure water
Stored at -20°C

Ethanol 98% (Absolute alcohol, BDH)

A4.4.2 Virus disruption buffer

20 ml 0.1 M DTT
5 ml 2% NP-40
5 ml 0.8 M Tris pH 8.1
5 ml 1 M KCl
Made up to 95 ml with ultrapure water
Stored at -20°C

A4.4.3 Tritiated thymidine triphosphate

Tritiated thymidine triphosphate (^{3}H-TTP) [methyl-^{3}H] thymidine 5'-triphosphate, ammonium salt (Amersham) 37 MBq/ml (1 mC_i/ml) dissolved in 50% ethanol and 50% water
Stored at 4°C

Appendix 4: (continued).

A4.5 Reagents for eucaryotic expression

100 mg puromycin (P-7255, Sigma)

5 g geneticin (G-418 sulphate, Life Technologies)

1 mM 4-hydroxytamoxifen (Tamoxifen, T-5648, Sigma)
100 mg dissolved in 27 ml absolute alcohol
Stored at -20°C

10 μM hydroxytamoxifen
50 μl 1 mM hydroxytamoxifen
450 μl absolute alcohol

A4.6 Reagents for propidium iodide staining

Acetic acid-alcohol fixative
5 ml glacial acetic acid
95 ml ethanol
Stored at room temperature

4 M HCl
34.5 ml concentrated HCl (specific gravity 1.18)
Made up to 100 ml with ultrapure water

0.1 M sodium borate
3.8 g sodium borate
Made up to 100 ml with ultrapure water

500 μg/ml propidium iodide
5 mg propidium iodide (Propidium I, Sigma)
Made up to 10 ml with PBS 1x

50 mg/ml RNase A (Sigma) in PBS 1x

Propidium iodide/RNase A staining solution
50 μl 500 μg/ml propidium iodide (final concentration: 5 μg/ml)
10 μl 50 mg/ml RNase A (final concentration: 100 μg/ml)
Made up to 5 ml with PBS 1x

Appendix 4: (continued).

A4.7 Reagents for mycoplasma testing

Hoescht 33258 [2(4-hydroxyphenyl)-5[5-(4-methylpiperazine-1-yl)-benzimidazol-2-yl]benzimidazole](bisbenzimide) (Sigma)
1 mg/ml in HBSS without phenol red or bicarbonate

Methanol:glacial acetic acid (3:1)

Appendix 5: List of commonly used reagents, stock solutions and buffers for molecular biology.

A5.1 Disposable plasticware

0.1-2 µl, 0.5-10 µl, 2-200 µl and 50-1,000 µl micropipettes (Gilson, Sealpette)
2-25 µl and 10-50 µl positive (direct) displacement pipettes (Gilson)
0.1-2 µl, 0.5-10 µl, 2-200 µl and 50-1,000 µl micropipette tips (Greiner)
0.1-2 µl, 0.5-10 µl, 2-200 µl and 50-1,000 µl filter-plugged micropipette tips (Greiner)
2-25 µl and 10-50 µl positive (direct) displacement tips (Gilson)
5-50 µl fine gel-loading pipette tips (Greiner)
1, 5, 10 and 25 ml disposable filter-plugged polycarbonate pipettes (Costar)
5, 15 and 50 ml screw-top polypropylene centrifuge tubes (Falcon and Greiner)
0.6 and 1.5 ml flip-top and screw-top polypropylene microcentrifuge tubes (Elkay and Treff)
20 ml screw-top polycarbonate Universal tube (Scotlab)
5 ml screw-top polycarbonate bijou (Scotlab)
1, 2.5, 5, 10 and 20 ml syringes (Scotlab)
Narrow-bore polypropylene bulb pastette (Alpha Labs)
Wide-bore polypropylene bulb pastette (Alpha Labs)
14 ml (17 mm x 100 mm) polypropylene 2059 tubes (Falcon)
Petri dishes (Fisons)

A5.2 General laboratory solutions and buffers

Tris-EDTA (TE) 1x
10 mM Tris.HCl pH 8.0
1 mM EDTA

Tris-boric acid-EDTA (TBE) 1x
90 mM Tris.Cl
90 mM boric acid
2.25 mM EDTA
Adjusted to pH 8.0

Tris-acetic acid-EDTA (TAE) 1x
40 mM Tris.Cl pH 8.0
20 mM sodium acetate
20 mM sodium chloride
2 mM EDTA
Adjusted to pH 8.0

Appendix 5: (continued).

Tris-NaCl-EDTA (TNE) 1x
10 mM Tris.Cl
100 MM NaCl
1 mM EDTA
Adjusted to pH 8.0

20% sodium dodecyl sulphate (SDS)

A5.3 Reagents for restriction endonuclease digestion of DNA

A5.3.1 Restriction endonucleases

Restriction endonucleases (Life Technologies). 10 or 40 U/µl
*Bam*HI
*Eco*RI
*Eco*RV
*Hin*dIII
*Pst*I
*Sst*I
*Xba*I
*Xho*I

A5.3.2 Additives for restriction endonuclease digests

100 mM (5 mg/ml) Sonicated salmon sperm DNA, GeneBloc, International Laboratory Services)
Boiled for 5 min then placed on ice before adding to RE digests

Restriction buffers 10 x (Life Technologies)

50 mg/ml RNase A (Sigma) in PBS 1x

A5.4 Reagents for the polymerase chain reaction

10 x dNTPs
10 mM each deoxynucleoside triphosphates (dATP, dGTP, dCTP, dUTP or dTTP) (Cambio)

10 x PCR buffer (Perkin-Elmer)
15 mM $MgCl_2$
500 mM KCl
100 mM Tris pH 8.2
0.5% Nonidet P-40 (NP-40)

Appendix 5: (continued).

Taq polymerase
5 U/µl *Thermus aquaticus* (*Taq*) thermostable DNA polymerase (Amplitaq DNA polymerase, Perkin-Elmer)

25 mM $MgCl_2$ (Perkin-Elmer)

A5.5 Reagents for processing cells and tissues for the polymerase chain reaction

Non-ionic detergent buffer (NIB)
PCR buffer 1x (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris pH 8.2, 100 µg/ml gelatin) (Perkin-Elmer)
0.45% Nonidet P-40 (NP-40) (Sigma)
0.45% Tween-20 (polyoxyethylene (20)-sorbitan monolaurate, BDH)

1 µg/µl proteinase K (Sigma)
10 mg in 10 ml ultrapure water
Stored at -20°C

10 µg/µl proteinase K (Sigma)
10 mg in 1 ml ultrapure water
Stored at -20°C

A5.6 Reagents for gel electrophoresis

A5.6.1 DNA Size Markers

100 ng/µl PhiX174 bacteriophage DNA digested with *Hae*III (ΦX174 RF DNA/*Hae*III Frag, Life Technologies) (1353, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp)

Lambda bacteriophage DNA digested with *Hind* III (λ*Hind*III, Life Technologies) (23100, 9400, 6600, 4400, 2300, 2000, 560 bp)

A5.6.2 Loading buffer/dye and ethidium bromide

Gel loading buffer/dye 5x
0.42% bromophenol blue
0.42% xylene cyanole
50% glycerol
Made up to 100% with deionised water

10 mg/ml ethidium bromide (Sigma)

Appendix 5: (continued).

A5.6.1 Polyacrylamide gel electrophoresis

8% polyacrylamide gels
26 ml 30% acrylamide (Northumbria Biologicals)
13 ml 2% bis-acrylamide (Northumbria Biologicals)
10 ml TBE 10x
2.2 ml 3% ammonium persulphate (APS)
30 µL N,N,N',N'-tetramethylethylenediamine (TEMED)
Made up to 100 ml with water

Elution buffer
0.5 M ammonium acetate
1 mM EDTA

A5.6.2 DNA agarose gel electrophoresis

Agarose (Sigma)

Low melting point agarose (Nusieve)

Diethylaminoethyl (DEAE) membrane (Schleicher & Schuell)

1 U/ml Gelase (Cambio)

5 M ammonium acetate

A5.7 Solutions for electroblotting and Southern blotting

Alkali (denaturing) buffer
438.3 g NaCl (final concentration: 1.5 M)
100 g NaOH (final concentration: 0.5 M)
Made up to 2 L with deionised water

Neutralising buffer pH 8.0
303.5 g Tris base
876.6 g NaCl
165 ml HCl
Made up to 5 L with deionised water
Adjusted to pH 8.0

Appendix 5: (continued).

Sodium saline citrate (SSC) 20x
3 M NaCl
0.3 M trisodium citrate
Adjusted to pH 7.0 with 10 M or 0.1 M NaOH

Transfer buffer SSC 10x
500 ml SSC 10x
Made up to 1 L with water

SSC 24x
1 kg NaCl (final concentration: 3.6 M)
500 g sodium citrate (final concentration: 0.36 M)
Made up to 4.7 L with water

Transfer buffer SSC 10x (9.6x)
400 ml SSC 24x
Made up to 1 L with water

Rinse buffer SSC 3x
250 ml SSC 24x
Made up to 1 L with water

A5.8 Reagents for radiolabelling probes

A5.8.1 Random priming

Multiprime Kit (RPN.1601Y, Amersham)
Multiprime buffer solution/nucleotide mix. dATP, dGTP and dTTP in concentrated buffer solution containing Tris.HCl pH 7.8, $MgCl_2$ and 2-mercaptoethanol (2ME)
Primer solution/primer-BSA containing random hexanucleotides and bovine serum albumin (BSA)
Enzyme solution/Klenow 1 U/µl containing 1 U/µl DNA polymerase I "Klenow" fragment in 50 mM potassium phosphate pH 6.5, 10 mM 2ME and 50% glycerol

α ^{32}P dCTP (R82075, Amersham)
370 MBq/ml (10 mCi/ml) α ^{32}P dCTP. Specific activity 30 TBq/mmol (800 Ci/mmol)

Appendix 5: (continued).

A5.8.2 5' end-labelling

T4 kinase buffer 10x
500 mM Tris.HCl pH 7.5
100 mM $MgCl_2$
50 mM dithiothreitol (DTT)
1 mM spermidine
1 mM EDTA pH 8.0

10 U/µl T4 polynucleotide kinase

γ ^{32}P ATP (R85132, ICN)
370 MBq/ml (10 mCi/ml) γ ^{32}P ATP. Specific activity 111 MBq/mmol (3000 Ci/mmol)

A5.9 Reagents for hybridisation

A5.9.1 Hybridisation solution

Standard hybridisation solution (50% formamide, SSC 3x)
500 ml 100% formamide (final concentratiobn: 50%)
50 ml Denhardt's solution 100x (final concentration: 5x)
5 ml 20% SDS (final concentration: 0.1%)
50 ml 1 M Tris pH 7.4 (final concentration: 50 mM)
20 ml 0.5 M EDTA (final concentration: 10 mM)
125 ml SSC 24x (final concentration: SSC 3x)
250 mg Sonicated salmon sperm DNA (GeneBloc, International Laboratory Services). Boiled for 5 min then placed on ice
Made up to 1 L with deionised water

Denhardt's solution 100x
2% Ficoll
2% bovine serum albumin (Fraction V)
2% polyvinylpyrolidine

A5.9.2 Wash solutions

SSC 3x, 0.1% SDS
30 ml SSC 24x
1.2 ml 20% SDS
Made up to 240 ml with water

Appendix 5: (continued).

SSC 2x, 0.1% SDS
100 ml SSC 20x
5 ml 20% SDS
Made up to 1 L with water

SSC 0.5x, 0.1% SDS
25 ml SSC 20x
5 ml 20% SDS
Made up to 1 L with water

A5.10 Media and reagents for cultivation of bacteria

A5.10.1 Media

Luria-Bertani (LB) broth
10 g tryptone (Unipath)
5 g yeast extract (Unipath)
10 g NaCl (BDH)
Made up to 1 L with water
Sterilised by autoclaving

LB agar
10 g tryptone (Unipath)
5 g yeast extract (Unipath)
10 g NaCl (BDH)
15 g bacteriological agar (Number 1, Unipath)
Made up to 1 L with water
Sterilised by autoclaving

SOC medium
20 g tryptone
5 g yeast extract
500 mg NaCl
200 mg KCl
10 ml 1 M $MgCl_2$
10 ml 1 M $MgSO_4$
1 ml 2 M glucose
Made up to 1 L with water
Sterilised by autoclaving

Appendix 5: (continued).

A5.10.2 Antibiotics

50 mg/ml ampicillin

12 mg/ml tetracycline

100 mg/ml chloramphenicol

20 mg/ml kanamycin

A5.10.3 Additives for blue-white screening

0.2 M isopropyl-1-thio-β-D-galactoside (IPTG)

40 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)
100 mg dissolved in 2.5 ml dimethyl formamide
Protected from light
Stored at -20°C

A5.10.4 Additives for bacterial transformation

500 mM 2-(β)-mercaptoethanol (2-ME) (Sigma)
5 µl 1.12 g/ml (14.3 M) 2-ME stock solution
138 µl sterile ultrapure water

A5.11 Reagents for extraction of plasmids from bacteria

A5.11.1 Modified alkaline lysis

Solution I
50 mM glucose
25 mM Tris.Cl pH 8.0
10 mM EDTA pH 8.0

Solution II
0.2 M NaOH
1% SDS

Solution III
3 M potassium acetate

Appendix 5: (continued).

3 M potassium acetate pH 4.8
60 ml 5 M potassium acetate
11.5 ml glacial acetic acid
Make up to 100 ml with water

A5.11.2 Boiling method

Sucrose-Triton X-100-EDTA-Tris (STET)
8% sucrose
0.5% Triton X-100
50 mM EDTA pH 8.0
10 mM Tris pH 8.0

A5.12 Reagents for sequencing

A5.12.1 Sequencing primers

M13 5′ > GTAAAACGACGGCCAGT < 3′

T3 5′ > AATTAACCCTCACTAAAGGG < 3′

T7 5′ > GTAATACGACTCACTATAGGGC < 3′

SP6 5′ > ATTTAGGTGACACTATAGAATAT < 3′

A5.12.2 ^{T7}Sequencing Kit (Pharmacia)

T7 DNA polymerase 8 U/μl in buffered glycerol

Sequencing mixes ('A', 'C', 'G' or 'T' mix-short) in 40 mM Tris.HCl pH 7.6 and 50 mM NaCl
'A' mix-short: 840 μM each dCTP, dGTP and dTTP, 93.5 μM dATP, 14 μM ddATP
'C' mix-short: 840 μM each dATP, dGTP and dTTP, 93.5 μM dCTP, 17 μM ddCTP
'G' mix-short: 840 μM each dATP, dCTP and dTTP, 93.5 μM dGTP, 14 μM ddGTP
'T' mix-short: 840 μM each dCTP, dGTP and dGTP, 93.5 μM dTTP, 14 μM ddTTP

Universal primer
5 μM M13

Annealing buffer
1 M Tris.HCl pH7.6
100 mM $MgCl_2$
160 mM DTT

Appendix 5: (continued).

Enzyme dilution buffer
20 mM Tris.HCl pH 7.5
5 mM DTT
100 μg/ml bovine serum albumin (BSA)
5% glycerol

Labelling mix-dATP
1.375 μM each dCTP, dGTP and dTTP
333.5 mM NaCl

Stop solution
97.5% formamide
10 mM EDTA pH 7.5
0.3% bromophenol blue
0.3% xylene cyanole

A5.12.3 ^{35}S-labelled dATP radioisotope

α ^{35}S dATP (SJ1304, Amersham)
9.25 MBq/μl (250 μCi/μl) α ^{35}S dATP. Specific activity 37 TBq/mmol (1000 Ci/mmol)

A5.12.4 Polyacrylamide sequencing gel

5% sequencing gel mix (non-setting)
10 ml Long Ranger DNA sequencing gel solution Hydro-Link (AT Biochem) 50% concentrate
6 ml TBE 10x
42 g urea
Make up to 100 ml with deionised water

5% polyacrylamide gel mix (setting)
100 ml 5% sequencing gel mix
500 μl 10% APS
50 μl TEMED

Appendix 5: (continued).

A5.13 Northern blot hybridisation

A5.13.1 Reagents for RNA extraction

0.05% diethyl pyrocarbonate (DEPC) in ultrapure water

EDTA-SDS-DEPC
1 mM EDTA pH 7.0
0.5% SDS
Made with 0.05% DEPC in ultrapure water

A5.13.2 Reagents for RNA gel electrophoresis

3-(N-morpholino)-2-hydroxypropane sulphonic acid (MOPS) buffer 10x
41.8 g MOPS
6.8 g sodium acetate (final concentration: 0.05 M)
3.7 g EDTA (final concentration: 5 mM)
Made up to 1 L with water
Adjusted to pH 7.0 with 10 M NaOH

1% agarose gel with MOPS 1x and 6% formaldehyde
2 g agarose dissolved in 147 ml water by boiling
20 ml MOPS 10x
33 ml 37% formaldehyde (Analar). Warmed to 37°C

Formamide buffer
500 µl MOPS 10x
830 µl 37% formaldehyde
2.5 ml formamide

RNA loading buffer/dye
50% glycerol
1 mM EDTA
0.4% bromophenol blue

Appendix 6: Complete nucleotide sequence of human T lymphotropic virus type I proviral DNA.

GenBank Accession number J02029 (9068 bp) (Seiki *et al* 1983). Oligonucleotide primer sequences for PCR are underlined (overlined, shaded or emboldened where overlapping) and annotated at right.

```
   1 ggaaaaactt ggagtgtagt tctgacaatg accatgagcc ccaaatatcc ccccgggggct
  61 tagagcctcc cagtgaaaaa catttccgag aaacagaagt ctgaaaaggt cagggcccag
 121 actaaggctc tgacgtctcc ccccggaggg cagctcagca ccggctcggg ctaggccctg
 181 acgtgtcccc ctgaagacaa atcataagct cagacctccg ggaagccacc aagaaccacc
 241 catttcctcc ccatgtttgt caagccgtcc tcaggcgttg acgacaaccc ctcacctcaa
 301 aaaactttte atggcacgca tatggctcaa taaactagca ggagtctata aaagcgtgga
 361 gacagttcag gagggggctc gcatctctcc ttcacgcgcc cgccgcccta cctgaggccg
 421 ccatccacgc cggttgagtc gcgttctgcc gcctcccgcc tgtgggtgcct cctgaactgc
 481 gtccgccgtc taggtaagtt taaagctcag gtcgagaccg ggcctttgtc cggcgctccc
 541 ttggagccta cctagactca gccggctctc cacgctttgc ctgaccctgc ttgctcaact 118
 601 ctacgtcttt gtttcgtttt ctgttctgcg ccgttacaga tcgaaagttc cacccctttc
 661 cctttcaatc acgactgact gccggcttgg cccacggcca agtaccggcg actccgttgg
 721 ctcggagcca gcgacagccc atcctatagc actctcagga gagaaattta gtacacagtt 119, 174
 781 gggggctcgt ccgggatacg agcgcccctt tattccctag gcaatgggcc aaatcttttc
 841 ccgtagcgct agccctattc cgcgaccgcc ccgggggctg gccgctcatc actggcttaa
 901 cttcctccag gcggcatatc gcctagaacc cggtccctcc agttacgatt tccaccagtt
 961 aaaaaaattt cttaaaatag ctttagaaac accggctcgg atctgtccca ttaactactc
1021 cctcctagcc agcctactcc caaaaggata ccccggccgg gtgaatgaaa ttttacacat
1081 actcatccaa acccaagccc agatcccgtc ccgtcccgcg ccaccgccgc cgtcatcccc
1141 cacccacgac ccccccggatt ctgatccaca aatccccccct cctctatgttg agcctacggc
1201 ccccccaagtc cttccagtca tgcatccaca tggtgctcct cctaaccatc gcccatggca
1261 aatgaaagac ctacaggcca ttaagcaaga agtctcccaa gcagcccctg ggagcccccca 175
1321 gttttatgcag accatccggc ttgcggtgca gcagtttgac cccactgcca aagacctcca
1381 agacctcctg cagtaccttt gctcctccch cgtggcttcc ctccatcacc agcagctaga 120, 122
1441 tagccttata tcagaggccg aaacccgagg tattacaggt tataacccat tagccggtcc
1501 cctccgtgtc caagccaaca atccacaaca acaaggatta aggcgagaat accagcaact 123
1561 ctggctcgcc gccttcgccg ccctgccggg gagtgccaaa gacccttcct gggcctctat
1621 cctccaaggc ctggaggagc cttaccacgc cttcgtagaa cgcctcaaca tagctcttga 121
1681 caatgggctg ccagaaggca cgcccaaaga ccccatctta cgttccttag cctactccaa
1741 tgcaaacaaa gaatgccaaa aattactaca ggcccgagga cacactaata gccctctagg
1801 agatatgttg cgggcttgtc agaccctgac ccccaaagac aaaaccaaag tgttagttgt
1861 ccagcctaaa aaaccccccc caaatcagcc gtgcttccgg tgcgggaaag caggccactg
1921 gagtcgggac tgcactcagc ctcgtccccc ccccgggcca tgccccctat gtcaagaccc
1981 aactcactgg aagcgagact gcccccgcct aaagcccact atcccagaac cagagccaga
2041 ggaagatgcc ctcctattag acctcccccgc tgacatccca cacccaaaaa acttcatagg
2101 gggggaggtt taacctcccc ccccacatta cagcaagtcc ttcctaacca agacccagca
2161 tctattctgc cagttatacc gttagatccc gcccgtcggc ccgtaattaa agcccaggtt
2221 gacacccaga ccagccaccc aaagactatc gaagctttac tagatacagg agcagacatg
2281 acagtcottc cgatagcctt gttctcaagt aatactcccc tcaaaaatac atccgtatta
2341 ggggcagggg gccaaaccca agatcactit aagctcacct ccctcctgt gctaatacgc
2401 ctcccctttcc ggacaacgcc tattgtttta acatcttgcc tagttgatac caaaaacaac
2461 taggccatca taggtcgtga tgccttacaa caatgccaag gcgtcctgta cctcctgag
2521 gcaaaaaggc cgcctgtaat cttgccaata caggcgccag ccgtccttgg gctagaacac
2581 ctcccaaggc ccccgaaat cagccagttc cctttaaacc agaacgcctc caggccttgc 176
2641 aacacttggt ccggaaggcc ctggaggcag gccatatcga accctacacc gggccaggga
2701 ataacccagt attcccagtt aaaaaggcca atggaacctg gcgattcatc cacgacctgc
2761 gggccactaa ctctctaacc atagatctct catcatcttc cccgggcccc cctgacttgt
2821 ccagcctgcc aaccacacta gcccacttgc aaactataga ccttagagac gccttttcc
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Appendix 6: (continued).

```
2881 aaatcccctt acctaaacag ttccagccct actttgcttt cactgtccca cagcagtgta
2941 actacggccc cggcactaga tacgcctgga aagtactacc ccaagggttt aaaaatagtc 296
3001 ccaccctgtt cgaaatgcag ctggcccata tcctgcagcc cattcggcaa gctttccccc
3061 aatgcactat tcttcagtac atggatgaca ttctcctagc aagcccctcc catgaggacc 297
3121 tactactact ctcagaggcc acaatggctt ccctaatctc ccatgggttg cctgtgtccg
3181 aaaacaaaac ccagcaaacc cctggaacaa ttaagttcct agggcagata atttcaccca
3241 atcacctcac ttatgatgca gtccccacgg tacctatacg gtcccgctgg gcgctacctg
3301 aacttcaagc cctacttggc gagattcagt gggtctccaa aggaactcct accttacgcc
3361 agccccttca cagtctctac tgtgccttac aaaggcatac tgatccccga gaccaaatat 1
3421 atttaaatcc ttctcaagtt caatcattag tgcagctgcg gcaggccctg tcacagaact 2
3481 gccgcagtag actagtccaa accctgcccc tcctaggggc tattatgctg accctcactg
3541 gcaccactac tgtagtgttc cagtccaagg agcagtggcc acttgtctgg ctacatgccc
3601 ccctacccca cactagccag tgccccctggg ggcagctact tgcctcagct gtgttattac
3661 tcgacaaata caccttgcaa tcctatgggc tgctctgcca aaccatacat cataacatct
3721 ccacccaaac cttcaaccaa ttcattcaaa catctgacca ccccagtgtt cctatcttac
3781 tccaccacag tcaccgattc aaaaatttag gtgcccaaac tggagaactt tggaacactt
3841 ttcttaaaac agctgcccca ttggctcctg tgaaagccct catgccagtg tttactcttt
3901 ccccggtgat tataaacacc gccccctgcc tgttttcaga cggatctacc tccccgggcag
3961 cctatattct ctgggacaag caaatattgt cacaaagatc attccccctt ccgccaccgc
4021 acaagtcggc ccaacgggcc gaacttctcg gacttttgca tggcctctcc agcgcccgtt
4081 cgtggcgctg tctcaacata tttctagact ccaagtatct ttatcattac cttcggaccc
4141 ttgccctggg caccttccaa ggcaggtcct ctcaggcccc ctttcaggcc cttctgcccc
4201 gcttactatc gcgtaaggtc gtctatttgc accacgttcg cagccatacc aatctacctg
4261 atcccatctc caggctcaac gctctcacag atgccctact aatcaccctt gtcctgcagc
4321 tctctcctgc agaactacac agtttcaccc attgcggaca gacggccctc acattgcaag
4381 gggcaaccac aactgaggct tccaatatcc tgcgctcttg ccacgcctgc cgcggaggca
4441 acccacaaca tcagatgcct cggggacaca tccgccgtgg cctacttcct aaccacatct
4501 ggcaaggcga cattacccat ttcaaatata aaaatacgct gtatcgcctt catgtatggg
4561 tagacacctt ttcaggagcc atctcagcta cccaaaagag aaaagaaaca agctcagaag
4621 ctatttcctc tttgcttcag gccattgccc atctaggcaa gcctagctac ataaacacag
4681 acaacggccc tgcctatatt tcccaagact tcctcaatat gtgtaccccc cttgctattc
4741 gccataccac ccatgtcccc tacaatccaa ccagctcagg actgtagaa cgctctaatg 67
4801 gcattcttaa aacctatta tataagtact ttactgacaa acccgacctа cccatggata
4861 atgctctatc catagcccta tggacaatca accacctgaa tgtgttaacc aactgccaca
4921 aaaccccatg gcagcttcac cactcccccc gactccagcc gatcccagag acacgttccc 68
4981 tcagcaataa acaaacccat tggtattatt tcaagcttcc tggtcttaat agccgccagt
5041 ggaaaggacc acaggaggct ctccaagaag ctgccggcgc tgctctcatc cuggtaagcg
5101 ctagttctgc ccagtggatc ccgtggagac tcctcaagcg agctgcatgc ccaagacccg
5161 tcggaggccc cgccgatccc aaagaaaaag acctccaaca ccatgggtaa gtttctgccc
5221 actttgattt tattcttcca gttctgcccc ctcatcttcg gtgattacag ccccagctgc
5281 tgtactctca caattggagt ctcctcatac cactctaaac cctgcaatcc tgcccagcca
5341 gtttgttcgt ggacctcga cctgctggcc ctttcagcag atcaggccct acagccccc
5401 tgccctaacc tagtaagtta ctccagctac catgccacct attcctata tctattccct
5461 cattggacta agaagccaaa ccgaaatggc ggaggctatt attcagcctc ttattcagac
5521 ccttgttcct taaagtgccc atacctgggg tgccaatcat ggacctgccc ctatacagga
5581 gccgtctcca gcccctactg gaagtttcaa cacgatgtca attttactca agaagtttca
5641 cgcctcaata ttaatctcca ttttttcaaaa tgcggttttc ccttctccct tctagtcgac
5701 gctccaggat atgaccccat ctggttcctt aataccgaac ccagccaact gcctcccacc
5761 gcccctcctc tactcccccа ctctaaccta gaccacatcc tcgagccctc tataccatgg 124
5821 aaatcaaaac tcctgaccct tgtccagtta acccтacaaa gcactaatta tacttgcatt
5881 gtctgtatcg atcgtgccag cctctccact tggcacgtcc tatactctcc caacgtctct
5941 gttccatcct cttcttctac ccccctcctt tacccatcgt tagcgcttcc agccccccac
6001 ctgacgttac catttaactg gacccactgc tttgaccccc agattcaagc tatagtctcc
6061 tccccctgtc ataactccct catcctgccc ccctttttcct tgtcacctgt tcccacccta 125
6121 ggatcccgct cccgccgagc ggtacccggtg gcggtctggc ttgtctccgc cctggccatg
6181 ggagccggag tggctggcgg gattacccgc tccatgtccc tcgcctcagg aaagagcctc
6241 ctacatgagg tggacaaaga tatttcccag ttaactcaag caatagtcaa aaaccacaaa
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Appendix 6: (continued).

```
6301 aatctactca aaattgcgca gtatgctgcc cagaacagac gaggccttga tctcctgttc
6361 tgggagcaag gaggattatg caaagcatta caagaacagt gccgttttcc gaatatttacc
6421 aattcccatg tcccaatact acaagaaaga ccccccottg agaatcgagt cctgactggc
6481 tggggcctta actgggacct tggcctctca cagtgggctc gagaggcctt acaaactgga
6541 atcacccttg ttgcgctact ccttcttgtt atccttgcag gaccatgcat cctccgtcag
6601 ctacgacacc tcccctcgcg cgtcagatac cccoattact ctcttataaa accigagtca
6661 tcoctgtaaa ccaagcacgc aattattgca accacatcgc ctccagcctc coctgccaat
6721 aatttaaccic tcccatcaaa tcctccttct cctgcagcaa cttcctccgt tcagcctcca
6781 aggactccac ctcgccttcc aactgtctag catagccatc aatcccccaac tcctgcattt
6841 tttctttcct agcactatgc tgtttcgcct tcccagcccc ttgtctccac ttgcgctcac
6901 ggcgctcctg ctcttcctgc ttcctcctag cgacgtcagc ggccttcttc tccgcccgcc
6961 tcctgcgccg tgccttctcc tcttccttcc ttttcaaata ctcagcggtc tgcttttcct
7021 cctcttttcc ccgctctttt tttcgcttcc tcttctcctc agcccgtcgc tgccgatcac
7081 gatgcgtttc cccgcgaggt ggcgctttct ccccctggagg gcccccgtcgc agcccggccgc
7141 ggctttcctc ttctaaggat agcaaaccgt caagcacagc ttcctcctcc tccttgtcct
7201 ttaactcttc ctccaaggat aatagcccgt ccaccaattc ctccaccagc aggtcctccg
7261 ggcatgacac aggcaagcat cgaaacagcc ctgcagatac aaagttaacc atgcttatta
7321 tcagcccact tcccagggtt tggacagagt cttcttttcg gataccagt ctacgtgttt 50, 282
7381 ggagactgtg tacaaggcga ctggtgcccc atctctgggg gactatgttc ggcccgccta
7441 catcgtcacg ccctactggc cacctgtcca gagcatcaga tcacctggga ccccatcgat 3, 51, 283
7501 ggacgcgtta tcggctcagc tctacagttc cttatccctc gactcccctc cttccccacc 190
7561 cagagaacct ctaagaccct caaggtcctt accccgccaa tcactcatac aacccccaac 4, 126
7621 attccaccct ccttcctcca ggccatgcgc aaatactccc ccttccgaaa tggatacatg
7681 gaacccaccc ttgggcagca cctcccaacc ctgtcttttc cagaccccgg actccggccc 127
7741 caaaacctgt acacccctcg gggaggctcc gttgtctgca tgtacctcta ccagcttttcc
7801 cccccccatca cctggcccct cctgccccac gtgatttttt gccaccccgg ccagctcggg 191
7861 gccttcctca ccaatgttcc ctacaagcga atagaagaac tcctctataa aatttcccctc
7921 accacagggg ccctaataat tctacccgaa gactgtttgc ccaccaccct tttccagcct
7981 gctagggcac ccgtcacgct aacagcctgg caaaacggcc tccttccgtt ccactcaacc
8041 ctcaccactc caggccttat ttggacattt accgatggca cgcctatgat ttccgggccc
8101 tgccctaaag atggccagcc atctttagta ctacagtcct cctcctttat atttcacaaa
8161 tttcaaacca aggcctacca cccctcattt ctactctcac acggcctcat acagtactct
8221 tcctttcata gtttacatct cctgtttgaa gaatacacca acatccccat ttctctactt
8281 tttaacgaaa aagaggcaga tgacaatgac catgagcccc aaatatcccc cgggggctta
8341 gagcctccca gtgaaaaaca tttccgagaa acagaagtct gaaaaggtca gggcccagac
8401 taaggctctg acgtctcccc ccggagggca gctcagcacc ggctcgggct aggcctgac
8461 gtgtcccccct gaagacaaat cataagctca gacctccggg aagccaccaa gaaccacccа
8521 tttcctcccc atgtttgtca agccgtcctc aggcgttgac gacaacccct cacctcaaaa
8581 aacttttcat ggcacgcata tggctcaata aactagcagg agtctataaa agcgtggaga
8641 cagttcagga gggggctcgc atctctcctt cacgcgcccg ccgccctacc tgaggccgcc
8701 atccacgccg gttgagtcgc gttctgccgc ctcccgcctg tggtgcctcc tgaactgcgt
8761 ccgccgtcta ggtaagttta aagctcaggt cgagaccggg cctttgtccg gcgctcccct
8821 ggagcctacc tagactcagc cggctctcca cgctttgcct gaccctgctt gctcaactct
8881 acgtctttgt ttcgttttct gttctgcgcc gttacagatc gaaagttcca ccccttcccc
8941 tttcattcac gactgactgc cggcttggcc cacggccaag taccggcgac tccgttggct
9001 cggagccagc gacagcccat cctatagcac tctcaggaga gaaatttagt acacatagtt
9061 ggaggtag
```

Appendix 7: Complete nucleotide sequence of human T lymphotropic virus type II proviral DNA.

GenBank Accession number M10060 (8952 bp) (Shimotohno *et al* 1985). Oligonucleotide primer sequences for PCR are underlined (shaded or emboldened where overlapping) and annotated at right.

```
   1 tgacaatggc gactagcctc ccaagccagc cacccagggc gagtcatcga cccaaaaggt
  61 cagaccgtct cacacaaaca atcccaagta aaggctctga cgtctccccc ttttttttagg
 121 aactgaaacc acggccctga cgtccctccc ccctaggaac aggaacagct ctccagaaaa
 181 aaatagacct cacccttacc cacttcccct agcgctgaaa aacaaggctc tgacgattac
 241 ccctgccca taaaatttgc ctagtcaaaa taaaagatgc cgagtctata aaagcgcaag
 301 gacagttcag gaggtggctc gctccctcac cgaccctctg gtcacggaga ctcaccttgg
 361 ggatccatcc tctccaagcg gcctcggttg agacgccttc cgtgggaccg tctcccggcc
 421 tcggcacctc ctgaactgct cctcccaagg taagtctcct ctcaggtcga gctcggctgc
 481 cccttaggta gtcgctcccc gagggtcttt agagacaccc gggttttcgc ctgcgctcgg
 541 ctagactctg ccttaaactt cacttccgcg ttcttgtctc gttctttcct ctttcgccgtc
 601 actgaaaacg aaacctcaac gccgccctct tggcaggcgt cccgggccca acatacgccg
 661 tggagccgcag caagggctag ggcttcctga acctctccgg gagaggtcta ttgctatagg
 721 caggcccgcc ctaggagcat tgtcttcccg gggaagacaa acaattgggg gctcgtccgg 174
 781 gatttgaatt cctccattct cacattatgg gacaaatcca cgggctttcc ccaactccaa
 841 taccccaaagc ccccaggggg ctatcaaccc accactggct taacttttcta caggctgctt
 901 accgcttgca gcctaggccc tccgatttcg acttccagca gctacgacgc tttctaaaac
 961 tagcccttaa aacgcccatt tggctaaatc ctattgacta ctcgctttta gctagcctta
1021 tcccccaaggg atatccagga agggtggtag agattataaa tatccttgtc aaaaatcaag
1081 tctcccctag cgccccccgcc gccccagttc cgacaccctat ctgccctact actactcctc
1141 cgccaccctcc cccccctttcc ccggaggccc atgttccccc cccttacgtg gaacccacca
1201 ccacgcaatg cttccctatc ttacatcccc caggagcccc ctcagctcat aggccctggc
1261 agatgaaaga cttacaggcc atcaagcagg aggtcagctc ctctgctctt ggcagccccc 175
1321 agttcatgca gaccctccgg ctggcggtac aacagtttga ccccaccgcc aaggacttac
1381 aagatctcct ccagtaccta tgctcctccc tcgtagtttc cttacaccat cagcagctta
1441 acacactaat taccgaggct gagacccgcg ggatgacagg ctacaacccc atggcagggc
1501 ccctaagaat gcaggctaat aaccccgccc agcaaggtct tagacgggag taccagaatc
1561 tttggctggc tgcttttctcc accctgccag gcaatacccg tgacccctct tgggcagcta
1621 tcctacaggg gctggaggaa ccctattgcg cgttcgtaga gcgccttaac gtggcccttg
1681 acaacggcct cccgagggt accccccaaag agcccatctt acgttcccta gcgtactcaa
1741 acgccaacaa agaatgccaa aaaatcttac aagcccgcgg acacactaac agcccccttg
1801 gggagatgct ccggacatgt caggcgtgga cacccaagga caaaaccaag gtccttgtgg
1861 tccaaccacg gaggcccccc cccacacagc cctgctttcg ttgtggcaag gtaggacact
1921 ggagtcggga ctgtacccag ccacgccccc ctcctggccc ctgccccccta tgccaagatc
1981 cttctcactg gaaaagggac tgcccacaac tcaaaccccc tcaggaggaa ggggaacccc
2041 tcctgttgga tctcccttcc acctcaggca ctactgagga aaaaaactcc ttaaggggggg
2101 agatctaatc tccccccatc ccgatcaaga catctcgata ctcccactca tccccctgcg
2161 gcagcaacag caaccaattc taggggtccg gatctccgtt atgggacaaa cacctcagcc
2221 tacccaagcg ctacttgaca caggagccga ccttacggtt atacccccaga cactcgtgcc
2281 cgggccggta aagctccacg acaccctgat cctaggcgcc agtgggcaaa ccaacaccca
2341 gttcaaactc ctccaaaccc ccctacacat attcttgccc ttccgaaggt cccccgttat
2401 cctttcctcc tgcctcttag acacccacaa caaatggacc atcattggaa gggacgccct
2461 acaacaatgc caggggcttc tatacctccc agacgacccc agccccccacc aattgctgcc
2521 aatagccact ccaaacacca taggcctcga acaccttccc ccacctcccc aagtggacca
2581 atttcctta aacctgagcg cctccaggcc ttaaatgacc tggtctccaa ggccctggag 176
2641 gctggtcaca ttgaaccata ctcaggacca ggcaataacc ccgtctttcc cgttaaaaaa
2701 ccaaatggta aatggaggtt catcatgtac ctaagagcca ccaatgccat tactaccacc
2761 ctcacctctc ctttcccagg gccccccgat ctcactagcc taccgacagc cttacccccac
2821 ctacagacca tagatcttac tgacgccttt ttccaaatcc cccttcccaa gcagtaccag
2881 ccatacttcg ccttcaccat tccccagcca tgtaactatg gccccgggac cagatatgca
```

Appendix 7: (continued).

```
2941 tggachgtcc tcccacaggg gtttaaaaac agccccaccc tcttcgaaca acaattagca
3001 gccgtcctca accccatgag gaaaatgttt cccacatcga ccattgtcca atacatggat
3061 gacatacttt tagccagccc caccaatgag gaattacaac aactctccca gctaaccctc
3121 caggcactga ccacgcatgg ccttccaatt tcccaggaaa aaacacaaca aaccccaggc
3181 caaatacgct tcttaggaca ggtcatctcc cctaatcaca ttacatatga gagtaccccc
3241 actattccca taaaatccca atggacactc actgaattac aagttatcct aggagagatc
3301 cagtgggtct ctaaaggaac acccatcctt cgcaaacacc tacaatccct atattctgcc
3361 cttcacgggt accgggaccc aagagcttgt atcaccctca ccccacaaca actccatgcg
3421 ttacatgcca ttcaacaagc tctacaacat aactgccgtg gccgcctcaa ccccgcccta
3481 cctctccttg gcctcatctc gttaagtaca tctggtacaa catctgtcat ctttcaaccc
3541 aagcaaaatt ggcccctggc ttggctccac acccccccac ctccgaccag tttatgtcct
3601 tggggtcacc tactggcctg caccatctta actctagaca aatataccct acaacattat
3661 ggccagctct gccaatcttt ccaccacaac atgtcaaagc aagccctttg cgacttcctg
3721 aggaactccc ctcatccaag tgtcggcatc ctcattcacc acatgggtcg attccataac
3781 cttggcagcc aacgtctgg tccgtggaag actctcttac acctcccaac ccttctccag
3841 gaaccacgac tcctcaggcc aatttttcacc ctctcccccg tcgtgcttga cacggccccc
3901 tgccttttt ccgatggctc ccctcaaaag gcagcgtacg ttctctggga ccagactatc
3961 cttcaacagg acatcactcc cctgccctct cacgaaacac attccgcaca aaaggggcag
4021 ctccttgcac ttatctgtgg actacgtgct gccaagccat ggccttcact taacatcttt
4081 ttagactcta aatatttaat caaatacctc cattcccteg ccattggggc cttcctcggc
4141 acttccgccc atcaaaccct ccaggcggcc ttgccacccc tactgcaggg caagaccatc
4201 taccttcacc atgtccgcag ccacaccaac ctccccgacc caatttccac cttcaatgaa
4261 tacacagact cccttatctt agctcccctt gttcccctga cgcccccaagg cctccacggc
4321 ctcacccatt gcaatcaaag ggctctagtc tcttttggcg ccacaccaag ggaagccaag
4381 tccccttgtac agacttgcca tacctgtcaa accatcaacl cacaacatca tatgcctcga
4441 gggtacattc gccggggcct cttgcccaac cacatatggc aaggtgatgt aacccattat
4501 aagtacaaaa aatacaaata ctgcctccac gtctgggtag acaccttctc cggtgcggtt
4561 tccgtctcct gtaaaaagaa agaaaccagc tgtgagacta tcagcgccgt tcttcaggcc
4621 atttcccttcc tagggaaacc actccacatt aacacagata atgggccagc cttcctatca
4681 caagaattcc aggagttttg tacctcctat cgcatcaagc attctaccca tataccatac
4741 aacccccacca gctcaggcct ggtcgagaga accaatggtg taatcaaaaa cttactaaat
4801 aaatatctac tagactgtcc taaccttccc ctagacaatg ccattcacaa agccctttgg
4861 actctcaatc agctaaatgt catgaacccc agtggtaaaa cccgatggca aatccaccac
4921 agtcctccac taccacccat tcctgaagcc tctacccctc ccaaaccacc tcccaaatgg
4981 ttctattata aactcccccgg ccttaccaat cagcggtgga aaggtccatt gcaatccctc
5041 caggaagcgg ccgggggcagc cttgctctcc atagacggct ccccccggtg gatcccgtgg
5101 cgattcctga aaaaagctgc atgcccaaga ccagacgcca gcgaactcgc cgagcacgcc
5161 gcaacagacc accaacacca tgggtaatgt tttcttccta ctttlattca gtctcacaca
5221 ttttccacta gcccagcaga gccgatgcac actcacgatt ggtatctcct cctaccactc
5281 cagcccctgt agcccaaccc aacccgtctg cacgtggaac ctcgacctta attcctaac
5341 aacggaccaa cgactacacc cccctgccc taacctaatt acttactctg gcttccataa
5401 gacttattcc ttatacttat tcccacattg gataaaaaag ccaaacagac agggcctagg
5461 gtactactcg ccttcctaca atgacccttg ctcgctacaa tgcccctact tgggctgcca
5521 agcatggaca tccgcataca cgggcccccgt ctccagtcca tcctggaagt ttcattcaga
5581 tgtaaatttc acccaggaag tcagccaagt gtcccttcga ctacacttct ctaagtgcgg
5641 ctcctccatg accctcctag tagatgcccc tggatatgat cctttatggt tcatcaccte
5701 agaacccact cagcctccac caacttctcc cccattggtc catgactccg accttgaaca
5761 tgtcctaacc cccccacgt cctggacgac caaaatactc aaatttatcc agctgacctt
5821 acagagcacc aattactcct gcatggtttg cgtggataga tccagcctct catcctggca
5881 tgtactctac acccccaaca tctccattcc ccaacaaacc tcctcccgaa ccatcctctt
5941 tcctttcctt gccctgcccg ctcctccatc ccaaccctte cchtggaccc attgctacca
6001 acctcgccta caggcgataa caacagataa ctgcaacaac tccattatcc tccccccttt
6061 ttcccctcgct cccgtacctc ctccggcgac aagacgccgc cgtgccgttc caatagcagt
6121 gtggcttgtc tccgccctag cggccggaac aggtatcgct ggtggagtaa caggctccct
6181 atctctggct tccagtaaaa gccttctcct cgaggttgac aaagacatct cccaccttac
6241 ccaggccata gtcaaaaatc atcaaaacat cctccgggtt gcacagtatg cagcccaaaa
6301 tagacgagga ttagacctcc tattctggga acaaggggct ttgtgcaagg ccatacagga
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Appendix 7: (continued).

6361 gcaatgttgc ttcctcaaca tcagtaacac tcatgtatcc gtcctccagg aacggccccc
6421 tcttgaaaaa cgtgtcatca ccggctgggg actaaactgg gatcttggac tgtcccaatg
6481 ggcacgagaa gccctccaga caggcataac cattctcgct ctactcctcc tcgtcatatt
6541 gtttggcccc tgtatcctcc gccaaatcca ggcccttcca cagcggttac aaaaccgaca
6601 taaccagtat tcccttatca acccagaaac catgctataa tagacctgct agcttctgca
6661 gcaaatcccc taggttcgtc ccctaccat tgacccatcc acagtcctct ataccagatg
6721 agtcgccccc gatgtccagc cctaactcga ttctgaataa ttgcctcaaa tagttcctct
6781 aaccccccgct cacattcctc ccataggacc ttctttttcc cttcaggaaa tccacataac
6841 cctgaagcaa gtcacaaaac ccatcaaaac ccaggagtcc tatacactcc aactgctgat
6901 gcctttcttc cctctcccgg cgcttttgat cctttttccg caggcgctcc tttctgcgcc
6961 gctcccgctc ctcacgctcc tgcagaagtt ttaagatctc ccgctgctcc tccgccaaca
7021 gttccgacg agagtctcgc acctgctcgc tgaccgatcc cgacccccaga gggcgacctt
7081 ttgctgtcct tctcggttcc tctccagggg gaggcacacc agatgtcaga ctcgcctctc
7141 cctggtctcc taacggcaat ctcctaaaat agtctaaaaa atcacacata attacaatcc
7201 tgtctcctct cagcccattt cccaggattt ggacagagcc tcctatatgg ataccccgtc 50
7261 tacgtgtttg gcgattgtgt acaggccgat tggtgtcccg tctcaggtgg tctatgttcc 288
7321 acccgcctac atcgacatgc cctcctggcc acctgtccag agcaccaact cacctgggac 3, 289
7381 cccatcgatg gacgcgttgt cagctcttcc ctccaatacc ttatccctcg cctccctcc 51
7441 ttccccaccc agagaacctc aaggacccta aaggtcctta ccccttccac cactcctgtc 4
7501 tcccccaagg ttccacctgc cttctttcaa tcaatgcgaa agcacacccc ctaccgaaat
7561 ggatgcctgg aaccaaccct ggggatcaag ctcccctccc tcgccttccc cgaacctggc
7621 ctccgtcccc aaaacatcta caccacctgg ggaaaaaccg tagtatgcct atacctatac
7681 cagctttccc cacccatgac atggccactt atacccccatg tcatatttctg ccaccccaga
7741 caattaggag ccttcctcac caaggtgcct ctaaaacgat tagaagaact tctatacaaa
7801 atgttcctac acacagggac agtcatagtc ctcccggagg acgacctacc caccacaatg
7861 ttccaacccg tgagggctcc ctgtatccag actgcctggt gtacaggact tctcccctat
7921 cactccatct taacaacccc aggtctaata tggaccttca atgacggctc accaatgatt
7981 tccggccctt acccccaaagc agggcagcca tctttagtag ttcagtcctc cctattaatc
8041 ttcgaaaaat tcgaaaccaa agcctttcat ccctcctatc tactctctca tcagcttata
8101 caatactcct ccttccataa ccttcacctt ctattcgatg aatacaccaa catccctgtc
8161 tctatttttat ttaataaaga agaggcggat gacaatggcg actagcctcc cgagccagcc
8221 acccagggcg agtcatcgac ccaaaaggtc agaccgtctc acacaaacaa tcccaagtaa
8281 aggctctgac gtctcccccct ttttttagga actgaaacca cggccctgac gtccctcccc
8341 cctaggaaca ggaacagctc tccagaaaaa aatagacctc acccttaccc acttcccctа
8401 gcgctgaaaa acaaggctct gacgattacc ccctgcccat aaaatttgcc tagtcaaaat
8461 aaaagatgcc gagtctataa aagcgcaagg acagttcagg aggtggctcg ctcctcaccc
8521 gacccctctgg tcacggagac tcaccttggg gatccatcct ctccaagcgg cctcggttga
8581 gacgccttcc gtgggaccgt ctcccggcct cggcacctcc tgaactgctc ctcccaaggt
8641 aagtctcctc tcaggtcgag ctcggctgcc ccttaggtag tcgctccccg agggtcttta
8701 gagacacccg ggttccgcc tgcgctcggc tagactctgc cttaaacttc acttccgcgt
8761 tcttgtctcg ttctttcctc ttcgccgtca ctgaaaacga aacctcaacg ccgcccctctt
8821 ggcaggcgtc ccggggccaa catacgccgt ggagcgcagc aagggctagg gcttcctgaa
8881 cctctccggg agaggtctat tgctataggc aggcccgccc taggagcatt gtcttcccgg
8941 ggaagacaaa ca

Appendix 8: Complete nucleotide sequence of bovine leukaemia virus proviral DNA.

GenBank Accession number K02120 (8714 bp) (Sagata *et al* 1985). Oligonucleotide primer sequences for PCR are underlined and annotated at right.

```
   1 tgtatgaaag atcatgccga cctaggagcc gccaccgccc cgtaaaccag acagagacgt
  61 cagctgccag aaaagctggt gacggcagct ggtggctaga atccccgtac ctcccaaact
 121 tcccctttcc cgaaaaatcc acaccctgag ctgctgacct cacctgctga taaatttaata
 181 aaatgccggc cctgtcgagt tagcggcacc agaagcgttc ttctcctgag accctcgtgc
 241 tcagctctcg gtcctgagct ctcttgctcc cgagaccttc tggtcggcta tccggcagcg
 301 gtcaggtaag gcaagcacgg tttggagggt ggttctcggc tgagaccacc gcgagctcta
 361 tctccggtcc tctgaccgtc tccacgtgga ctctctcctt tgcctcctga ccccgcgctc
 421 caagggcgtc tggcttgcac ccgcgtttgt ttcctgtcct actttctgtt tctcgcggcc
 481 cgcgctctct ccttcggcgc cctctagcgg ccaggagaga ccggcaaaca attgggggct 174
 541 cgtccgggat tgatcacccc ggaaccctaa caactctctg gacccacccc ctcggcggca
 601 ttttgggtct ctccttcaaa ttatatcatg ggaaattccc cctcctataa ccccccccgct
 661 ggtatctccc cctcagactg gctcaacctt ctgcaaagcg cgcaaaggct caatccgcga
 721 cctctcccta gcgattttac cgatttaaag aattacatcc attggtttca taagacccag
 781 aaaaaaccat ggactttcac ttctggtggc cccacctcat gtccacccgg gagattcggc
 841 cgggttcccc tcgtcttggc cacccctaaac gaagtactct caaacgaagg gggcgccccg
 901 ggtgcatcgg ccccagaaga acaaccccccc ccttatgacc ccccccgccat tttgccaatc
 961 atatctgaag ggaatcgcaa ccgccatcgt gcttgggcac tccgagaatt acaagatatc 175
1021 aaaaaagaaa ttgaaaataa ggcaccgggt tcgcaagtat ggatacaaac actacgactt
1081 gcaatcctgc aggccgaccc tactccggct gacctagaac aactttgcca atatattgct 194
1141 tccccggtcg accaaacggc ccatatgacc agcctaacgg cagcaatagc cgccgctgaa
1201 gcggcaacac cctccagggt tttaacccccc aaaacgggta ccctaacccca acaatcagct
1261 cagcccaacg ccgggggatct tagaagtcaa tatcaaaacc tctggcttca ggccggaaaa 195
1321 atctccctac tcgtccttca gctacaacct tggtccacca tcgtccaagg ccccgccgaa
1381 agctctgtag agtttgtcaa ccggttacaa atttcattag ctgacaacct tcccgacgga
1441 gtcctaagga acccattatt gactcccctta gttatgcaaa tgctaacaga gagtgtcagc
1501 aaattttgca ggggcgaggc cagtggccgc ggtggggcaa aaactgcagg cttgcgcaca
1561 attgggcccc caagaatgaa acagcctgca cttctcgtcc acaccccagg gcccaagatg
1621 cccgggcctc ggcaaccggc ccccaaaagg cctcccccag gaccatgcta tcgatgcctc
1681 aaagaaggcc attgggcccg ggattgtcct accaaggcca ccggcccacc tccgggacct
1741 tgccccatat gtaaagatcc ttcccattgg aaacgagact gtccaacctt caaatcaaaa
1801 aactaataga ggggggactt agcgccccccc aaaccataac acctataacg gattctctta
1861 gtgaggccga attagaatgc ttacttttta ttcctctggc tcgcagccgt ccctccgtgg
1921 ctgtatacct gtctggcccc tggctgcagc cctctcagaa tcaagcccta atgcttgtgg
1981 acaccggggc tgaaaatacg gttctcccac aaaattggct ggttcgagat tacccacgga
2041 tcccccgccgc agtgctcgga gcaggggggag tctcccggaa cagatacaat tggctacaag
2101 gccctctgac cctggctcta aaaccagagg gtcccctttat caccatccca aaaatttttag
2161 ttgacacttc cgacaaatgg caaattttag gacgggacgt ccctcccgcc tacaggcttc
2221 tatctccata cctgaggaag tacgcccccc tgtggtaggc gtcttggata ccccccgcgag
2281 ccacattgga ttagaacatc tgccccccccc acctgaggtg cctcaattcc ctttaaacta 176
2341 gaacgcctcc aggcccttca agacctggtc catcgctctc tggaggcagg ttatatctcc
2401 ccctgggacg ggccaggcaa taatccagtc ttcccggtac ggaaaccaaa tggcgcctgg
2461 aggtttgtgc atgacctacg agctacaaat gctcttacaa agcccattcc ggcactctct
2521 cccggaccgc cagaccttac cgctatccct acgcaccctc cacatatcat ttgcctagat
2581 ctcaaagatg cctttttcca gattccagtc gaagaccgct tccgcttcta cttgtctttt
2641 accctcccat ccccccgggg actccaacct catagacgct ttgcctggcg ggtcctacct
2701 caaggcttca ttaacagccc agctctttttc gaacgagcac tacaggaacc tcttcgccaa
2761 gttttccgccg ccttttccca gtctctttctg gtgtcctata tggacgatat cctttacgct
2821 tcgcctacag aagaacagcg gtcacaatgt tatcaagccc tggctgcccg cctccgggac
2881 ctagggtttc aggtggcatc cgaaaagact agccagacgc cttcgcccgt ccccttttttg
2941 ggacaaatgg tccatgagca gattgtcacc taccagtccc tacctacctt gcagatctca
3001 tccccaattt ctcttcacca attacaggcg gtcttaggag acctccaatg ggtctctagg
3061 ggcacaccca ctacccgccg gccctgcaa cttctctact cttcccttaa aaggcatcat
```

Appendix 8: (continued).

3121 gaccctaggg ccatcatcca gctttccccg gaacagctgc aaggcattgc agagcttcga
3181 caagccctgt cccacaacgc aagatctaga tataacgagc aagaaccccc gctagcctac
3241 gtacacctaa cccgggcggg gtccaccctg gtactcttcc aaaagggcgc tcaatttccc
3301 ctggcctact ttcagacccc cttgactgac aaccaagcct caccttgggg cctcctttct
3361 ctgctgggat gccaatacct gcagactcag gccttaagct cgtatgccaa gcccatactt
3421 aaatattatc acaatcttcc taaaacctct ctagacaatt ggattcaatc atctgaggac
3481 cctcgagtcc aggagttgct gcaattgtgg ccccagattt cctctcaggg aatacagccc
3541 ccgggccctt ggaagacctt aatcaccagg gcagaggttt ttttgacgcc ccagttctcc
3601 cctgatccga ttcctgcggc cctttgcctc tttagtgacg gggctacagg acgaggagca
3661 tattgcttgt ggaaagacca cctttttagac tttcaggccg ttccggcccc agaatccgct
3721 caaaagggag aactagcagg tctcttggcg ggcttagcag ccgccccgcc tgaacctgta
3781 aatatatggg tagattccaa atacctgtac tctttgctca gaaccctagt tctgggagct
3841 tggcttcaac ctgaccccgt accctcctac gccctcctat ataaaagcct cctccgacat
3901 ccagcaatcg ttgttggtca tgtccggagc cactcttcag catcccaccc tattgcttcc
3961 ctgaacaatt atgtagatca actgcttccc ttagaaactc cagagcaatg gcataagctc
4021 acccactgca actctcgggc cttgtctcga tggccgaacc cacgtatctc tgcctgggac
4081 cccgttccc cggctacgct gtgtgaaacc tgccaaaagc ttaatccaac tggaggagga
4141 aagatgcgaa ctattcagag agggtgggcc ccgaatcata tttggcaggc cgatataacc
4201 cattataaat acaaacagtt cacctacgct ctgcatgtgt ttgtagatac ttactctgga
4261 gctactcatg cctcggcgaa gcgtgggctc accactcaaa cgaccattga gggccttctt
4321 gaggccatag tgcatctggg tcgcccaaaa aagctaaaca ctgaccaagg tgcaaactac
4381 acctccaaaa cctttgtcag gttttgccag cagttcggag tttcccttte tcatcatgtt
4441 ccctacaacc ccacaagttc ggggttagat gaacggacaa atggactgct caaacttctt
4501 ctatctaaat atcacctaga cgaaccccac cttcccatga ctcaggccct ttctcgagcc
4561 ctctggactc acaatcagat taacctccta ccaattctaa agaccagatg ggagctacac
4621 cattcacccc cacttgctgt catttcagag ggcggagaaa cacccaaggg ctctgataaa
4681 ctctttttgt acttgctccc cgggcaaaac aatcgtcggt ggctaggacc actcccggcc
4741 ctagtcgaag cctcgggagg cgctctcctg gctactgacc cccccgtgtg ggttccctgg
4801 cgtttgctga aagccttcaa atgcctaaag aacgacggtc ccgaagacgc ccacaaccga
4861 tcatcagatg ggtaagtctc actctcactc tcctcgctct ctgtcggccc atccagactt
4921 ggagatgctc cctgtcccta ggaaaccaac aatggatgac agcatataac caagaggcaa
4981 aatttccat ctccattgac caaatactag aggctcataa tcagtcacct ttctgtgcca
5041 agtctcccag atacaccttg gactctgtaa atggctatcc taagatctac tggccccccc
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5161 gcccttatgt gggggcagat cggttcgact gccccccactg ggacaatgcc tcccaggctg
5221 atcaaggatc cttttatgtc aatcatcaga ttttattcct gcatctcaaa caatgtcatg
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5401 ctgtcagatc atgggccctg cttttaaatc aaacagcacg ggccttcccc gactgtgcta
5461 tatgttggga accttccccc cccctgggctc ccgaaatatt agtatataac aaaaccatct
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5581 cgtttaacac cacccaagga tggcaccacc cttcccagag gttgttgttc aatgtttctc
5641 aaggcaacgc cttgttatta cctcctatct ccctggttaa tctctctacg gcttcctccg
5701 cccctcctac ccgggtcaga cgtagtcccg tcgcggccct gaccttaggc ctagccctgt
5761 cagtggggct cactggcatt aatgtggccg tgtctgccct tagccatcag agactcacct
5821 ccctgatcca cgttctggag caagatcagc aacgcttgat cacagcaatt aatcagaccc
5881 actataattt gcttaatgtg gcctctgtgg ttgcccagaa ccgacggggg cttgattggt
5941 tgtacatccg gctgggtttt caaagcctat gtcccacaat taatgagcct tgctgtttcc
6001 tgcgcattca aaatgactcc attatcctcc gcggtgatct ccagcctctc tcgcaaagag
6061 tctctacaga ctggcagtgg ccctggaatt gggatctggg gctcactgcc tgggtgcgag
6121 aaaccattca ttctgttcta agcctgttcc tattagccct ttttttgctc ttcctggccc
6181 cctgcctgat aaaatgcttg acctctcgcc ttttaaagct cctccggcag gctccccact
6241 tccctgaaat ctccttaacc cctaaacccg attctgatta tcaggccttg ctaccatctg
6301 caccagagat ctactctcac cctctccccg tcaaacccga ttacatcaac ctccgaccct
6361 gcccttgata ccccccgcgtt tcacgcaccc ccaggctgtg gtggtgcact ggcttagtgg
6421 agtagtcagt gtaccatcac aagcctcttc ttgctgccag caccgagttc gaacacagct
6481 ctaccctgag cctctctgag tgcatgactg agtgtagcgc agagagattg tcgcttctgc

Appendix 8: (continued).

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6541 gtgtcgctca gtcatttttt atagccgatt ggggttcgcg ccctttcgtt gcctgtgaca
6601 cagataagac ctctctcact tctgcttcac catccccctg ccagcgttgg tctagtggaa
6661 agaactaacg ctgacggggg cgatttcttg cagctgtgct agcgggaggc tctggtgctg
6721 gggataagat gtggcccttа gcaccacagt ctctgcgcct tttgggttcg aatcttcccc
6781 acgcagcttc cgctttttac gccctgttgc acacccttтc tagagatacc tgaaaatctc
6841 agctcgcacc ctgaggaagg ttgtggctca gaggttaaaa tagctgagc cgcaacctcc
6901 ctttctttтt attccaccct cgcaaggccc cgggttctga gccccctaac ggaggttcaa
6961 aatttcctct acaaggggat gctcgggtcc aagtgtgcac aatatctctt ccaaaaggtc
7021 ctgatgaacg tcttcccatg taacaagccc cagcagagac attccagcca catccagcag
7081 catttgggcc gccttttcta acagtgcccа taaagtccct tccgtttcca caacggctgc
7141 ctctgcatct tctatttcca cctcggcacc gactcccccg ccgagccctt cgagctcttc
7201 gggatccatt acctgataac gacaaaatta tttcttgtct tttaagcaag tgttgttggt
7261 tgggggcccc actctctaca tgcctgcccg gccctggttt tgtccaatga tgtcaccatc
7321 gatgcctggt gccccctctg cgggccccat gagcgactcc aattcgaaag gatcgacacc
7381 acgctcacct gcgagaccca ccgtatcaac tggaccgccg atggacgacc ttgcggcctc
7441 aatggaacgt tgttcctcg actgcatgtc tccgagaccc gccccaagg gccccgacga
7501 ctctggatca actgcccccт tccggccgtt cgcgctcagc cgggcccggt ttcactttcc
7561 cccttcgagc ggtccccctt ccagccctac caatgccaat tgccctcggc ctctagcgac
7621 ggttgcccca ttatcgggca cggccttctt cctggaaca acttagtaac gcatcctgtc
7681 ctcggaaaag tccttatatt aaatcaaatg gccaattttt ccttactccc ctccttcgat
7741 acctcccttg tggacccccт ccggctgtcc gtctttgccc cagacaccag gggagccata
7801 cgttatctct ccacccttтt gacgctatgc ccagctactt gtattctacc cctaggcgag
7861 cccttctctc ctaatgtccc catatgcccc tttccccggg actccaatga acccccccтt
7921 tcagaattcg agctgccccc catccaaacg cccggcctgt cttggtctgt ccccgcgatc
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8041 caggccttac agcgcttcct tcatgacccт acgctaacct ggtccgaatt ggttgctagc
8101 agaaaaataa gacttgattc ccccttaaaa ttacaactgc tagaaaatga atggctctcc
8161 cgcctttttt gaggggagt catttgtatg aaagatcatg ccgacctagg agccgccacc
8221 gcccccgtaaa ccagacagag acgtcagctg ccagaaaagc tggtgacggc agctggtggc
8281 tagaatcccc gtacctcccc aacttcccct ttcccgaaaa atccacaccc tgagctgctg
8341 acctcacctg ctgataaatt aataaaatgc cggccctgtc gagttagcgg caccagaagc
8401 gttcttctcc tgagacccтc gtgctcagct ctcggtcctg agctctcttg ctcccgagac
8461 cttctggtcg gctatccggc agcggtcagg taaggcaagc acggtttgga gggtggttct
8521 cggctgagac cacccgagc tctatctccg gtcctctgac cgtctccacg tggactctct
8581 cctttgcctc ctgacccccc gctccaaggg cgtctggctt gcacccgcgt ttgtttcctg
8641 tcttactttc tgtttctcgc ggcccgcgct ctctccttcg gcgccctcta gcggccagga
8701 gagaccggca aaca
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Appendix 9: Human β-globin partial sequence.

GenBank Accession number NM_000518 (626 bp) (Proudfoot and Brownlee 1976). Oligonucleotide primer sequences for PCR are underlined and annotated at right.

```
  1 acatttgctt ctgacacaac tgtgttcact agcaacctca aacagacacc atggtgcacc 18
 61 tgactcctga ggagaagtct gccgttactg ccctgtgggg caaggtgaac gtggatgaag 52
121 ttggtggtga ggccctgggc aggctgctgg tggtctaccc ttggacccag aggttctttg
181 agtcctttgg ggatctgtcc actcctgatg ctgttatggg caaccctaag gtgaaggctc
241 atggcaagaa agtgctcggt gcctttagtg atggcctggc tcacctggac aacctcaagg
301 gcacctttgc cacactgagt gagctgcact gtgacaagct gcacgtggat cctgagaact
361 tcaggctcct gggcaacgtg ctggtctgtg tgctggccca tcactttggc aaagaattca
421 ccccaccagt gcaggctgcc tatcagaaag tggtggctgg tgtggctaat gccctggccc
481 acaagtatca ctaagctcgc tttcttgctg tccaatttct attaaaggtt cctttgttcc
541 ctaagtccaa ctactaaact gggggatatt atgaagggcc ttgagcatct ggattctgcc
601 taataaaaaa catttatttt cattgc
```

Appendix 10: Alignments of conserved regions of retroviral and hepadnaviral Pol amino acid sequences and derived consensus oligonucleotides.

A10.1: Comparison of conserved regions of retroviral and hepadnaviral reverse transcriptase (Pol) amino acid sequences.

Alignments derived from GenEMBL database using Genetics Computer Group 1994 PileUp programme (Altschul *et al* 1997).

```
Retroviruses
                1                                                  46
      BIV       WNVLPQGWVCSPAIYQTTTQKIIENIKKSHPDVMLYQYMDDLLIGS
      FIV       WCSLPQGWILSPLIYQSTLDNIIQPFIRQNPQLDIYQYMDDIYIGS
      EIAV      WNCLPQGFVLSPYIYQKTLQEILQPFRERYPEVQLYQYMDDLFVGS

     Visna      WKVLPQGWKLSPAVYQFTMQKILRGWIEEHPMIQFGIYMDDIYIGS
      CAEV      WKVLPQGWKLSPSVYQFTMQEILGEWIQEHPEIQFRIYMDDIYIRS

    SIVagm      FNCLPQGWKGSPTIFQNTASKILEEIKKELKQLTIVQYMDDLWVGS
    SIVmac      YKVLPQGWKGSPAIFQYTMRHVLEPFRKANPDVTLVQYMDDILIAS
     SIVsm      YKVLPQGWKGSPAIFQYTMRNILEPFRRANPDVTLIQYMDDILIAS
     HIV-1      YNVLPQGWKGSPAIFQSSMTKILEPFRKQNPDIVIYQYMDDLYVGS
     HIV-2      YKVLPQGWKGSPAIFQHTMRQVLEPFRKANKDVIIIQYMDDILIAS

       BLV      WRVLPQGFINSPALFERALQEPLRQVSAAFSQSLLVSYMDDILYAS

    HTLV-II     WTVLPQGFKNSPTLFEQQLAAVLNPMRKMFFTSTIVQYMDDILLAS
     HTLV-I     WKVLPQGFKNSPTLFEMQLAHILQPIRQAFPQCTILQYMDDILLAS
     HTLV-I     WRVLPQGFKNSPTLFEMQLAHILQPIRQAFPQCTILQYMDDILLAS

       LPDV     WTVLPQGMKNSPYICQQVVAEVIRPIRERFRDAVIIHYMDDILIAA
       MMTV     WKVLPQGMKNSPTLCQKFVDKAILTVRDKYQDSYIVHYMDDILLAH

       SRV1     WKVLPQRMANSPTLCQKYVATAIHKVRHAWKQMYIIHYMDDILIAG

       SRV2     WKVLPQGMANSPTLCQKYVAAAIEPVRKSWAQMYIIHYMDDILIAG
       MPMV     WKVLPQGMANSPTLCQKYVATAIHKVRHAWKQMYIIHYMDDILIAG
      SHIAP     WKVLPQGMANSPTICQLYVQEALEPIRKQFTSLIVIHYMDDILICH

      MuIAP     WKVLPQGMSNSPTMCQLYVQEALLPVREQFPSLILLLYMDDILLCH
     Pr-RSV     WKVLPQGMTCSPTICQLVVGQVLEPLRLKHPSLCMLHYMDDLLLAA

    Fr-MuLV     WTRLPQGFKNSPTLFDEALHRDLADFRIQHPDLILLQYVDDLLLAA
    Mo-MuLV     WTRLPQGFKNSPTLFDEALHRDLADFRIQHPDLILLQYVDDLLLAA
       BAEV     WTRLPQGFKNSPTLFDEALHRDLTDFRTQHPEVTLLQYVDDLLLAA
       FELV     WTRLPQGFKNSPTLFDEALHSDLADFRVRYPALVLLQYVDDLLLAA
       GALV     WTRLPQGFKNSPTLFDEALHRDLAPFRALNPQVVLLQYVDDLLVAA

      MuRRS     WMW*PQEFKNSPTLFDEALHRDLASFRAENSQVTLLQYVDDLLLAA Endogenous

      HERV4     WTQLPQRFKNSPTIFGEALARDLQKFPTRDLGCVLLQYVDDLLLGH Endogenous
      HERVC     WTQLPQRFKNSPTIFGEALARDLQKFPTRDLGCVLLQYVDDLLLGH Endogenous

       SFV3     WTRLPQGFLNSPALFTADVVDLLKEVPN......VQVYVDDIYISH
       SFV1     WTRLPQGFLNSPALFTADVVDLLKEIPN......VQAYVDDIYISH
        CFV     WTRLPQGFLNSPALFTADAVDLLKEVPN......VQVYVDDIYLSH

Hepadnaviruses

       DHBV     FRKAPMGVGLSPFLLHLFTTALGSEISRRF.NVWTFTYMDDFLLCH
   HeronHBV     FRKAPMGVGLSPFLLHLFTTAIGAEIASRF.NVWTFSYMDDFLLCH
        HBV     FRKIPMGVGLSPFLLAQFTSAICSVVRRAFPHCLAFSYMDDVVLGA
        WHV     FRKLPMGVGLSPFLLAQFTSALASMVRRNFPHCVVFAYMDDLVLGA
        WHV     FRKLPMGVGLSSSLLAQFTSALASMVRRNFPHCVVFAYMDDLVLGA
       GSHV     FRKLPMGVGLSPFLLAQFTSALTSMVRRNFPHCLAFAYMDDLVLGA
```

Appendix 10: (continued).

Abbreviations

BAEV	Baboon endogenous virus
BIV	Bovine immunodeficiency virus
BLV	Bovine leukaemia virus
CAEV	Caprine arthritis-encephalitis virus
CFV	Chimpanzee foamy virus
DHBV	Duck hepatitis B virus
EIAV	Equine infectious anaemia virus
FELV	Feline leukaemia virus
FIV	Feline immunodeficiency virus
Fr-MuLV	Friend murine leukaemia virus
GALV	Gibbon ape leukaemia virus
GSHV	Ground squirrel hepatitis virus
HBV	Hepatitis B virus
HeronHBV	Heron hepatitis B virus
HERV4	Human endogenous retrovirus 4
HERVC	Human endogenous retrovirus C
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HTLV-I	Human T lymphotropic virus type I
HTLV-II	Human T lymphotropic virus type II
LPDV	Lymphoproliferative disease virus of turkeys
MMTV	Mouse mammary tumour virus
Mo-MuLV	Moloney murine leukaemia virus
MPMV	Mason-Pfizer monkey virus
MuIAP	Murine intracellular type A particles
MuRRS	Murine retrovirus-like sequence
Pr-RSV	Prague Rous sarcoma virus
SFV1	Simian foamy virus 1
SFV3	Simian foamy virus 3
SHIAP	Syrian hamster intracellular type A particles
SIVagm	Simian immunodeficiency virus (African green monkey)
SIVmac	Simian immunodeficiency virus (rhesus macaque)
SIVsm	Simian immunodeficiency virus (sooty mangabey)
SRV1	Simian retrovirus type 1
SRV2	Simian retrovirus type 2
Visna	Maedi-visna virus
WHV	Woodchuck hepatitis virus

Appendix 10: (continued).

A10.2 Comparison of nucleotide and amino acid sequences of conserved regions of retroviral reverse transcriptase (*pol*) genes (Donehawer *et al* 1990).

Nucleotides are designated by single letter codes (Appendix 5). The single letter code for each amino acid is centred underneath the three nucleotides that encode it.

	5' sequence	3' sequence
HTLV-I	GTACTACCCCAAGGGTTTAAA V L P Q G F K	CTTCAGTACATGGATGAC L Q Y M D D
HTLV-II	GTCCTTCCACAGGCGTTTAAA V L P Q G F K	GTCCAATACATGGATGAC V Q Y M D D
BLV	GTCCTACCTCAAGGCTTCATT V L P Q G F I	GTGTCCTATATGGACGAT V S Y M D D
HIV-1	GTGCTTCCACAGGGATGGAAA V L P Q G W K	TATCAATACATGGATGAT Y Q Y M D D
EIAV	TGTTTACCACAAGGATTCGTG C L P Q G F V	TATCAATATATGGATGAT Y Q Y M D D
Mo-MuLV	AGACTCCCACAGGGTTTCAAA R L P Q G F K	CTACAGTACGTGGATGAC L Q Y V D D
Pr-RSV	GTCTTGCCCCAAGGGATGACC V L P Q G M T	TTGCATTATATGGATGAT L H Y M D D
MMTV	GTTTTGCCCCAGGGTATGAAA V L P Q G M K	GTGCATTACATGGATGAC V H Y M D D
	5' > GTNYTNCCNCARGG < 3'	5' > RTCRTCCATRTA < 3'
	5' consensus primer	3' consensus primer

Appendix 10: (continued).

A10.3 Comparison of 5' conserved regions of retroviral and hepadnaviral reverse transcriptase (Pol) amino acid sequences and derivation of consensus sense (forward) oligonucleotide primers for PCR (Mack and Sninsky *et al* 1988).

Amino acids and nucleotides are designated by single letter codes (Appendix 5). Potential three-nucleotide codons are listed under each amino acid alignment. Consensus nucleotide sequences derived from these reverse translations are shown using the standard nomenclature for incompletely specified bases.

Group 1 viruses

```
HTLV-1        L  P  Q  G  F  K  N  S  P  T  L  F
HTLV-1I       L  P  Q  G  F  K  N  S  P  T  L  F
BLV           L  P  Q  G  F  I  N  S  P  A  L  F
MuLV          L  P  Q  G  F  K  N  S  P  T  L  F
FeLV          L  P  Q  G  R  K  N  S  P  T  L  F
              *  *  *  *        *  *  *     *  *
            TTGCCTCAAGGTTTTAAAAATTCTCCTACTTTGTTT
            TTACCCCAGGGCTTCAAGAACTCCCCCACCTTATTC
            CTTCCA   GGACGTATT   TCACCAACACTT
            CTCCCG   GGGCGCATC   TCGCCGACGCTC
            CTA         CGAATA   AGT   GCTCTA
            CTG         CGG      AGC   GCCCTG
                        AGA            GCA
                        AGG            GCG
             * ** ** **    *  **    **  *  * **
      5' > YTNCCNCARGGNHKNAWNAAYWSNCCNRCNYTNTTY < 3'

      5' > YTNCCNCARGGNHKNAWNAA < 3'

      5' > YTNCCNCARGG < 3'
```

Group 2 viruses

```
MMTV          L  P  Q  G  M  K  N  S  P  T  L  C  Q
SRV-1         L  P  Q  R  M  A  N  S  P  T  L  C  Q
MPMV          L  P  Q  G  M  A  N  S  P  T  L  C  Q
SHIAP         L  P  Q  G  M  A  N  S  P  T  I  C  Q
RSV           L  P  Q  G  M  T  C  S  P  T  I  C  Q
              *  *  *     *        *  *  *     *  *
            TTGCCTCAAGGTATGAAAAATTCTCCTACTTTGTGTCAA
            TTACCCCAGGGC   AAGAACTCCCCCACCTTATGCCAG
            CTTCCA   GGA   GCTTGTTCACCAACACTT
            CTCCCG   GGG   GCCTGCTCGCCGACGCTC
            CTA      CGT   GCA   AGT      CTA
            CTG      CGC   GCG   AGC      CTG
                     CGA   ACT            ATT
                     CGG   ACC            ATC
                     AGA   ACA            ATA
                     AGG   ACG
              * ** **   * ***          ** **  * ** **
      5' > YTNCCNCARVGNATGRMNWRYWSNCCNACNHTNTGYCAR < 3'

      5' > YTNCCNCARVGNATG < 3'

                          5' > YWSNCCNACNHTNTGYCAR < 3'
```

Appendix 10: (continued).

Group 3 viruses

```
HIV-1        L  P  Q  G  N  K  G  S  P
HIV-2        L  P  Q  G  W  K  G  S  P
SIV-1        L  P  Q  G  W  K  G  S  P
EIAV         L  P  Q  G  F  V  L  S  P
CAEV         L  P  Q  G  W  K  L  S  P
Visna        L  P  Q  G  W  K  L  S  P
             *  *  *  *           *  *
           TTGCCTCAAGGTAATAAAGGTTCTCCT
           TTACCCCAGGGCAACAAGGGCTCCCCC
           CTTCCA   GGATGGGTTGGATCACCA
           CTCCCG   GGGTTTGTCCGGTCGCCG
           CTA         TTCGTATTGAGT
           CTG            GTGTTAAGC
                             CTT
                             CTC
                             CTA
                             CTG
            * ** ** **             **
      5' > YTNCCNCARGGNWDBRWNBKNWSNCCN < 3'
```

Group 4 viruses

```
HBV          L  P  M  G  V  G  L  S  P  F  L  L  A  Q  F  T
WHV          L  P  M  G  V  G  L  S  P  F  L  L  A  Q  F  T
GSHV         L  P  M  G  V  G  L  S  P  F  L  L  A  Q  F  T
DHBV         A  P  M  G  V  G  L  S  P  F  L  L  H  L  F  T
             *  *  *  *  *  *  *  *  *  *  *        *  *
           TTGCCTATGGGTGTTGGTTTGTCTCCTTTTTTGTTGGCTCAATTTACT
           TTACCC   GGCGTCGGCTTATCCCCCTTCTTATTAGCCCAGTTCACC
           CTTCCA   GGAGTAGGACTTTCACCA   CTTCTTGCATTG   ACA
           CTCCCG   GGGGTGGGGCTCTCGCCG   CTCCTCGCGTTA   ACG
           CTA                CTAAGT      CTACTACATCTT
           CTG                CTGAGC      CTGCTGCACCTC
           GCT                                   CTA
           ATT                                   CTG
           ATC
           ATA
              ** ***** ** **  *    ** **  *  *       ** **
      5' > NYNCCNATGGGNGTNGGNYTNWSNCCNTTYYTNYTNSMNYWNTTYYACN < 3'

         5' > CCNATGGGNGTNGGNYTNWSNCCNTT < 3'
```

Appendix 10: (continued).

A10.4 Comparison of 3' conserved regions of retroviral and hepadnaviral reverse transcriptase (Pol) amino acid sequences and derivation of consensus antisense (reverse) oligonucleotide primers for PCR (Mack and Sninsky *et al* 1988).

Amino acids and nucleotides are designated by single letter codes (Appendix 5). Potential three-nucleotide codons are listed under each amino acid alignment. Consensus nucleotide sequences derived from these reverse translations are shown using the standard nomenclature for incompletely specified bases. The complementary sequences are used to derive antisense (reverse) oligonucleotide primers.

Group 1 viruses	MuLV	G Y V D D L
	FcLV	Q Y V D D L
		* * *
		TATGTTGATGAT
		TACGTCGACGAC
		GTA
		GTG
		** ** * **
		5' > TAYGTNGAYGAY < 3'
Group 2 viruses	HTLV-I	Q Y M D D I
	HTLV-II	Q Y M D D I
	BLV	S Y M D D I
		* * *
Group 3 viruses	MMTV	H Y M D D I
	SRV-1	H Y M D D I
	MPMV	H Y M D D I
	SHIAP	H Y M D D I
	RSV	H Y M D D L
		* * * * *
Group 4 viruses	HIV-1	Q Y M D D L
	HIV-2	Q Y M D D I
	SIV-1	Q Y M D D I
	EIAV	Q Y M D D L
	CAEV	I Y M D D I
	Visna	I Y M D D I
		* * * *
Group 5 viruses	HBV	S Y M D D V
	WHV	A Y M D D L
	GSHV	A Y M D D L
	DEBV	T Y M D D F
		* * * *
		TATATGGATGAT
		TAC GACGAC
		** ***** **
		5' > TAYATGGAYGA < 3'
		3' > ATRTACCTRCT < 5'
		5' > TCRTCCNTRTA < 3'
	Consensus:	TATGTTGATGAT
		TACGTCGACGAC
		GTA
		GTG
		ATG
		** ** * **
		5' > TAYRTNGAYGA < 3'
		3' > ATRYANCTRCT < 5'
		5' > TCRTCNAYRTA < 3'