AN ANALYSIS OF THE FUNCTIONS OF THE EPSTEIN-BARR VIRUS LATENT PROTEINS, LMP1 AND EBNA3.

A dissertation submitted for the degree of Ph.D.

by

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DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Pamela Morgan ID No. 96970760

Pamela Morgan

Date: 13/9/2000

I dedicate this thesis to Mam and Dad

for the love and support you have always given me.

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Abstract

EBV is a prevalent human herpesvirus which is implicated in the aetiology of several human malignancies, including Burkitt's Lymphoma and several other cancers of lymphoid/epithelial origin. Infection of primary B lymphocytes in vitro with EBV leads to expression of a restricted set of EBV latent genes and subsequent immortalisation of cells into continuously proliferating lymphoblastoid cell lines (LCLs). Eleven viral genes are expressed in latently-infected (immortalised) B cells, of which just six are critical for transformation. These include latent membrane protein 1 (LMP1) and five nuclear antigens (EBNA1, -2, -3A, -3C and -LP).

The first part of this study was undertaken in order to investigate the mechanism of EBV-mediated deregulation of cell growth by examining its effects on the mRNA levels of a range of cell cycle inhibitor genes using ribonuclease protection assay (Chapter 3). Significantly elevated p21 mRNA levels was found to be a characteristic feature of the transition from EBV latency type I infection (expressing EBNA1 only) to type III infection (expressing all 11 latent EBV genes) of Burkitt Lymphoma (BL) cells, with elevated expression detected in EBV-immortalised lymphoblastoid cell lines, consistent with previous reports. Western blot analysis confirmed a similar degree of upregulation at the protein level. p21 (WAF1/CIP1) is an important nuclear protein with cyclin-dependent kinase (cdk) inhibitory activity, which can promote cytostasis by blocking cell cycle progression at the G_1 and/or G_2 phases of the cell cycle and by inhibiting PCNA-dependent DNA replication. As EBNA2 and LMP1 are both central to the immortalisation process, the contributions of each of these proteins to the observed p21 upregulation was investigated using a tetracycline-regulatable gene expression system in an EBV-negative BL background. This revealed an important role for LMP1, but not for EBNA2 when expressed singly. LMP1 is defined as a classical oncogene and its profound effects on cell growth are well-documented. The observed LMP1-mediated upregulation of p21 was found to be a B cell-specific effect, and was not detected in a second BL-derived cell line which lacks the characteristic *c-myc* translocation. In addition, the effect is likely to be p53-independent. On further investigation into the mechanism of upregulation, no transactivation of the p21 promoter was detected while enhanced p21 mRNA

stability was found to be important in the LMP1-mediated effect. Further studies will be required to characterise the molecular basis of this stabilisation.

The precise functions of the EBNA3 proteins are unclear, although persistent expression of these genes against negative selective pressure by cytotoxic T lymphocytes in vivo is consistent with important roles for all three members of this protein family. In attempting to identify potential protein binding partners for EBNA3B, the yeast two hybrid system (YTHS) was employed to screen two cDNA libraries. Both libraries yielded only false positives, including two EBNA3B-specific interactions. However, this type of result is well-documented as a recurring problem associated with use of YTH systems.

Abbreviations

A	Adenosine
aa	Amino acid
Abs	Absorbance
AD	Activation domain
AIDS	Acquired Immune Deficiency Syndrome
amp	Ampicillin
AP	Alkaline phosphatase
APS	Ammonium persulphate
3-AT	3-Aminotriazole
ATP	Adenosine tri-phosphate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
β-gal	β-galactosidase
BL	Burkitt's Lymphoma
BSA	Bovine serum albumin
C	Cystidine
CD44	Cluster of differentiation 44
cDNA	Complementary DNA
CIP	Calf Intestinal Phosphatase
CMV	Cytomegalovirus
DEPC	Diethylpyrocarbonate
d H_2O	Distilled water
dATP	Deoxy Adenosine tri-phosphate
DBD	DNA binding domain
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EA-D	Early antigen-diffuse
EA-R	Early antigen restricted
EBER	Epstein-Barr virus Encoded RNA
EBNA	Epstein-Barr virus Nuclear Antigen

EBNALP	EBV Nuclear Antigen Leader Protein
EBV	Epstein-Barr Virus
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FCS	Fetal calf serum
G	Guanosine
G/Glu	Glucose
HA	Haemagglutinin antigen
HEPES	N-Hydroxyethyl]piperazine N'-[2-ethanesulfonic acid]
H/HIS	Histidine
HPV	Human Papilloma virus
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL	Interleukin
IM	Infectious Mononucleosis
IPTG	Isopropyl β-D-Thiogalactopyranoside
L/Leu	Leucine
LB	Luria-Bertrani broth
LCL	Lymphoblastoid cell line
LiAc	Lithium Acetate
LMP	Latent Membrane Protein
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MCS	Multiple Cloning Site
mRNA	Messenger RNA
NBT	Nitroblue tetrazolium
NPC	Nasopharengeal Carcinoma
OD	Optical density
OHL	Oral Hairy leukoplakia
ori	Origin of Replication
p	Plasmid

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating-cell nuclear antigen
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl Flouride
POD	Peroxidase
RB	Retinoblastoma protein
RBP	Recombination Signal binding protein
RNA	Ribonucleic acid
RNase	Ribonucleases
RPA	Ribonuclease protection assay
RT-PCR	Reverse transcription PCR
SA-PMPs	Streptavidin paramagnetic particles
SDS	Sodium dodecyl sulphate
SssDNA	Sheared salmon sperm DNA
STP	Signal transduction pathway
SV40	Simian virus 40
T	Thymidine
T/Trp	Tryptophan
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBS	Tris buffer saline
TBST	Tris buffer saline plus tween 20
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TF	Transcription factor
tRNA	Transfer RNA
upH_2O	Ultra pure water
U/Ura	Uracil
UAS	Upstream activation site

UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
X-Gal	5-Bromo-4-chloro-3indoyl-β-D-galactoside
YNB	Yeast Nitrogen Base
YTHS	Yeast Two Hybrid System

UNITS

bp	Base pairs
Ci	Curies
Kb	Kilobases
KD	Kilodaltons
µg	Micrograms
µl	Microliters
°C	Degrees Celsius
cm	Centimetres
g	g force
g	Grams
h	Hours
Kg	Kilograms
L	Litres
M	Molar
mA	Milliamps
mg	Milligrams
min	Minutes
ml	Millilitres
mM	Milimolar
mol	Moles
ng	Nanograms
nM	Nanomolar
pmole	Picomolar
s	Second
U	Enzyme units
V	Volts

FIGURES

TABLES

Acknowledgements

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CHAPTER 1
INTRODUCTION

1.0 Epstein-Barr Virus

1.1 Classification and Structure

Epstein-Barr virus is a ubiquitous member of the herpesvirus family. The herpesviruses are a family of almost 100 DNA viruses found commmonly in humans and animals. Classification of this family, established on the basis of similarity in biological properties, places EBV in the gammaherpesvirus subfamily, the other two main subfamilies being alpha- and betaherpesviruses. EBV is the most extensively studied and the only human herpesvirus in the gammaherpesvirus subfamily and is the prototype virus of the genera lymphocryptovirus. EBV was originally discovered in 1962 when it was suggested that EBV may be important in the aetiology of African Burkitt's Lymphomas (Burkitt, 1962). Characteristic of gammaherpesviruses, EBV exhibits a tropism for lymphoid cells and a capacity to induce cell proliferation *in vivo*, resulting in transient or chronic lymphoproliferative disorders. *In vitro*, many gammaherpesviruses, like EBV, can immortalise the infected cell. The current classification of herpesviruses does not help in determining evolutionary relatedness based on genome organisation and structural similarities. The distinction between alpha, beta and gamma herpesviruses has been somewhat blurred by more detailed molecular studies and by the discovery of new viruses that co-express the structural features of one subfamily and at least some biological properties of another. Thus, taxonomists have renamed EBV human herpervirus 4 (HHV-4).

Like other herpesviruses, a mature EBV virion has a toroid-shaped protein core that is wrapped with double-stranded DNA this is surrounded by an icosahedral capsid with 162 capsomers (Figure 1.1). The capsid is surrounded by an amorphous material, the tegument, composed of globular proteins. The envelopes of herpesviruses have numerous glycoprotein spikes, but EBV differs from most other herpesviruses in the predominance of a single glycoprotein in the outer envelope (Kieff, 1996).

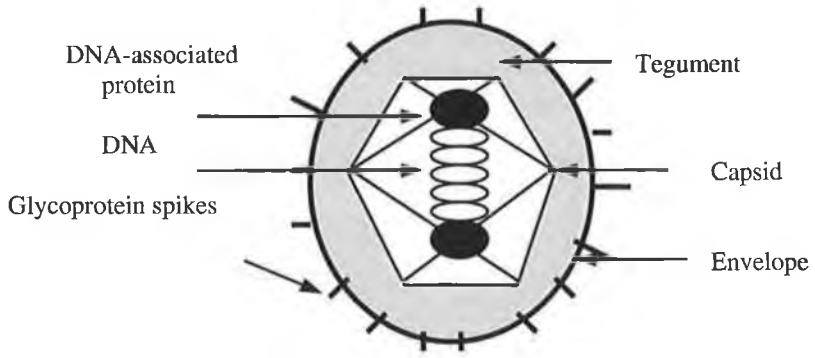


Fig. 1.1. Schematic representation of herpes virus structure.

1.2 EBV genome structure

The EBV genome is a linear, double stranded 172 Kb DNA with a guanine/cytosine content of 60% (Kieff, 1996). The genome encodes an estimated 100 genes, but like many viruses there is complicated differential splicing of RNA transcripts and the number of proteins produced may be greater (Kieff, 1996). The EBV genome was completely sequenced from the EBV strain B95-8, initially cloned as a *Bam*H1 fragment library. For this reason, nomenclature of open reading frames (ORFs), for transcription or RNA processing, is based on their location within specific *Bam*H1 fragments (Baer *et al.*, 1984). For example, the BARF1 ORF is found in the *Bam*H1 A fragment (BA) and it is the first ORF (F1) extending in a rightward (R) direction. On the basis of the presence and location of repeated sequences greater than 100 base pairs, herpesviruses can be divided into 6 structurally distinct groups, identified as A – F. As such, EBV is classed as a group C virus, where both terminal and internal repeat sequences are present throughout the viral genome, which sub-divide it into well-defined unique seqences. A simplified schematic representation of the EBV genome shown below (Figure 1.2) illustrates this feature.

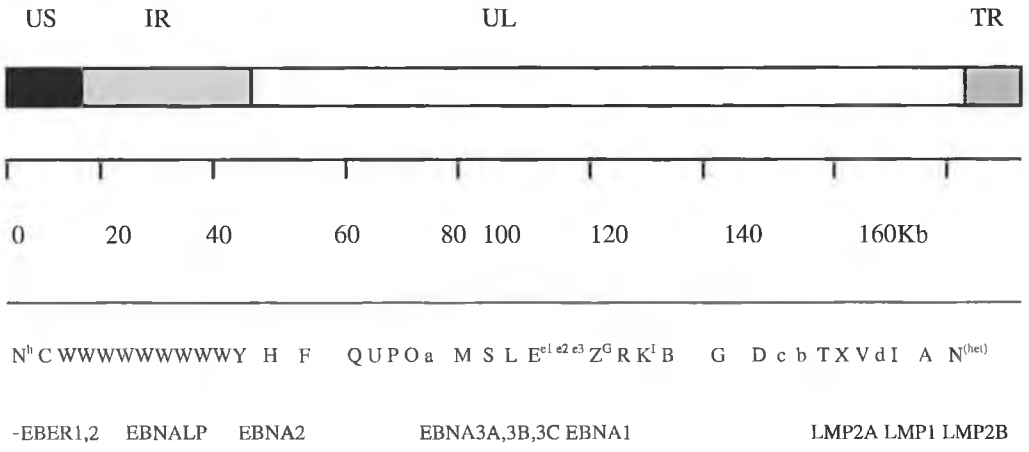


Figure 1.2. A schematic representation of the EBV genome (adapted from IARC Monographs, 1997). The genome is divided into repeat regions, 0.5 Kb terminal direct repeats (TR) and 3.0 Kb internal direct repeats (IR) that divide the genome into short and long largely unique sequence domains (US and UL). The *Bam*H1 fragments are represented by the letters below the red line. The *Bam*H1 fragment location of the EBV latent genes are indicated below the fragments. EBER: EBV-encoded RNA; EBNA: EBV nuclear antigen; LMP: latent membrane protein; LP: leader protein.

The major DNA repeat elements serve as landmarks on the EBV genome map, however, serial passage of virus infected cells frequently results in differences in the number of tandem repeat reiterations (Dambaugh *et al.*, 1980; Heller *et al.*, 1981; Brown *et al.*, 1986; Siaw *et al.*, 1986). Some of these repeats encode proteins and this can explain differences in protein sizes observed on immunoblots and can also serve as an important marker in identifying virus strains, or virus infected cells (Kieff, 1996). It has been found that some EBV genes expressed during latent and lytic infection have no homology with other herpesvirus genes and may have arisen from cellular DNA. The EBV latent gene BCRF1 is the most striking example of an acquisition from the cell gene pool. BCRF1 is nearly identical to human interleukin 10 (IL-10) in primary amino acid sequence (Moore *et al.*, 1993).

Two EBV types circulate in most human populations (Gerber *et al.*, 1976; Young *et al.*, 1987; Rowe *et al.*, 1989). These genomes formerly known as type A and type B are now referred to as type-1 and type-2. The genomes are almost identical except for the

genes that encode some of the Epstein-Barr nuclear antigens (EBNAs) such as EBNA2, EBNA3A, 3B, 3C and EBNALP, in latently infected cells (Bornkamm *et al.*, 1980). Apart from these genes, the genomes appear to have little differences beyond those which characterise individual EBV strains. The differences in type-1 and type-2 EBV genomes are reflected in type-specific and type-common epitopes for antibodies (Young *et al.*, 1987) and T-cell recognition (Moss *et al.*, 1988). As type-1 EBV is more common in developed societies, most EBV immune human sera from these countries react preferentially or exclusively with type-1 EBNA2, EBNA3A, 3B, 3C and EBNALP. African sera are almost evenly split in their serological reactivity, however, the recovery of type-2 virus from blood is unusual (Young *et al.*, 1987; Rowe *et al.*, 1989), perhaps because EBV type-2 infected lymphocytes grow less efficiently *in vitro* than their type-1 infected counterparts (Rickinson *et al.*, 1987). Different viral strains within types-1 and -2, based on significant DNA sequence heterogeneity have been found within the genome of EBV isolated in certain geographical areas or even from the same area. These polymorphisms may cause amino acid substitution in viral proteins and may even affect peptides that are important for the immune control of viral infection.

1.3 EBV strategy of infection

Under normal circumstances EBV infection is restricted to humans. Target cell tropism is usually limited to B cells and epithelial cells, although the expanding list of virus-associated tumours serves to illustrate that the target cell tropism of EBV *in vivo* is much broader than was originally anticipated. The conditions and mechanisms that allow EBV infection of these diverse cell types are, however, in most cases unknown. Nevertheless, EBV infection of primary B-lymphocytes *in vitro* involves binding of CD21 on the B-lymphocyte plasma membrane. CD21 (also known as CR2) is the receptor for the C3d component of complement. After binding, aggregation of CD21 in the plasma membrane, co-aggregation of surface immunoglobulins (sIg) and internalization of EBV into cytoplasmic vesicles occurs (Nemerow and Cooper, 1984; Carel *et al.*, 1990). The virus envelope then fuses with the vesicle membrane, releasing the nucleocapsid and tegument into the cytoplasm. Penetration is usually complete within 1-2 h.

Superinfection of established BL cell lines is somewhat different in that EBV binding does not result in as significant a patching of CD21 and sIg and the envelope fuses with the plasma membrane, releasing the nucleocapsid and tegument into the plasma membrane. The observed differences in mode of infection between primary B-lymphocytes and BL cells are likely to be due to the cytoskeletal abnormalities of the tumour cells (Kieff, 1996). The EBV outer envelope glycoprotein gp350 and gp220 form the CD21 ligand. The interaction of CD21, gp350 and gp220 mediates EBV adsorption (Tanner *et al.*, 1987; Nemerow *et al.*, 1987; 1989). Another EBV glycoprotein gp85, has been implicated in the fusion of the EBV envelope with the vesicle membrane. Monoclonal antibodies to gp85 inhibit the fusion of the EBV envelope and the cell membrane (Miller and Fletcher, 1988). Little is known about EBV capsid dissolution, genome transport to the cell nucleus or DNA circularization. By comparing EBV to other DNA viruses that replicate in the nucleus it may be suggested that the cytoskeleton is likely to mediate EBV capsid transport to the nucleus (Dales and Chardonet, 1973).

Two forms of EBV-cellular infection are recognised, latent and replicative (or lytic). Cell transcription factors probably determine if latent or lytic infection ensues after the genome enters the nucleus and circularizes (Kieff, 1996). *In vitro* experiments show that most human peripheral blood B-lymphocytes are susceptible to EBV infection. The virus does not usually replicate in recently-infected B-lymphocytes, which instead become stably latently infected. In latent infection, virus penetrates the cell and remains present either as circular episomal DNA (formed through fusion of the terminal repeats) or, less frequently, as linear DNA integrated into the host genomic DNA. Episomes, present in low copy numbers in the host cell nucleus, are copied by host cell DNA replicating enzyme and pass to daughter cells in mitosis (Joske and Knecht, 1993). Episomal DNA is also likely to be necessary for lytic cycle EBV DNA replication.

A specific set of nuclear (EBNA) and membrane (LMP) protein and small RNA (EBERs) viral gene products maintain the latent infection and cause the previously resting B-lymphocytes to continuously proliferate (Mark and Sugden, 1982). The effect

on cell growth is immediate and efficient, with most cells entering DNA synthesis 48-72 hours after EBV infection. The EBV infected proliferating B-lymphocytes are similar to activated B-lymphocytes in their secretion of immunoglobulin and their adherence to each other (Klein, 1987; Zhang *et al.*, 1991). Approximately 1 in every 10^5-10^6 of the B-lymphocytes purified from the peripheral blood of previously infected people are latently infected with EBV. These latently infected B-lymphocytes may be cultured and will proliferate into long-term lymphoblastoid cell lines (LCL) (Sixbey and Pagano, 1985). LCL outgrowth is the simplest means for establishing immortal cell lines from individual humans for chemical, biological and genetic analysis. Although epithelial cells are fully permissive for lytic EBV infection *in vivo*, infection of epithelial cells *in vitro* has proved inefficient, and thus, most of our knowledge of latent or lytic EBV infection *in vitro* is based on infection of B lymphocytes.

1.4 EBV Latent Infection

Because EBV-infected lymphocytes are growth-transformed by the virus, they can be grown indefinitely in culture and are amenable to detailed biochemical analyses including investigation of the mechanism of latent genome persistence and of cell growth transformation. At least 11 EBV genes are expressed in latent infection. Two of these encode small, non-polyadenylated RNAs (EBER1 and EBER2), six encode nuclear proteins (EBNA1, 2, 3A, 3B, 3C and LP) and three encode integral membrane proteins (LMP1, 2A and 2B). Six of these genes are essential for the immortalization of primary B cells (EBNA1, 2, 3A, 3C, -LP and LMP1). Transcription of nuclear proteins is initiated at RNA polymerase II-dependent promoters in the *Bam*H1 C (Cp) and *Bam*H1 W (Wp) regions of the viral genome (Rogers *et al.*, 1992).

The EBV genome circularises in the infected cell nucleus within 12-16 hours of infection. At about the same time, the Wp promoter initiates rightward transcription. The first viral proteins to be expressed in B cells upon EBV infection, namely EBNA2 and EBNALP are believed to play critical roles in the early stages of the immortalization process (Alladay *et al.*, 1989; Rooney *et al.*, 1989; Alfieri, *et al.*, 1991). EBNA2 and

EBNALP are initially transcribed from the very strong promoter Wp, which is present in multiple copies in the major internal repeat and are detectable within 12-16 hours post-infection. Once immortalization is established, Wp activity declines and transcription of the EBNA genes switches to using the Cp promoter (Woisetschlaeger *et al.,* 1990). All EBNA coding mRNAs are derived from the same transcriptional unit by alternative splicing and alternative polyadenylation (Figure 1.3.). The coding exons for most of the EBNAs are towards the 3' end of the mRNAs and are preceded by the highly spliced leader exons, which are encoded within the major internal repeat of the genome (Farrell, 1995). The infected B cells enter the first S phase approximately 40 h after the virus penetration (Sample and Kieff, 1990; Alfieri *et al*, 1991), at which stage all the EBNA and LMP proteins have reached detectable levels, with all 11 latent proteins being detectable by 72 h post-infection.

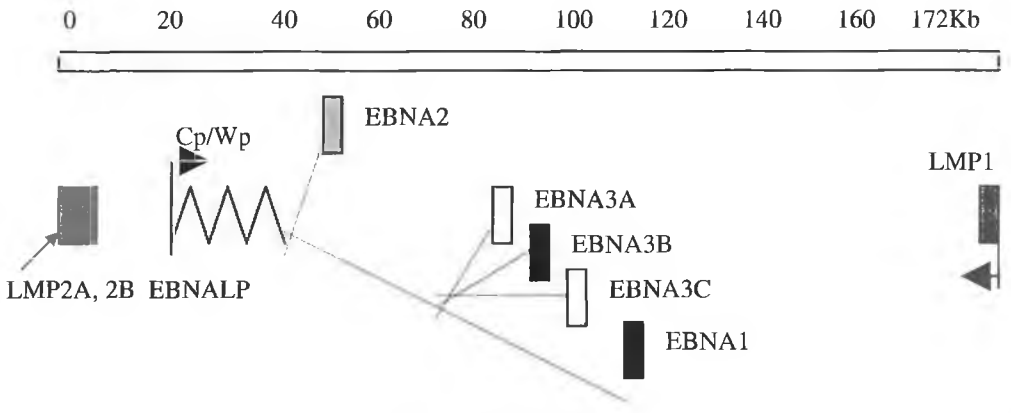


Figure 1.3 A simplified outline of the splicing of the EBV nuclear antigen coding mRNAs. Transcription initiation is shown to arise from the Cp promoter. The EBNA gene mRNAs all derive from the same transcription unit by alternative splicing and alternative polyadenylation.

The usual outcome of B-lymphocyte infection with EBV is a persistent latent infection. Three forms of latent infection have been characterised in EBV-carrying B-cell lines and EBV-carrying tumour biopsy samples. Thus, latency types I, II and III may be distinguished on the basis of expression of EBV latent genes and promoter usage (Sample *et al.,* 1986; 1991; Rowe *et al.,* 1986; 1987). Type-I latency is characterised by the expression of a single EBV protein, EBNA1 (Rowe *et al.,* 1987), together with high copy numbers of EBER1 and EBER2 (Rymo, 1979; Howe and Shue, 1989). The classic

features of latency I are exhibited in endemic (BL) biopsies and in early passage cell lines derived from these tumours (Rowe *et al.*, 1987). Cells in latency II resemble latency I cells in that they express EBNA1 and the EBER RNAs, but also express LMP1, LMP2A and LMP2B. Nasopharyngeal carcinoma (NPC) and Hodgkin's disease (HD) are two EBV-related clinical conditions which exhibit the latency 2 program. Explanted BL cells grow continuously in culture and on serial passage some retain the phenotype of the original biopsy (type-I). However, during prolonged culture *in vitro* many BL cell lines show a dramatic phenotypic drift, with increased expression of B-cell activation antigens and adhesion molecules and the appearance in the culture of clumps of more lymphoblastoid-like cells (type-III). As the group-III phenotype cells dominate the culture, they frequently lose expression of CD10 and CD77 (which are BL-associated markers), while other LCL associated markers, such as CD40, intercellular adhesion molecules and Bcl-2 are up-regulated (Rooney *et al.,* 1986; Rowe *et al.,* 1987; Henderson *et al.,* 1991). Type-III cells express the full set of EBV latent genes as well as cellular genes such as CD23 and a ligand for the EBV receptor CD21 (Wang *et al.,* 1987). Two EBV-associated diseases best exemplify the latency III program, infectious mononucleosis (IM) and post-transplantation lymphoproliferative disorder (PTLD) which is a potentially fatal immunoblastic lymphoma in transplant patients. The pattern of EBV latent gene expression is illustrated in Table 1.1 below.

Type of latency	Gene Product	Examples	Reference
I	EBERs, EBNA1	Burkitt's lymphoma	Rowe *et al.*, (1987)
		Gastric Carcinoma	Imai *et al.*, (1994)
II	EBERs, EBNA1,	Hodgkin's disease	Deacon *et al.*, (1993)
	LMP1, 2A, 2B,	Nasopharyngeal carcinoma	Hitt *et al.*, (1989)
	BARFO		Brooks *et al.*, (1992)
III	All EBV latent genes	PTLD, IM	Young *et al.*, (1989)
			Tierney *et al.*, (1994)
Other	EBERs, EBNA1, 2	Smooth muscle tumours	Lee *et al.*, (1995)

Table 1.1. Pattern of EBV latent gene expression. (adapted from the IARC monograph, 1997). PTLD, Post-transplant lymphoproliferative disorder, IM infectious mononucleosis.

1.5 EBV Latent Genes

1.5.1 EBNA1

EBV nuclear antigen 1 is required both for latent replication of the EBV genome and as a regulator of viral gene transcription (Speck and Strominger, 1987; Sugden, 1989) and is the only EBV latent gene which is detectable in all EBV infected cells. This 73 kDa protein consists of a short amino-terminal region a 20 kDa - 40 kDa, glycine alanine repetitive sequence flanked by arginine rich sequences and a highly charged acidic carboxy terminal sequence (Hennessy and Kieff, 1983). During latent infection of human host cells, EBV genomes are maintained as double-stranded DNA episomes that replicate once every cell cycle (Adams, 1987, Yates and Guan, 1991). The carboxy terminus of EBNA1 determines its nuclear localisation by interacting with a specific protein that is homogeneously distributed on chromosomes (Harris *et al.*, 1985; Petti *et al.*, 1990). This property is likely to be important for segregation of episomes into progeny nuclei during mitosis. Part of EBNA1 is also associated with the nuclear

matrix. EBNA1 is the only EBNA that continues to be made during lytic infection (IARC Monograph, 1997).

Although most of the EBNAs bind to DNA cellulose, only EBNA1 has sequence-specific DNA binding properties. The specific EBNA1 cognate sequence is a partial palindrome: TGGATAGCATATGCTATCCA for which EBNA1 has a high affinity. EBNA1 binds as a dimer to two components of the latent cycle origin of replication, *ori P* and it is the only virus encoded trans-acting factor required for episomal maintenance of the EBV genome (Ring, 1994). The replication origin is composed of 20 tandem repeats of the EBNA1 binding site, spaced about 1 Kb away from the 20 repeats are a further 4 copies of the binding site, 2 in dyad symmetry and two in tandem. The dyad symmetry component is stringently required for episome replication. The interaction of EBNA1 with the tandem repeats and dyad symmetry sites is co-operative and results in high-order structures that lead to bending of the DNA, distortion of the duplex and looping out of the intervening sequences (Frappier and O'Donnell, 1991; Orlowski and Miller, 1991; Frappier and O'Donnell, 1992). Regions of the protein important for DNA binding and transactivation of *ori P* are located in the carboxy-terminal third of the protein (Ambinder *et al.,* 1991) (see Figure 1.4). Furthermore, *ori P* acts as an EBNA1 dependent enhancer and plays a crucial role in the regulation of viral transcription from both the C and the LMP1 promoter in growth-transformed cells (Sugden and Warren, 1989; Gahn and Sugden 1995).

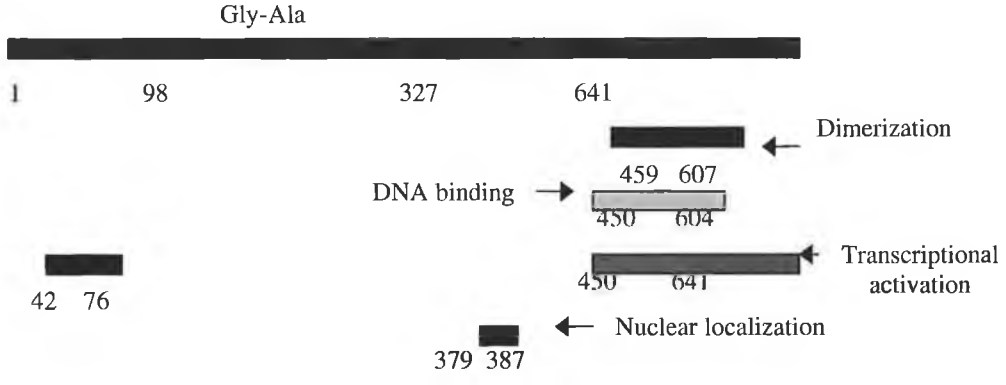


Figure 1.4 Functional domains of EBV nuclear antigen 1 (EBNA1). The Gly-Ala box is a repetitive region composed entirely of glycine and alanine, it varies in length between viral strains. Adapted from Farrell, 1995.

The promoter from which the EBNA1 gene is transcribed differs between cell types. In EBV transformed LCLs all the EBNA genes are derived from a highly spliced transcript that is generated by transcription from the C or W promoters located on the *Bam*H1 C and W fragments respectively (Middelton *et al.,* 1991). A recent study has indicated that RNA transcripts from latently infected early passage type-I BL cells, in the absence of a stimulus to induce virus replication, is initiated from a promoter distinct from Fp located in the adjacent *Bam* H I-Q fragment (Nonkwelo *et al.,* 1995). This promoter is designated Qp. Qp does not contain a recognisable TATA box, which is consistent with multiple sites of transcription initiation from Qp (Nonkwelo *et al.,* 1996). Additionally, EBNA1 can negatively autoregulate expression within receptor plasmids containing both Fp and Qp through two binding sites downstream of the 3'-most Qp start site (Sample *et al.,* 1992; Snug *et al.,* 1994). Following the switch from latent to lytic infection EBNA1 transcription is controlled by the Fp upstream of Q (Lear *et al.,* 1992).

EBNA1 can bind RNA *in vitro* through arginine/glycine motifs (Snudden *et al.,* 1994). EBNA1 also activates expression of the lymphoid recombinase genes (RAGs) through an as yet unidentified mechanism (Srinivas and Sixbey, 1995). Activation of the RAGs could promote chromosomal rearrangements and translocations and possibly also facilitate viral integration. This may indicate that EBNA1 can activate expression of critical cellular genes and affect cellular growth control. Expression of EBNA1 in EBV negative cell lines has no obvious effect upon cellular growth characteristics. However, the expression of EBNA1 in the B cells of transgenic mice has been shown to be associated with the development of lymphocytic lymphoma and leukaemia suggesting that EBNA1 predisposes the mouse lymphocytes to oncogenic change (Wilson and Levine, 1992).

1.5.2 EBNA2

EBNA2 plays a central role in the immortalisation of primary B lymphocytes by EBV, and is one of the first genes to be expressed during this process. The EBNA2 gene encodes an 83 kD protein which localises in large nuclear granules and is associated with nucleoplasmic chromatin and nuclear matrix fractions (Petti *et al*, 1990). The EBNA2 protein is overall acidic, containing a polyproline region, a glycine-arginine repeat and a highly acidic carboxy terminus (Dambaugh *et al.,* 1984). Like EBNA-LP and EBNA1, EBNA2 is phosphorylated on serine and threonine residues and must undergo significant post-translational modification in addition to phosphorlyation as the size of the nascent protein is smaller than that of the stable intranuclear EBNA2 (Kieff, 1996). EBNA2 is a specific *trans*-activator of latent viral genes and certain cellular genes including the B cell activation marker, CD23 (Wang *et al*, 1987, 1990, 1991), the B lymphocyte differentiation marker, CD21 (Cordier *et al*, 1990) and the *c-fgr* oncogene (Knutson, 1990). Viral genes transactivated by EBNA2 include LMP1 (Abbot *et al.,* 1990; Ghosh and Kieff, 1990; Wang *et al.,* 1990b; Tsang *et al.,* 1991; Fahraeus *et al.,* 1993), LMP2 (Tsang *et al.,* 1991) and the *cis*-acting element upstream of the Cp promoter (Walls and Perricaudet, 1991; Sjoblom *et al.,* 1995).

Three regions have been located which appear to be stringently required for transformation and the *trans*-activating activity of EBNA2, between amino acid residues 95-110, 280-337 and 425-462 (see Figure 1.5). While the role of the 95-110 region is unclear, the 425-462 region is essential due to its acidic *trans*-activating characteristics. Detailed analysis of this region indicates that it is similar in many respects to the prototype VP16 acidic domain (Cohen and Kieff, 1991, Cohen, 1992) The 425-462 domain shares with VP16 an affinity for the transcription factors TFIIB, TAF40, TFIIH and RPA70 suggesting a critical role for this region in recruiting these factors to EBNA2-responsive promoters. The main function of the 280-337 region is to mediate interactions with DNA sequence-specific binding proteins as EBNA2 is unable to interact directly with its responsive elements. Thus, targeting of EBNA2 to specific DNA sequences is achieved through the exploitation of a ubiquitously-expressed cellular

DNA-binding protein, RBP-Jκ (CBF1). Interaction of EBNA2 with RBP-Jκ has been demonstrated in vitro (Grossman *et al*, 1994; Henkel *et al*, 1994) and in vivo (Yalamanchilli *et al*, 1994).

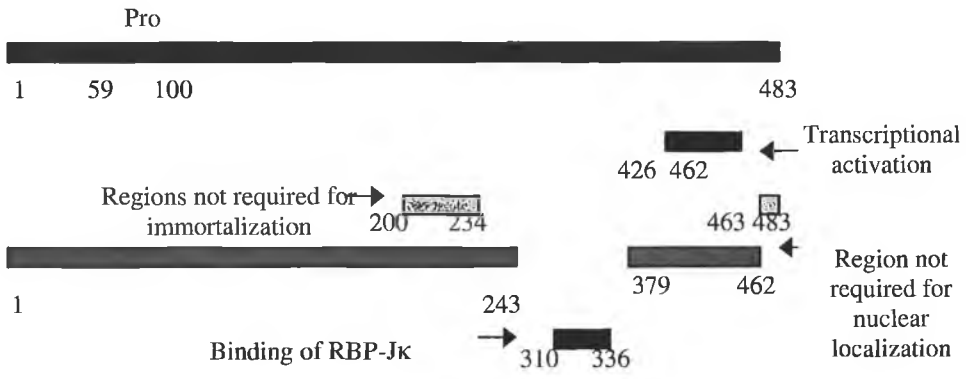


Figure 1.5 Functional domains of EBV nuclear antigen 2 (EBNA2). The pro box is a region composed entirely of proline, it varies in length between viral strains. Adapted from Farrell 1995.

EBNA2 activates gene expression through a common cis-regulatory element found in both viral and cellular promoters. EBNA2 response elements (E2RE) have been characterised upstream of the EBV LMP-1, LMP-2A and Cp promoters as well as the CD23 promoter. Each E2RE includes MNYYGTGGGAA, which includes the cognate sequence for RBP-Jκ, CGTGGGAA. Additional protein binding sites are present in E2REs: for example, mutation of the Spi-1 (a member of the ets family of transcription factors, also known as PU.1) binding site has a profound effect on the responsiveness of the LMP1 promoter (Johannsen *et al*, 1995; Laux *et al*, 1994a). Spi-1/PU.1 is also likely to be an important member for many of the cellular genes that are activated by EBNA2 , as it is frequently involved in B lymphocyte-specific gene transcription.

RBP-Jκ is a highly-conserved repressor protein which is part of a signaling pathway initiated at the Notch receptor (Tun *et al.,* 1994). Upon ligand binding, the cytoplasmic domain of Notch is assumed to be cleaved off the membrane and translocated to the nucleus where it activates target genes by converting the RBP-Jκ repressor protein to an activator (Artavanis-Tsakonas *et al.,* 1995). Notch1 is a human Notch first identified at

the breakpoint of a recurrent chromosomal translocation associated with a subset of human T-cell acute lymphoblastic leukaemia/lymphomas (T-ALL) (Aster *et al.,* 1997). A truncated oncogenic form of Notch 1 has been identified in human T-ALLs and EBNA2 has been found to mimic this constitutively activated Notch in its mode of action through binding of RBP-Jκ, thus linking EBNA2 to cell transformation. Mutational analysis of the EBNA2-responsive regions of the various promoters reveals a high similarity between EBNA2- and Notch1-transactivation regarding the crucial cis-elements. In a recent study, activated Notch1 stably introduced into EBNA2-negative BL cell lines, in a regulatable fashion, modulated expression of most but not all EBNA2-regulated cellular genes (Strobl *et al*, 2000). In the same study, stable introduction of an activated Notch1 expression construct into an LCL with an oestrogen-dependent EBNA2-oestrogen receptor fusion protein (EREB2-5 cells) revealed that after oestrogen withdrawal activated Notch1 was not sufficient to maintain proliferation. From these data, it may be concluded that EBNA2 exerts additional functions apart from triggering the Notch pathway, in the process of B cell immortalisation.

A consistent feature of BL cells is the transcriptional activation of the proto-oncogene c-*myc* by chromosomal translocation (Bornkamm *et al.,* 1988; Spencer and Groudine, 1991). The most frequent translocation t(8;14) fuses the c-*myc* gene locus on chromosome 8 to the constant region of the Ig heavy chain gene locus on chromosome 14. Since BL cells are thought to proliferate through activation of the c-*myc* gene the growth promoting function of EBNA2 may not be required in the setting of BL. A novel function of EBNA2 has been described using an oestrogen responsive system whereby the expression of EBNA2 is controlled by the presence or absence of oestrogen (Jochner *et al.,* 1996). EBNA2 down-regulates surface IgM expression and transcription of the Ig-μ locus very efficiently. In BL cell lines with the t(8:14) translocation, down-regulation of Ig-μ is associated with concomitant transcriptional shut-off the c-*myc* gene, reflecting the fact that c-*myc* is under the control of Ig heavy chain locus in these cells. The function of EBNA2 as a negative regulator of Ig-μ provides an explanation for the growth inhibiting effect of EBNA2 in cells carrying a t(8;14) translocation (Jochner *et al.,* 1996). The down regulation of IgM expression by EBNA2 may also provide an

explanation for the long standing observation that EBV negative BL cells in culture tend to have higher levels of Ig expression than their EBV positive counterparts (Benjamin *et al.,* 1982; Cohen *et al.,* 1987; Magrath *et al.,* 1990). Ig-μ and c-*myc* are down-regulated by EBNA2 at the transcriptional level and the transcription of Ig-μ and c-*myc* are affected by EBNA2 simultaneously. This suggests that EBNA2 is mediating its effect on expression through a common target, presumably a transcription factor (Jochner *et al.,* 1996).

EBNA2 not only plays a key role in the cascade of events leading to B cell transformation but is also essential for the maintenance of the transformed state. Using an LCL conditional for functional EBNA2 expression in the presence of oestrogen, it has been found that cells deprived of functional EBNA2 entered a quiescent non-proliferative state reminiscent of normal resting B cells or die by apoptosis (Kempkes *et al*, 1995). Functional EBNA2 was shown to be required at both the G_1 and G_2 phases of the cell cycle, while a role in terminating S phase could not be excluded. It could be concluded from the findings of this study that EBNA2 induces B-cell activation and entry into the cell cycle by inducing and maintaining the expression of early G_1-regulating proteins.

A more recent study using an LCL conditional for functional EBNA2 expression (as described above) has found that the transcription of the proto-oncogene c-*myc* was activated by EBNA2, but the precise mechanism of this transcription activation remains to be elucidated (Kaiser *et al.,* 1999). It was also concluded that in contrast to c-*myc* and LMP1, neither cyclin D2 nor cdk4 is a direct EBNA2 target. Cyclin D2 and cdk4 are both elements of the basic cell cycle machinery and drive cell cycle progression in early G_1. Since it has been shown that different B cell activation protocols can induce cyclin D2 and cdk4, it may be that the induction of proliferation by EBNA2 is a secondary event potentially driven by the primary viral and cellular EBNA2 targets (Kaiser *et al.,* 1999).

1.5.3 EBNA-LP

The EBNA leader protein (also known as EBNA-5) is so named because it is encoded by the 5' leader sequence of bicistronic mRNAs specifying the other EBNAs. The translation initiation codon for EBNA-LP is created by a splicing event that occurs near the 5' end of the message (Sample *et al.,* 1986; Speck *et al.,* 1986; Rogers *et al.,* 1990). The EBNA-LP protein is composed of amino-terminal repetitive segments followed by a unique carboxy-terminal sequence. Thus, EBNA-LP is frequently observed as a ladder of proteins in gel electrophoresis which differ in the number of amino-terminal repeats due to the fact that the repetitive part of the protein is derived from exons in the major internal repeat of the virus, which varies in copy number in any EBV population (Hammerschmidt and Sugden, 1989). The protein is strongly associated with the nuclear matrix, with an unusual distribution as illustrated by immunofluorescence microscopy - a proportion is diffusely spread through the nucleus while the rest is concentrated in a few granules frequently distributed in curved linear arrays (Petti *et al.,* 1990; Jiang *et al.,* 1991).

Although EBNA-LP appears to be important at least for initiation of B cell transformation, it's precise role has not been well characterised. Previous work has focused attention on cell cycle-related effects of EBNA-LP expression (Allan *et al,* 1992; Inman and Farrell, 1995; Kitay and Rowe, 1996). A role in cell cycle regulation is suggested by the finding that, along with EBNA2, EBNA-LP is the first viral gene product detected upon primary infection of resting lymphoblasts Also, EBNA-LP mutant virus-immortalised cells show delayed transit through the G_1 phase of the cell cycle. Association of EBNA-LP with pRb and p53 has been suggested based on *in vitro* biochemical interaction and colocalization of EBNA-LP with pRb as detected with one antibody and not another, however the *in vivo* relevance of this is unknown (Jiang *et al.,* 1991; Szekely *et al.,* 1993). Transient transfection of EBNA-LP and EBNA2 into primary B-lymphocytes co-stimulated with gp350 indicated that the two proteins co-operate in the induction of G_0 to G_1 transition as marked by induction of cyclin D_2, however, the mechanism of action remains unclear (Sinclair *et al.,* 1994). Furthermore,

EBNA-LP phosphorylation is dependent on the cell cycle stage (Kitay and Rowe, 1996). Recent studies suggest that this protein greatly enhances EBNA2-induced trans-activation of LMP1 expression through interaction with the EBNA2 acidic trans-activation domain (Harada and Kieff, 1997).

1.5.4 LMP1

Latent membrane protein 1 (LMP1) is one of six EBV proteins essential for immortalisation of normal resting B cells. LMP1 demonstrates the features of a classical oncogene, as defined by its ability to transform rodent fibroblast cell lines and render them tumorigenic. LMP1 is the only EBV gene that has transforming effects in non-lymphoid cells. In both Rat-1 (Wang *et al*, 1985) and BALB/c 3T3 (Baichwal and Sugden, 1988) cells, LMP1 expression permits growth at lower serum concentrations, and promotes anchorage-independence and loss of contact inhibition. Furthermore, LMP1 has pleiotropic effects when expressed in cells that are natural targets for EBV infection, human lymphocytes and epithelial cells. There is also evidence of synergy of LMP1 with EBNA2 in upregulating certain cellular proteins (Rowe, 1995; Wang *et al*, 1990). In epithelial cells, LMP1 blocks normal differentiation, a property which, together with the upregulated levels of CD40 and the epidermal growth factor receptor (EGFR) (Fahraeus *et al*, 1988), may be important in the pathogenesis of undifferentiated carcinoma of the nasopharynx. In primary NPC biopsies, the expression of EBV latent genes is more restricted than in latently infected B lymphocytes. One study of rare preinvasive NPC lesions has demonstrated LMP1 expression in all cells in 100% of cases, suggesting an important role for this protein in the development of this malignancy (Rajadurai *et al*, 1995). LMP1 is toxic when expressed at high levels in both lymphoid and epithelial cell lines, a shared phenotype that indicates that one or more of it's activities or its level of expression must be regulated to permit survival of the host cell (Farhaeus *et al*, 1988).

LMP1 mRNA is the second most abundant viral transcript in latently infected cells (Fennewald *et al*, 1984; Sample and Kieff, 1990). Expression of LMP1 in B lymphoma cell lines induces many of the phenotypic changes characteristic of EBV-mediated immortalisation of primary lymphocytes. LMP1 induces cell size increase, cell clumping, increased villous projections, increased vimentin expression and modulates expression of a range of cell surface receptors that are mediators of signaling events that affect cellular activation or proliferation. These include CD23 (low affinity IgE receptor) and CD21 (C3d/EBV receptor), CD39, CD40, CD44 and class II major histocompatibility complex (MHC II), adhesion molecules such as ICAM-1, LFA-1 and LFA-3 and the B cell promoting cytokine, IL-10 (Wang *et al*, 1988b; Birkenbach, 1989; Wang *et al*, 1990a; Liebowitz *et al*, 1992; Peng and Lundgren 1992; Nakagomi *et al*, 1994; Zhang *et al*, 1994a; Kieff, 1996). In contrast, expression of CD10 is decreased by LMP1 (Wang *et al*, 1990). Although LMP1 obviously plays an important role in EBV-mediated B cell immortalisation, the signal transduction pathways leading to modulation of cellular gene expression have not been fully elucidated.

1.5.4.1 LMP1 structure

The LMP1 gene product, encoded by 3 exons, consists of a 20 amino acid hydrophilic amino-terminal cytoplasmic domain, 6 markedly hydrophobic transmembrane domains (alpha helicase transmembrane segments each 20 amino acids long) separated by short reverse turns (each 10 amino acids in length) and a 200 amino acid carboxy-terminal cytoplasmic terminus that is rich in acidic residues (Figure 1.6).

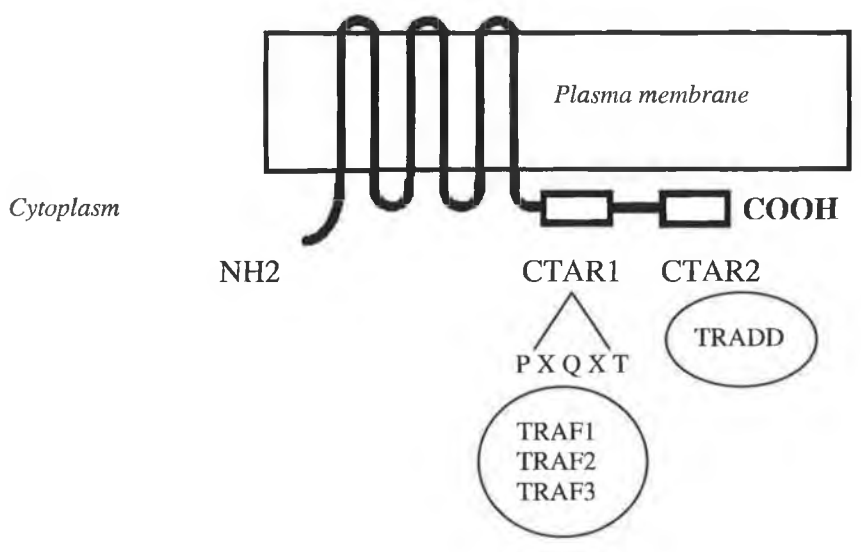


Figure 1.6 Schematic representation of LMP1 (adapted from Puls and others, 1999). LMP1 consists of an N terminal cytoplasmic domain, six hydrophobic transmembrane domains separated by reverse turns and a 200 amino acid C-terminal domain (CTD). Two signaling domains, CTAR1 and CTAR2 are located in the CTD and has been shown to interact with TNFR-associated factors TRAF1-3 and TRADD, respectively as indicated in the figure.

LMP1 migrates on SDS-PAGE between 58 and 63 kDa depending on the EBV strain. The transmembrane domains enable LMP1 to posttranslationally insert into membranes and to accumulate in aggregates (Hennessy *et al*, 1984; Liebowitz *et al*, 1986). Live-cell protease cleavage experiments indicate a cytoplasmic orientation of the amino- and carboxy-terminal domains and an extrinsic plasma membrane orientation of the first outer reverse turn domain (Liebowitz *et al*, 1986). Shortly after being synthesised, LMP1 is phosphorylated on serine and threonine residues in the carboxy-terminal domain and becomes tightly bound to the cell cytoskeleton (Liebowitz *et al*, 1987). A substantial proportion of LMP1 (at least half) localises to a patch at the periphery, where it is closely associated with vimentin intermediate filaments (Liebowitz *et al*, 1987). Unbound, nascent LMP1, as determined by detergent-solubility, has a half-life of less than 2 hours, while the insoluble, phosphorylated, cytoskeleton-associated form, has a half life of 3-15 hours (Moorthy and Thorley-Lawson, 1990, 1993a). LMP1 is transcribed during lytic infection and full size LMP1 is incorporated into virions, indicating that virion-associated products may affect the growth of newly-infected cells (Mann *et al*, 1985).

1.5.4.2 Signal Transduction by LMP1

The structure of LMP1 as a membrane protein with cytoplasmic amino-terminal and carboxy-terminal domains as outlined above, is just one of several characteristics which are consistent with it stimulating existing cellular signal transducing pathways. LMP1's oncogenic activity correlates with its ability to attach to the cytoskeleton, localise in patches in the plasma membrane and turn over rapidly (Liebowitz *et al*, 1996; Mann and Thorley-Lawson, 1987). These are properties which are shared with activated growth factor receptors. As an integral membrane protein, LMP1 acts like a constitutively active receptor (Gires *et al*, 1997) and shares certain characteristics with members of the TNFR family (reviewed by Kieff *et al*, 1996). In a manner similar to other members such as CD30, CD40 and TNFR1, LMP1 binds specific TNFR associated factors (TRAFs) which have been found to associate directly with it's carboxy-terminal region

(Figure1.6). Moreover, LMP1 activates the transcription factor NFκB and the c-Jun N-terminal kinase 1 (JNK1, also known as stress activated protein kinase) cascade by pathways which involve TRAF molecules. Since LMP1 acts in a ligand-independent manner, it replaces the T cell-derived activation signal necessary to sustain indefinite B cell proliferation.

Although LMP1 and CD40 share very little protein homology, their shared functions have led to suggestions that LMP1 may mimic B cell activation processes which are physiologically triggered by CD40-CD40 ligand signals. Recently, it was reported that LMP1 activates B cells to secrete Ig and IL-6 and rescues them from B cell receptor-mediated growth arrest analagous to CD40 signaling, and that LMP1 and CD40 signaling pathways interact cooperatively in inducing B cell effector functions (Busch and Bishop, 1999). Also, in EBV-immortalised B cells lacking a functional EBNA2 protein, CD40 activation and LMP1 expression result in the same phenotype of prolonged cell survival and DNA synthesis (Zimbler-Strobl *et al*, 1996). These and other similarities, including the ability to upregulate genes such as CD54 (ICAM1) and to affect cell growth and apoptosis have led to the suggestion that LMP1 signaling is similar or even identical to CD40 signaling. However, in Jurkat T cells, while ligand-induced CD40 signaling was found to be impaired, LMP1 was demonstrated to be fully functional, therefore indicating that signaling by LMP1 and CD40 differ (Floettmann *et al*, 1998). Two regions within the cytosolic domain of LMP1 have been found to effect cell signaling. One of these, the carboxy-terminal activation region-1 (CTAR1), binds members of the TRAF family of proteins and the other, CTAR2, binds the TNFR associated death domain protein (TRADD). Using mutated LMP1 genes in Jurkat cells, it has been shown that LMP1 loses it's ability to upregulate the CD54 cell surface marker, when either the CTAR1 or CTAR2 domain is non-functional, thus behaving like CD40. However, the CTAR1 domain of LMP1, which shares a TRAF binding sequence motif with CD40, differs from CD40 in being unable to activate NFκB in Jurkat cells (Floettmann *et al*, 1998).

Activation of NFκB involves two independent domains in the cytoplasmic C-terminal tail; the TRAF-interacting site which associates with TRAFs 1,2,3 and 5 via a PXQXT/S core motif and a TRADD interacting site. Although NFκB appears to mediate the upregulation of many genes by LMP1 eg. A20 (Laherty *et al*, 1992), it has become clear in recent years that NFκB has only a partial role to play in LMP1 induced B cell activation. For example, ICAM-1 and CD71 were shown to be upregulated in the presence of a dominant inhibitory IκB mutant. The characteristic LMP1-mediated cell size increase was similarly unaffected in the absence of NFκB activation (Liljeholm *et al*, 1998). Also, induction of EGFR in epithelial cells by LMP1 or CD40 is mediated through a signaling pathway which is distinct from NFκB (Miller *et al*, 1997).

LMP1 expression also results in activation of the c-Jun N-terminal kinase (JNK) cascade (Kieser *et al*, 1997; Epiopolos, 1998), an effect which is mediated exclusively through CTAR2 and can be dissociated from NFκB induction. A recent study identified the extreme 8aa of the CTAR2 region as important for JNK signaling via a mechanism involving TRAF2 and TRADD (Eliopoulos *et al*, 1999). Using a tetracycline-regulated LMP-1 allele, JNK was shown to be an effector of non-toxic LMP1 signaling in B cells. JNK was also shown to mediate activation of the AP-1 transcription factor, a dimer of Jun/Jun or Jun/Fos proteins (Kieser *et al*, 1997) which is readily acitivated by growth factors and mitogens. The JNK cascade is complicated by having at least eleven known upstream MAP kinase kinase kinases (ASK1, Tpl-2, DLK, TAK1, MEKK1,2,3,4 and MLK1,2,3) that may regulate the pathway in different cell types and in response to different stimuli (Ip and Davis, 1998). In addition to activation of NFκB/JNK1, LMP1 engages the p38 mitogen activated protein kinase cascade, leading to activation of the transcription factor, ATF2. While, like NFκB activation, p38 is induced via the CTAR1 and CTAR2 regions, the 2 pathways are primarily independent, with evidence suggesting a divergence of signals downstream of TRAF2. Using a highly specific inhibitor of p38, SB203580, LMP1-mediated IL-6 and IL-8 expression was found to utilise the p38 pathway (Eliopoulos *et al*, 1999).

As outlined above, LMP1 appears to mimick the molecular functions of TNFR1. However, TNFR1 regulates a wide range of cellular responses including apoptosis, whereas LMP1 constitutes a transforming protein. The molecular function of TRADD in LMP1 signaling has been found to differ from its role in TNFR1 signal transduction. JNK activation by TNFR1 involves Cdc42, whereas LMP1 signaling to JNK is independent of p21 Rho-like GTPases (Cdc42 has been implicated in several STPs, including organisation of the actin cytoskeleton and activation of both JNK and NFκB). Thus, although both LMP1 and TNFR1 interact with both TRADD and TRAF2, the different topologies of the signaling complexes correlate with substantial differences between LMP1 and TNFR1 signal transduction to JNK (Kieser *et al*, 1999). In support of this, activation of JNK and NFκB by the TNFα and IL-1 receptors and by LMP1 in fibroblasts was found to occur independently of Cdc42. Moreover, signaling to Cdc42 and to JNK/NFκB occurred through distinct pathways and members of the TRAF and TRADD families were not required for Cdc42 activation (Puls *et al*, 1999). Further studies are in progress in an effort to identify molecules capable of interacting with the transmembrane or loop regions of LMP1 as potential candidates for mediating Cdc42 activation.

LMP1 expression in a continuous human B-lymphoblast cell line causes steady-state increases in intracellular free calcium and acid production (Wang *et al*, 1998). A transient increase in intracellular free calcium is a prominent immediate manifestation of B lymphocyte activation (reviewed by Cambier *et al*, 1987). As a plasma membrane protein with multiple membrane-spanning domains, LMP1 could be a channel protein or could interact with a channel protein. Thus, modification of an ion channel could directly or indirectly lead to the observed increase in intracellular free calcium. Since measurements of radioactive calcium uptake or efflux in LMP1 expressing versus nonexpressing cells do not reveal a difference, the mechanism is more likely to be indirect (Wang *et al*, 1998). Many of the effects of increased intracellular free calcium are transmitted by the calcium receptor calmodulin which, upon binding calcium, activates a diverse family of effector molecules. An important component of the calcium signaling cascade in mammalian cells is a family of serine-threonine-specific, calcium-

calmodulin-dependent protein kinases, whose members have been implicated in a broad array of cellular processes including cell cycle progression and gene expression (reviewed by Hanson and Schulman, 1992). One member of this family of kinases, CaM kinase-Gr (a multifunctional calcium-calmodulin-dependent protein kinase type Gr) has been found to be upregulated by LMP1 in the EBV-negative cell line, BJAB. This kinase is absent from primary human B lymphocytes but is expressed in EBV-transformed B-lymphoblastoid cell lines (Mosialos *et al*, 1994). CaM kinase Gr was reported to mediate cell cycle progression in Xenopus eggs (Lorca *et al*, 1993) and may have a similar role in EBV-transformed B lymphocytes. The LMP1-induced kinase is activated in B lymphocytes by increased intracellular free calcium in response to surface IgM crosslinking (Mosialos *et al*, 1994).

1.5.5 LMP2A and 2B

The LMP2 gene is transcribed as two alternatively-spliced mRNAs which encode LMP2A and -2B proteins, also known as terminal proteins -1 and –2 (Laux *et al.,* 1988; Sample *et al.,* 1989). LMP2A and –2B are found in latently-infected, growth transformed lymphocytes in vitro, in different human tumours and in latently infected B cells in vivo. LMP2A, but not LMP2B, is detectable in B lymphocytes isolated from peripheral blood of EBV sero-positives (Qu and Rowe, 1992) and LMP2 transcripts as well as antibodies against the proteins have been detected in patients with nasopharyngeal carcinoma (Brooks *et al*, 1993). The LMP2 mRNAs are formed by transcription and splicing across the joined termini of the circularised EBV genome as it is present in latently infected cells. (Laux *et al.,* 1988; Sample *et al.,* 1989). The two messages consist of different 5' exons and eight common exons and are predicted to encode nearly identical proteins differing only in the length of their hydrophilic amino termini (Ring, 1994). Transcription of the LMP2A starts 3Kb downstream of the LMP1 transcription start site (Laux *et al.,* 1988; Sample *et al.,* 1989). The LMP2B and LMP1 promoters form bi-directional transcription units containing a common EBNA2 responsive element, while a separate EBNA2 response element regulates LMP2A transcription (Zimber-Strobl, *et al.,* 1993). LMP2A and 2B are 54 and 40 kDa in size respectively. Both proteins are predicted to encode twelve highly hydrophobic

membrane-spanning domains and are localized to patches in the plasma membrane of infected cells, in close association with LMP1 (Longnecker and Kieff, 1990). LMP2A and 2B are not required for EBV mediated immortalization but they do improve efficiency of transformation (Longnecker *et al.,* 1992; Brielmeir *et al.,* 1996). The LMP2 proteins are phosphorylated on serine, threonine and tyrosine residues (Longnecker *et al.,* 1991) and have been shown to interact with *src*-family tyrosine kinases in EBV-infected B cells (Burkhardt *et al.,* 1992) They have also been associated with another stably phosphorylated tyrosine kinase *syk (Miller et al, 1995).* These interactions suggest that the LMP2s play a role in transmembrane signal transduction (Kieff, 1996). LMP2A has been shown to inhibit anti-immunoglobulin-mediated Ca^{2+} mobilization, PKCγ2 activation and anti-immunoglobulin-induced reactivation of the lytic cycle, which can be bypassed by TPA with Ca^{2+} ionophores (Miller *et al.,* 1994b, 1995a). These data are consistent with a model in which LMP2A sequesters the receptor associated tyrosine kinase, blocking its autophosphorylation and downstream signaling events (Miller *et al.,* 1995a).

1.5.6 EBV-encoded RNAs (EBERs)

EBER expression lags behind that of the other EBNA and LMP proteins and is delayed until after the initiation of DNA synthesis (Alfieri *et al,* 1991). The two EBV-encoded, small nonpolyadenylated RNAs, EBER1 and EBER2, are by far the most abundant EBV RNAs in latently infected cells, with an estimated abundance of 10^7 copies per cell. They are usually transcribed by RNA polymerase III although polymerase II may also be involved. Most EBERs are located in the nucleus and are associated at the 3' terminus with the cellular La antigen (Howe and Steiz, 1986; Howe and Shu; 1989). EBER 1 and 2 have extensive sequence similarity to adenovirus VA1 and VA2 and cell U6 small RNAs, both of which form similar secondary structures and complex with La protein (Rosa *et al.*, 1981; Glickman *et al.*, 1988). The role of the EBERs is unclear but based on the functions of VA and U6 RNAs two alternative roles have been proposed for the EBERs. Firstly, the EBERS can partially complement VAI-mediated inhibition of activation of an interferon-induced protein kinase, which blocks transcription by

phosphorylating the protein-synthesis initiator factor eIF-2α. Secondly a possible role of the EBERs is the splicing of the primary EBNA and LMP1 mRNA transcripts (suggested by the partial complementarity to RNA splice sites). Although the delayed expression of the EBERs appears to be incompatible with both of these proposed functions, nevertheless, the earlier events in primary B-cell infection are sensitive to interferon (IF) and EBERs may play a role in blocking eIF-2 kinase (Thorley-lawson, 1980; 1981). EBV recombinants in which the EBERs have been deleted can initiate primary B cell infection and growth transformation in the same way as wild-type virus, and no differences have been observed in the growth of LCLs infected with EBER-deleted and control virus or in the permissiveness of these cells for lytic infection (Swaminthatan *et al*, 1991).

1.5.7 EBNA-3A, -3B and –3C

Examination of the coding sequences of the EBNA-3A, EBNA-3B and EBNA-3C (also known as EBNA3, EBNA4 and EBNA6, respectively) genes reveals that each is composed of one short and one long exon (of similar sizes in all three genes). The genes are similar in structure and are tandemly located in the *Bam*H1 region of the EBV genome (Bodescot *et al*, 1986; Joab *et al*, 1987; Peit *et al*, 1988; Kerdiles *et al*, 1990). These features have led to the proposal that the EBNA-3 genes may have arisen by gene duplication, although there is little overall homology between the protein sequences. The proteins are remarkably hydrophilic overall, contain unusual clusters of charged amino acids, and have similarly positioned clusters of negatively charged amino acids. The type 1 EBNA-3A, -3B and -3C genes encode proteins which migrate on SDS gels at 145, 165 and 155 kDa, respectively. Comparison of the primary sequences of EBNA-3 proteins reveals a similar organisation (Le Roux *et al*, 1994) as follows (see Figure 1.6).

1. a ca. 90 amino acid hydrophilic N-terminal end,
2. a region of 220 -230 residues that shows 23 - 28% amino acid identity,
3. a region of short sequences rich in negatively- and positively-charged amino acids,
4. a proline-rich C-terminal half containing repeated polypeptide domains.

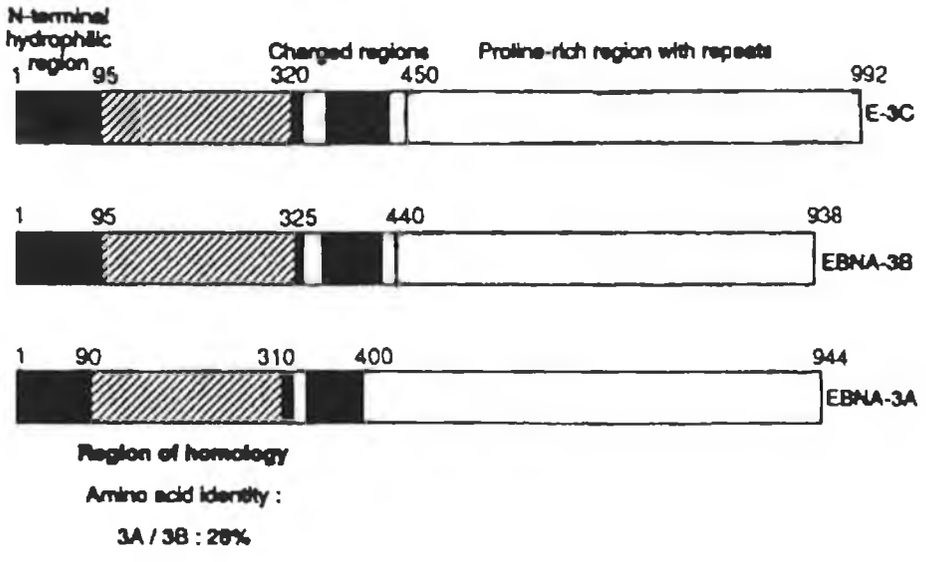


Figure 1.7 Structural and sequence similarites among the EBNA3 family of proteins. In the charged regions, sequences rich in basic amino acids are shown in white, sequences rich in acidic amino acids are shown in black, sequences rich in both types of charged residuesare checkered. Amino acid identity was scored between residues 90 and 320 of each EBNA3 protein using Kanehisa's sequence comparison program (Fortini and Artavanis-Tsakonas, 1994).

The EBNA-3s encode more than half of the translated open reading frames in latently infected growth-transformed lymphoctes. However, their precise functions in vivo are as yet poorly understood. It is considered likely that the three proteins have unique functions since recombinant EBVs that carry null mutations in either EBNA-3A or EBNA-3C are nonimmortalising (Tomkinson and Kieff. 1992; Tomkinson *et al*, 1993). This implies that EBNA-3A and -3C individually possess different functions that are essential for immortalisation and which cannot be complemented by other members of

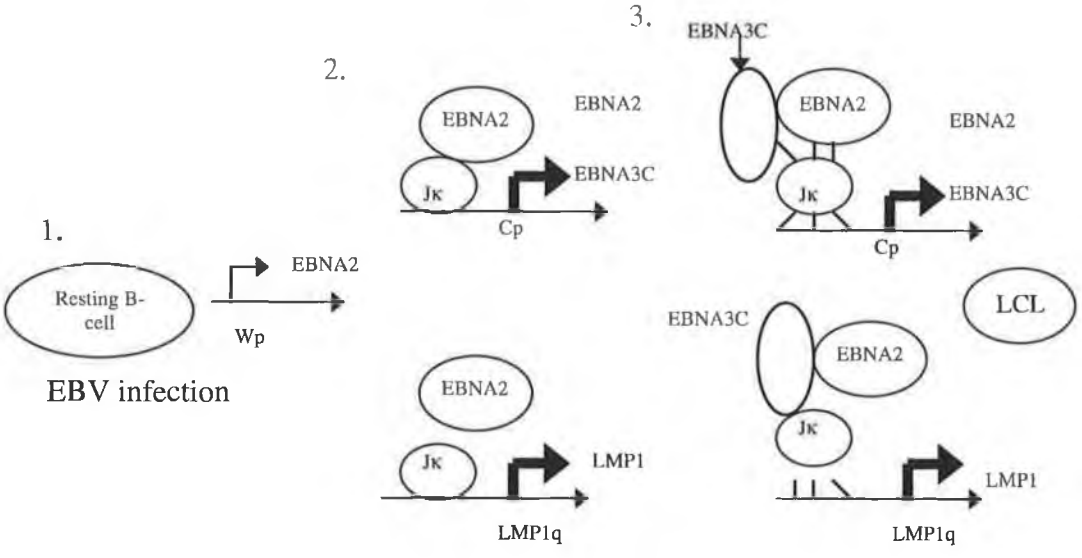
the EBNA-3 family. In constrast, in vitro experiments have shown that EBNA-3B is not critical for EBV latent infection, cell growth transformation or lytic virus replication in B lymphocytes (Tomkinson and Kieff, 1992). EBNA-3A, -3B and -3C each include epitopes that are frequently recognised by EBV-immune cytotoxic T lymphocytes (CTLs). Given the importance of EBNA-3A, -3B and -3C epitopes in CTL recognition of EBV-infected lymphocytes (Gavioli *et al.*, 1992) strains with deletions of all or part of each of the EBNA-3 proteins would have been expected to have arisen and been identified if these proteins were not important for infection in vivo.

There is increasing evidence that EBNA3C, like EBNA2, functions as a trans-activator of both cellular and viral genes. Transfection of an EBNA3C expression construct into an EBV negative BL cell line has been shown to result in the upregulation of the EBV receptor CD21 (Wang *et al.*, 1990). Furthermore, expression of EBNA3C in the Raji cell line (in which the EBV genome is deleted for most of the EBNA3C open reading frame) induces an up-regulation of LMP1 and the cellular proteins CD23 and vimentin (Allday *et al.*, 1993; Ring, 1994). EBNA-3C can co-operate with activated Ha-ras in the transformation of primary murine fibroblasts. EBNA-3C has a glutamine-proline-rich domain which can substitute for the acidic transactivation domain of EBNA2 and a similar glutamine-proline-rich domain has been found in EBNA-3B (Cohen and Kieff, 1991). A novel transcription activation function for EBNA-3A, perhaps analagous to that reported previously for EBNA-3C, was recently revealed although there is no substantial sequence homology between the two proteins in the parts involved in transcription activation (Marshall and Sample, 1995).

When stably expressed in group I DG75 BL cells, EBNA-3B, has been found to upregulate vimentin as well as surface expression of the activation antigen, CD40, while the Burkitt's Lymphoma-associated antigen BLA/CD77 is down-regulated (Sillins and Sculley, 1994). Functional analogies have been drawn between EBNA-3B and the EBV latent membrane protein, LMP-1. It is thought that EBNA-3B and LMP-1 may co-ordinately regulate CD40 and vimentin levels, as LMP1 has also been found to upregulate both these proteins. In addition, a further study found that DG75 cells

induced to undergo apoptosis in response to serum starvation were protected in the presence of EBNA-3B (Sillins and Sculley, 1995), a feature also common to LMP1. Cell enlargement has been observed as a prominent feature of EBNA-3B-expressing BL clones (Wang *et al*, 1988). Thus, EBNA-3B may have the potential to contribute in several different ways to the transformation process.

The most widely documented and common function of the EBNA3 proteins is their capacity to inhibit EBNA2-mediated activation of the TP1 and LMP1 promoters in transient expression assays (Le Roux *et al*, 1994; Marshall and Sample, 1995; Robertson *et al*, 1995). EBNA2 is a key transactivator of a wide spectrum of viral and cellular genes (see section 1.5.2) and interaction with the RBP-Jk transcription factor is critical for the specific targeting of EBNA2 to the responsive elements of many of these genes. By destabilizing RBP-Jκ and EBNA2/RBP-Jκ complexes from binding to their cognate RBP-Jκ binding sites (see Figure 1.8), EBNA3A, 3B and 3C proteins have been shown to inhibit the transcriptional activation of EBNA2 responsive promoters (Le Roux *et al.*, 1994). EBNA3 proteins are thus believed to counter balance and finely tune the action of EBNA2 (Waltzer *et al.*, 1996; Robertson *et al.*, 1996; Zhao *et al.*, 1996).



1. EBNA transcription 2. EBNA2 transactivation of transcription 3. EBNA3C modulation of transcription.

Figure 1.8 A schematic model of the mechanism by which EBNA3C counteracts EBNA2-mediated transactivation. EBNA3C destabilises the interaction of RBP-Jκ and RBP-Jκ/EBNA2 complexes binding DNA (Adapted from Roberston *et al.*, 1995).

As EBNA2 can also be recruited to promoters through interaction with other factors such as the proteins from the PU.1 family, inhibition of EBNA2-mediated transcriptional activation by the EBNA3 proteins could be restricted to promoters activated through RBP-Jκ binding sites. This could represent a way to differentially regulate certain viral or cellular genes (Waltzer *et al.*, 1996). For example, by inhibiting transactivation of the Cp promoter, a feedback loop of inhibition by EBNA3 proteins of their own synthesis is created. Furthermore, it has been suggested that by inhibiting EBNA2-mediated transactivation, EBNA-3A, -3B and -3C could down-regulate the expression of EBV latent proteins that bear epitopes provoking the anti-EBV cytotoxic T cell response, thus allowing EBV to escape host immune surveillance. It could also be important to down-regulate LMP1 expression, as LMP1 has been shown to be toxic to the cells when overexpressed. However, all the data demonstrating the inhibitory effect of the EBNA-3

proteins on EBNA-2 activation are based on over-expression assays and may not pertain specifically to effects at physiologic levels of protein.

RBP-Jk was originally identified and erroneously named as a potential recombinase that appeared to bind to the immunoglobulin kappa J region (Matsunami *et al*, 1989). RBP-Jk is now known to be a ubiquitous sequence-specific DNA-binding repressor protein that is a key mediator of the transcriptional regulatory effects of certain proteins, including EBNA2 (Abbott *et al.*, 1990; Cohen and Kieff, 1991; Henkel *et al*, 1994; Laux *et al*, 1994; Ling *et al.*, 1994; Zimbler-Strobl *et al*, 1994) and is a key component of the Notch signaling pathway. By using RBP-Jk as a target for EBNA2 and the EBNA3s, EBV effectively subverts the ability of B cells to control the expression of particular genes. Moreover, EBNA2 mimics cellular Notch which normally binds RBP-Jk and activates B cell genes in response to stimuli. RBP-Jk is known to be a key mediator of signaling from activated notch receptors in neural and muscular development (Fortini and Artavanis-Tsakonas, 1994; Jarriault, 1995; Goodburn, 1995; Artavanis-Tsakonas, 1995). Since constitutive notch activation is an important aetiologic factor in human T cell leukaemia (Ellison *et al.*, 1991), RBP-Jk is also implicated in leukaemogenesis. Thus the level of steady state association of EBNAs with RBP-Jk is likely to be important in EBV-driven B lymphocyte proliferation.

Since RBP-Jk is a critical component of EBNA-2 interaction with response elements, the binding of EBNA-3A, -3B and -3C to RBP-Jk likely indicates that RBP-Jk is also a critical mediator of EBNA-3 interactions with response elements (Le Roux *et al*, 1994; Allday and Farell, 1994; Robertson, 1995; Marshall and Sample, 1995; Robertson *et al*, 1996). In addition it has been suggested that the EBNA3 proteins could transactivate transcription by regulating the interaction of RBP-Jk with DNA. Thus, dissociation of the RBP-Jk repressor protein from DNA could activate promoters with RBP-Jk sites. A weakness in this model, however, is the lack of an apparent need for the size and complexity of the EBNA-3 gene family, since the N-terminal domain of one EBNA-3 protein would suffice for this effect.

All three EBNA3 family members have been shown to bind RBP-Jk domains (preferentially the smaller isoform) via their amino-terminal in B lymphoblasts (Robertsonet *et al.*, 1996). In vitro-translated EBNA-3A binds about 30-fold less efficiently than EBNA-3B and about 4-fold less efficiently than EBNA-3C to GST-RBP-Jk, but in vivo-expressed EBNA-3A, -3B and -3C bind similarly to RBP-Jk. The lower efficiency of binding of full-length vitro-translated EBNA-3A appears to be due to an inhibitory effect of the C-terminus when the protein is translated in vitro (Robertson *et al.*, 1996). The yeast two hybrid system has been used to delineate the sequences of both EBNA-3C and RBP-Jk mediating the interaction between these two proteins. It was found that a Jk domain of 56 amino acids (aa 125-181) was sufficient to bind EBNA-3C, while a conserved 74 aa domain of EBNA-3C and –3B (aa 181-257), was sufficient to interact with the Jk protein (Hsieh *et al*, 1996). Another report suggests that the EBNA3 site lies between amino acids 90 and 138. It was found that, EBNA-3A amino acids:1-138, EBNA-3B amino acids:1-311 and EBNA-3C amino acids:1-183 were sufficient for RBP-Jk interaction, while EBNA3B amino acids:1-109 showed less or no binding (Robertson *et al.*, 1996). These interacting domains overlap with the most highly conserved domain (aa 90-320) among the EBNA-3 proteins.

An alternative interpretation of the significance of the binding of EBNA3 proteins to RBP-Jk which may be equally relevant is the proposal that RBP-Jk acts to buffer the levels of active EBNA3 proteins, where RBP-Jk-EBNA3 complexes represent inactive forms of the EBNA3 proteins. There is considerable evidence that the levels of EBV transforming proteins have to be tightly regulated in LCLs and although the binding of EBNA3 proteins to RBP-Jk counterbalances the activation effects of EBNA2 on the Cp promoter (which dives transcription of the EBNA3 genes), Cp is in fact only partially dependent on EBNA2-RBP-Jk in LCLs (Evans *et al*, 1996). This alternative model supposes that there are novel functions unrelated to RBP-Jk binding for EBNA-3A and EBNA-3C in EBV transformation and is supported by the limited sequence homology exhibited between the EBNA3 proteins. The relationship of the exon structures and protein sequences is only significant in the N-terminal parts of the proteins and although all three proteins have repetetive sequence elements in the C-terminal regions, these are

unrelated at the sequence level. Also, it is unlikely that EBV would require four large viral proteins comprising 79% of the sequence complexity of the six viral proteins required for transformation to control signaling through RBP-Jk.

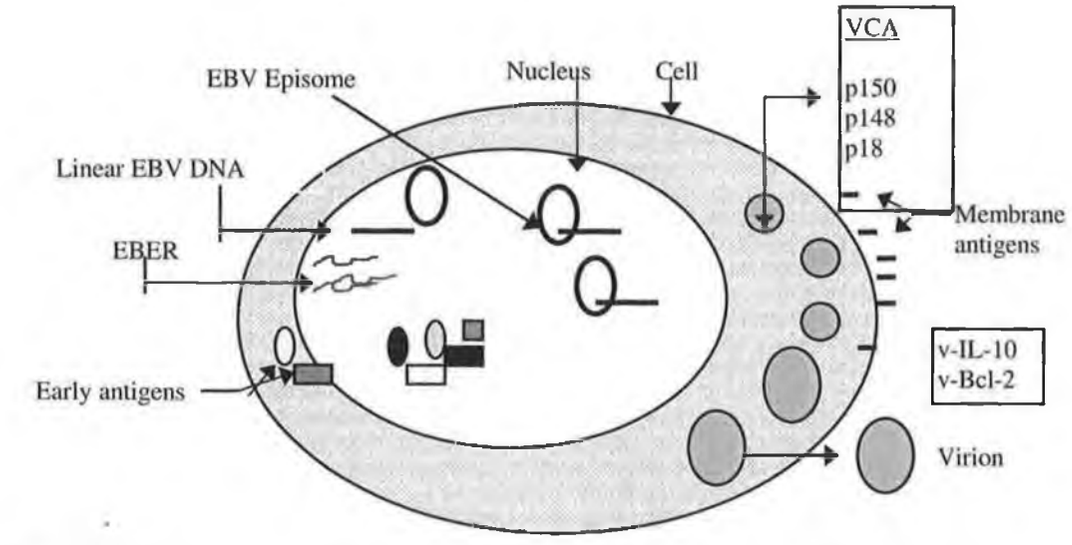
As outlined above, much evidence exists to indicate that the EBNA3 proteins should be regarded as transcriptional regulators (Bourillot *et al*, 1996; Cludts and Farrell, 1998). However, the effects of each of the EBNA3 proteins on cell promoters differ and are distinct from those of EBNA2 (Wang *et al.*, 1990; Sillins and Sculley, 1994). Also, the different effects of EBNA-3C, -3B and EBNA2 observed in non-EBV-infected BL cells likely indicate a role for other protein-protein interactions as determinants of transcriptional effects of the EBNA3 proteins on specific cell genes. Thus, like EBNA2, EBNA3 proteins may be dependent on interactions with factors other than RBP-Jk, and the role of the EBNA3 proteins can be expected to be more complex than a retro-control on RBP-Jk-EBNA2-activated genes. The identification and characterisation of other potential cellular partners for the EBNA3 proteins is required for a better understanding of their precise role in EBV immortalisation.

1.6 Genes of the Lytic Viral Cycle

In the study of viral replication, lytic infection is usually induced by chemicals (Luka *et al.,* 1979; Saemundsen *et al.,* 1980; Laux *et al.,* 1988b) as only a small fraction of latently infected B-lymphocytes spontaneously enters the productive cycle. In these cells the viral DNA is amplified several hundred fold by a lytic origin of DNA replication, *ori Lyt* (Hammerschmidt and Sugden, 1988). Phorbol esters are among the most reproducible and most broadly applicable inducers, their effect is probably mediated by protein kinase C activation of Jun-fos interactions with AP-1 upstream of the immediate early virus genes (Farell *et al.,* 1983; 1989; Farell 1992; Laux *et al.,* 1988). Some LCL cell lines can be induced to permit viral replication in about 10% of cells. Alternatively, the Akata cell line which carries an LMP2A-deleted virus can be induced by cross-linking of surface immunoglobulins (sIg) to the extent that more than 50% of the cells enter the lytic cycle (Takada, 1984; Takada and Ono, 1989). A second approach to

investigating viral replication is to induce the lytic cycle by superinfection of Raji cells with defective EBV from the P3HR-1 cell line (Mueller-Lantzsch *et al.,* 1980). Raji is an EBV-positive BL cell line with an unusually high EBV episome copy number, it is defective for DNA replication and late gene expression thus is tightly latent (Polack *et al.,* 1984a). Defective virions from P3HR-1 contain rearranged DNA molecules in which the intermediate early *trans*-activator of the lytic cycle are expressed after superinfection (Cho *et al.,* 1984; Miller *et al.,* 1984). After induction, cells that have become permissive to viral replication undergo cytopathic changes characteristic of herpesviruses, including migration of nuclear chromatin, synthesis of viral DNA, assembly of nucleocapsids, envelopment of the virus by budding through the inner nuclear membrane and inhibition of host macromolecular synthesis (IARC Monograph, 1997).

Studies with such cell lines has allowed the division of EBV replicative proteins into early antigens (EA), membrane antigens (MA) and virus capsid antigens (VCA). Early antigens are further subdivided into EA-D (diffuse) and EA-R (restricted) due to a different sensitivity to methanol fixation (Henle *et al.,* 1971a; 1971b) (see Figure 1.8). Virus gene expression follows a temporal and sequential order (Farrell, 1992; Takada and Ono, 1989). Some virus genes are expressed independently of new protein synthesis, early after induction and are classified as immediate early genes. Early lytic virus genes are expressed slightly later and their expression is not affected by inhibition of viral DNA synthesis (Kieff, 1996).



EA	ORF
EBV DNA polymerase	**BALF5**
Thymidine Kinase	**BXLF1**
Ribonucleotide reductase	**BORF2 & BARF1**
Alkaline DNase	**BGLF5**
DNA binding protein p188	**BALF2**

MA	ORF
gp350/220	**BLLF1**
gp150	**BDLF3**
gp110	**BALF4**
gp85	**BXLF2**
gp78	**BILF2**
gp42	**BZLF2**
gp25	**BKRF2**

Figure 1.9 A schematic representation of early and late EBV gene expression. The VCA the MA and the EA are illustrated, their open reading frames are written in bold.

1.6.1 Immediate Early genes

After P3HR-1 superinfection of Raji or sIg cross-linking of Akata cells in the presence of protein synthesis inhibitors, three leftward mRNAs are transcribed. The BZLF1, BRLF1 and BI'LF4 encoded proteins are potent transactivators of early EBV lytic gene expression (Takada and Ono, 1989; Marschall *et al.*, 1991; Kieff, 1996). The functional and physical interaction of BZLF1 with NFκB is an important mediator of LMP1 effects in EBV latent infection. BZLF1 can also downregulate the EBNA Cp promoter perhaps facilitating the transition from latent to lytic infection (Kenny *et al.*, 1989; Sinclair *et al.*, 1992). A recent study has found that BZLF1 inhibits both cellular diffentiation and cell

cycle progression in epithelial cells. In HeLa and SCC12F (human keratinocyte) cells, BZLF1 induced a G2/M block, whereas in fibroblasts a G1/S block was induced. The exact mechanism of this block is unknown, but activation of the cell cycle inhibitors p21 and p27 was not observed (Mauser *et al*, 1998). In a different study, BRLF1 was shown to bind Rb in vivo shortly after induction of the viral lytic cycle in EBV-infected Akata cells. This interaction may initiate cell cycle progression and facilitate viral DNA synthesis during lytic replication (Zacny *et al*, 1998).

1.6.2 Early genes

The early genes are expressed when the lytic cycle is induced in the presence of inhibitors of DNA synthesis. By this criterion at least 30 EBV mRNAs are early gene products (Hummel and Kieff, 1982a,b; Baer *et al.,* 1984). Two very abundant early proteins have been mapped to specific DNA sequences. The BALF2 protein is homologous to the HSV DNA binding protein ICP8 and is important in DNA replication (Hummel and Kieff, 1982a; Kieff, 1996). The BHRF1 protein, which is expressed in moderate abundance, has extensive collinear homology with *bcl-2* (Pearson *et al.,* 1983a; Austin *et al.,* 1988). BHRF1 can protect EBV negative BL cells from apoptosis (Mc Carthy *et al.,* 1996), however, EBV recombinants lacking the BHRF1 ORF are fully capable of initiating and maintaining cell growth transformation and they can also enter the lytic cycle and produce virus (Lee and Yates, 1992; Marchini *et al.,* 1991). Several of the early genes are linked to DNA replication, as indicated in Figure 1.9. Transfection experiments demonstrate that some of these genes are activated in the process of cell differentiation in the absence of other viral gene products, suggesting a possible role of cellular factors in regulating the productive cycle, at least in certain cell types (Marshall *et al*, 1991).

1.6.3 Late genes

The late genes code for structural glycoproteins or proteins that modify the infected cells in order to permit viral envelopment or egress (IARC Monograph, 1997). Among the

non-glycoproteins, the major nucleocapsid protein is encoded by BCLF1, BNRF1 encodes the major external nonglycoprotein of the virion and BXRF1 is likely to encode a basic core protein. The BFRF3 ORF encodes the tegument protein, (see figure 1.1) and VCA p18, which is strongly immunogenic in humans (Kieff, 1996). The genes encoding the EBV glycoproteins are illustrated in bold in figure 1.8. The late BCRF1 gene, which is located in the middle of the EBNA regulatory domain between *ori-P* and Cp, is a close homologue of the human IL-10 gene, with nearly 90% collinear identity in amino-acid sequence (Moore *et al.*, 1990; Vieira *et al.*, 1991; Touitou *et al.*, 1996). BCRF1 has most of the activity of human IL-10, including negative regulation of macrophages and NK cell functions and inhibition of IF γ production. Thus, virally expressed IL-10 may have a local effect on these responses to reactivate infection (IARC Monograph, 1997).

1.7 EBV-associated non-malignant diseases

1.7.1 Infectious mononucleosis

Infectious mononucleosis (IM) is an acute disease associated with primary EBV infection, characterised by fever, pharyngitis and lymphadenopathy. The illness, commonly known as glandular fever, is the classical syndrome caused by EBV. Primary infection with EBV during early childhood is normally sub-clinical (Henle and Henle, 1970), but the severity of disease increases with advancing age, and infection during or after adolescence can give rise to IM in up to half of the infected individuals (Henle and Henle, 1979). Clinically apparent IM tends to be a disease of the socio-economic advanced countries where a greater number of people escape infection in childhood, with a peak incidence occurring in people from 15-25 years of age (Strauss, 1988). Hormonal changes and maturation of the immune response are thought to be possible reasons for this maturation-related incidence of disease. After infection there is a 30-50 day incubation period, followed by a 3-5 day period where mild symptoms are

experienced, these include headache, malaise and fatigue. In more than 80% of cases a sore throat will occur during the first week. Fever with temperatures reaching 39.5°C or higher lasts for about 10 days and then falls gradually over an additional 7-10 days. Although the tetrad of fever, fatigue, pharyngitis and lymphadenopathy are typical, patients may have all or only some of these features. Splenomegaly is observed in about 50% of IM cases, chemical hepatitis is present in most patients and a few develop frank jaundice (Merck Manual, 1992). The disease generally runs its course within a few weeks, but more protracted cases of greater than a few weeks occur occasionally. Prolonged symptoms of fatigue are also associated with chronic fatigue syndrome (CFS) and there has been some speculation that EBV plays a role in the pathogenesis of CFS, although little objective evidence supports this hypothesis. While IM is usually a benign, self-limiting disease, complications may ensue, including rupture of the spleen. Neurological complications, interstitial nephritis with renal failure and interstitial pneumonitis have also been reported (Imoto *et al.*, 1995; Mayer *et al.*, 1996; Morgenlander, 1996; Sriskandan *et al.*, 1996). Fatal mononucleosis usually occurs only in individuals showing severe immunodeficiency (Miller, 1990), for example, renal transplant recipients may develop fulminant mononucleosis or monoclonal B cell malignancy.

The acute phase of virus infection is characterised by a well-defined serological pattern, which includes the absence of antibodies to EBNA and the presence of IgM antibodies to structural components of the virion, anti-VCA (viral capsid antigen) and anti-MA (anti-membrane or envelope antigen). Antibodies to early components of the viral replication cycle called early antigens (EA) are also readily detected (Henle and Henle, 1979). IgM antibodies to VCA evolve quickly with infection, persist for weeks to months and do not reappear. Thus their detection is presumptive evidence of recent primary infection. Antibodies to EA of the diffuse or restricted types develop in most primary infections and wane with time (Horwitz *et al.*, 1985). The appearance of antibody to EBNAs usually occurs weeks to months after infection. EBNA is present in all cells containing the viral genome, whether latently or productively infected (Rickinson, 1986). Transcriptional analysis suggests that a type-III EBV latency prevails

in infectious mononucleosis, with expression of the full set of EBV latent genes, including Cp/Wp-driven EBNA1 (Falk *et al.*, 1990; Tierney *et al.*, 1994). A more detailed analysis of EBV gene expression at the level of the single cell reveals, however, a more heterogeneous picture. Only a subset of cells coexpress EBNA2 and LMP1, characteristic type-III latency. Most cells appear to be EBER positive but negative for EBNA2 and LMP1, suggesting a type-I latency and some large immunoblasts are seen which appear to express LMP1 in the absence of EBNA2 - type-II latency. There are also many small lymphocytes that express EBNA2 but no detectable LMP1. It is uncertain if this represents a new type of latency or a transitory phenomenon (Niedobitek *et al.*, 1997b).

1.7.2 X-linked Lymphoproliferative syndrome

Also known as Duncan's syndrome, the X-linked Lymphoproliferative syndrome (XLP) is a hereditary immunodeficiency disorder characterised by a self-destructive immune response to primary EBV infection (Provisor *et al.*, 1975; Purtilo, 1976). Patients are usually asymptomatic until they encounter EBV, but may present symptoms of immunodeficiency prior to EBV infection. After primary EBV infection, the majority of patients (approximately 63%) develop IM with a fatal outcome. Patients who survive the primary infection are at high risk of developing malignant lymphoma (24%), hypogammaglobulinaemia or aplastic anaemia (29%). The XLP gene has been localised to Xq25 and the region spanning the smallest deletion in patients has been cloned (Lamartine *et al*, 1996; Lanyi *et al*, 1995). Identification of the function of this gene is of prime importance for a better understanding of the complex interaction between EBV and its host (IARC Monograph, 1997). The only curative treatment for X-linked lymphoproliferative syndrome is allogenic bone-marrow transplantation (Williams *et al.*, 1993). In the future it should be possible to identify carriers of the genetic defect, provide appropriate genetic counselling, and diagnose the disease *in utero*.

1.7.3 Oral hairy leukoplakia

Oral hairy leukoplakia is an epithelial lesion of the tongue which was originally described in HIV-infected individuals but was subsequently found in immunosuppressed transplant patients. Detection of this lesion is a significant indicator of HIV-induced immunosuppression and is highly predictive of the subsequent development of AIDS. Oral hairy leukoplakia manifests itself as a raised white lesion, typically located at the lateral border of the tongue, but which may extend to other parts of the oral mucosa (Merck Manual, 1992). Although the histopathologic features are characteristic of human papillomavirus, clear evidence for the presence of EBV has come from immunocytochemistry with monoclonal antibodies, from electron microscopic morphology, and from DNA studies with EBV probes. Southern blot hybridisation provided clear evidence for the presence of EBV in complete linear form and in very high copy number, localised to the superficial epithelial cells. The expression of viral lytic cycle antigens e.g. BZLF1 and VCA, have been shown, indicating that epithelial cells may support EBV replication (Greenspan *et al.*, 1985; Gilligan *et al.*, 1990a; Young *et al.*, 1991). Expression of BZLF1 and VCA, are restricted to the more differentiated upper epithelial cell layer (Greenspan *et al.*, 1985; Young *et al.*, 1991). In contrast to the abundance of the virus in the upper epithelial cells, viral genomes and EBV gene products associated with latent infection are absent from the basal or parabasal epithelial cells of oral hairy leukoplakia (Thomas *et al.*, 1991). Together with the absence of a detectable episomal population of EBV genomes, this indicates that oral hairy leukoplakia is an isolated focus of lytic EBV infection, with no detectable latent phase (IARC Monograph, 1997). Regression of oral hairy leukoplakia can be induced by treatment with acyclovir, indicating that this lesion is indeed caused by EBV (Resnick *et al.*, 1988).

1.8 EBV-associated malignant diseases.

1.8.1 Burkitt's Lymphoma

Although the incidence in Europe and the USA is low (2-3 cases per million children per year in the USA), Burkitt's lymphoma (BL) is the most common childhood cancer in certain parts of equatorial Africa and Papua New Guinea, with an annual incidence of more than 50 cases per million children below the age of sixteen. In fact, Burkitt's lymphoma now accounts for 30-70% of childhood cancers in equatorial Africa. The high incidence of BL in these locations is associated with geographic and climatic features, determined by altitude in East Africa and by rainfall in West Africa, coincident with holoendemic malaria (Haddow,1963; Burkitt, 1969, 1983; O'Connor, 1970). The fact that malaria infection might be a cofactor in the development of BL is supported by the observation that individuals with malaria have a reduced T-cell response to EBV-infected cells. This disease is more common in males than in females with an average age at onset of seven years. BL is a poorly differentiated malignant lymphoma in which the tumour cells show little variation in size or shape. The tumour cells are monoclonal B lymphocytes and they contain characteristic chromosomal translocations (Manolov and Manalova, 1972; Manalova *et al.*, 1979; Rowe and Gregory, 1989). The jaw is the most frequently involved site for tumours and the commonest presenting feature in patients with BL in equatorial Africa (Burkitt, 1958; 1970a) and Papua-New Guinea (Burkitt, 1967). Jaw tumours seem to be age dependent, occurring most frequently in young children, very young children often have orbital or maxillary tumours (Olurin and Williams, 1972). Involvement of the CNS (about 33 % of cases), ovaries, kidneys, liver and mesentery are also prominent in BL.

Burkitt's lymphoma is classified as a non-Hodgkin's lymphoma, invariably of B-cell origin, with B-cell markers such as CD19, CD20, CD22 and CD79a and surface immunoglobulin always detectable. The surface immunoglobulins are usually IgM (IARC Monograph, 1997). Other surface markers that are expressed in most BLs include CD10 and CD77 but CD23 and CD5 are absent (Harris *et al.*, 1994). BL cells

express low levels of HLA class I adhesion and activation molecules such as CD54, CD11a/18 and CD58 (Massucci *et al.*, 1987; Billuad *et al.*, 1989; Anderson *et al.*, 1991).

In the areas of Africa where BL is endemic, about 95 % of the tumours contain EBV DNA and express EBNA1 (Geser *et al.*, 1983). However, in parts of the world where BL is sporadic (Western Europe and the Americas), only about 15-20% of BL tumours contain EBV DNA, indicating that EBV is not essential for formation of the tumour. Therefore, EBV may not play a direct role in the pathogenesis of BL, but may simply increase the risk of development of BL by virtue of it's ability to immortalize B cells (including the cell population that gives rise to Burkitt's lymphoma) (Klein, 1979). This hypothesis is consistent with the lack of expression of EBV latent genes (e.g. EBNA2, EBNA3 and LMP) known to be necessary for the transformation of B cells (Alfeiri *et al.*, 1991; Woisetschlaeger, *et al.*, 1991). The only latent gene invariably expressed in Burkitt's lymphoma, EBNA1, has never been shown to have transforming functions (Rowe *et al.*, 1988; Rowe *et al.*, 1987; 1992; Sample *et al.*, 1991; Magrath *et al.*, 1993).

The discovery of non-random chromosomal translocations associated with Burkitt's lymphoma (Bernheim *et al.*, 1981) paved the way to an understanding of the genetic derangements that are a central component of its pathogenesis. It has been observed that the chromosomal breakpoint on chromosome 8, band q24 is common to all three of the observed translocations in BL and that the breakpoints are located on chromosome 14, 2 and 22, at the heavy- and light-chain immunoglobulin loci (Croce *et al.*, 1979; Lenoir *et al.*, 1982; Mc Bride *et al.*, 1982). The t(8;14) is the most frequent location of a breakpoint in African BL occurring in 75% of tumours and in 50% of Brazilian tumours (Gutierrez *et al.*, 1992). The net consequence of translocation appears to be that c-myc is regulated as if it were an immunoglobulin gene, i.e. it is constitutively expressed in these immunoglobulin-synthesising tumour cells.

1.8.2 Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is a rare malignant tumour in most populations, however, it is highly prevalent in Southern China where it represents the most common tumour in males. Within China itself, the rate of NPC decreases from south to north (Parkin *et al.*, 1997) with more moderate rates seen in the Inuit population and in other parts of Southeast Asia and North Africa. (Ho, 1978). NPC is a disease with a remarkable racial and geographical distribution. It constitutes 75-95% of all malignant tumours occurring in the nasopharynx in low risk populations and virtually all of those in high risk populations (Ho, 1971; Levine and Connelly, 1985). The rates of NPC are higher in men than in women in most populations studied and the number of cases increases steadily with age with a peak incidence at around 45-54 years of age in high-risk populations (Parkin *et al.*, 1997), although in low-risk populations a peak incidence in young adults has been observed (Doll *et al*, 1970).

The high incidence of NPC among the Cantonese population of China was first described by Ho in 1971, who also observed that salted fish is the principal source of supplemented food in the diet of these people (which consists mainly of rice). Further studies revealed that salted fish consumption was significantly related to the risk for developing NPC tumours and increasing frequency of intake was consistently associated with increased risk. The association with salted fish was stronger when exposure occurred during childhood as compared with adulthood (Huang *et al.*, 1981). Carcinogenic volatile nitrosamines have been detected in Chinese salted fish, however, their precise role in NPC has yet to be determined (IARC Monograph, 1997).

NPC is derived from poorly differentiated epithelial cells and arises in the surface epithelium of the posterior pharynx (Parkin *et al.*, 1986). In about half the cases of NPC the presenting sign is a cervical mass resulting from spread to regional lymph nodes. Other symptoms may include nasal obstruction, postnasal discharge, impairment of hearing, tinnitus or otitis media. NPC may metastasise to the skeleton, the spine, the liver, lung and skin as well as to the peripheral lymph nodes (Miller, 1990). A strong association between NPC and EBV is suggested by detectable DNA sequences in almost all cases of types-2 and –3 NPC as ascertained by DNA/DNA or cRNA/DNA

hybridisation in biopsy samples (Pagano *et al*, 1975). EBV infection is an essential step in the progression to malignancy. It has been shown that EBV DNA in NPC is clonal, arising from a single EBV infected cell (Raab-Traub and Flynn, 1986b). EBV has been detected in dysplastic lesions of the nasopharynx in individuals with high IgA titres the cell suggesting involvement of EBV prior to the carcinomatous state (Pathmanathan *et al*, 1995). NPC is unlikely to be the result of primary EBV infection as it occurs primarily in adults in high-incidence areas where initial EBV infection occurs during childhood (Parkin *et al*, 1984). Studies have shown that NPC patients frequently possess elevated serum antibodies to two EBV lytic cycle antigens, viral capsid antigen (VCA) and early antigen (EA) (Henle and Henle, 1976; Ho *et al.*, 1976). Serum detection of these antibodies is a routine diagnostic test for NPC in South-east Asia.

The detection of EBV DNA and EBERs has been useful in identifying carcinomas that have metastasised to lymph nodes when the primary tumour has not been identified (Ohshima *et al.*, 1991; Chao *et al.*, 1996). Transcriptional expression of EBV latent genes in NPC cells has been studied by northern blotting/hybridization (Raab-Traub *et al.*, 1983; Gilligan *et al.*, 1990; Karran *et al.*, 1992). BARFO, LMP2, EBER and EBNA1-coding transcripts are always expressed in NPC cells and LMP1 is detected in 50% of tumours (Fahraeus *et al.*, 1988; Brook *et al.*, 1992). Occasionally lytic cycle early genes are also detected in a few cells (Luka *et al.*, 1988; Cochet *et al.*, 1993).

1.8.3 Hodgkin's disease

Hodgkin's disease is the commonest variant of the malignant lymphomas, accounting for about half of all cases. The disease is most common between the ages of 20 and 40 but may occur at any age, usually presenting with lympadenopathy, but sometimes with fever, night sweats, weight loss and pruritis. Though the cell of origin of this tumour is still not known, histologically, HD is characterised by mononuclear Hodgkin cells (HC) and their multinucleated variants, the Reed-Sternberg cells (RS). The Rye classification distinguishes four major types of Hodgkin's disease: nodular lymphocyte-predominant, nodular sclerosis, mixed cellularity and lymphocyte-depleted (Luka and Butler, 1966,

Harris *et al.*, 1994). It is now accepted that lymphocyte-depleted HD represents a separate tumour entity and is considered separately from the other three classical forms of HD. Increasing evidence suggests that HD is not a single entity but rather a heterogeneous group of diseases (Harris *et al.*, 1994). The clinical representation of HD varies with geographical location and in the western world HD usually arises as a unifocal lesion in cervical lymph nodes. Continuous spread of the tumour to adjacent lymph nodes gives rise to enlarged nodes. With spread of the tumour through lymphatic channels, other organs are involved, the preferential sites of involvement including the spleen and distant lymph nodes. Subsequently as the disease becomes more aggressive, other organs are involved, including the liver and the kidney. Bone-marrow involvement in HD is indicative of extensive tumour infiltration (Kaplan, 1980).

In most western populations, very few cases occur among children, a rapid increase in incidence among teenagers is seen followed by a peak at about age 25, the incidence then plateaus with a second peak with increased age. There is an excess in males which is more pronounced at older ages (Mac Mahon, 1957). In poorer populations there is an initial peak in childhood only among boys with a relatively low abundance among young adults followed by a late peak in those of advanced age (Correa and O'Connor, 1971). There is evidence that the risk factor for HD in young adulthood through middle age is associated with higher education, higher social class, fewer siblings, less crowded housing and early birth rank. All of these factors lead to increased susceptibility to late infections with the common childhood infections, which tend to be more severe (IARC Monogragh, 1997).

Following a report by Weiss and colleagues of EBV DNA in 50% of Hodgkin's disease (HD) tissues (Weiss *et al.*, 1987), the role of EBV in HD has been subjected to intense scrutiny (Joske and Knecht, 1993). In situ hybridisation has disclosed the presence of the virus in virtually all tumour cells in EBV-positive cases, consistent with the detection of monoclonal EBV genomes in DNA extracted from most HD tissues. These findings indicate that EBV infection of Hodgkin-Reed-Sternberg cells takes place before clonal expansion. Pallesen *et al.*, (1991a) and Herbst *et al.*, (1991) reported that the EBV

in HD has a restricted latent phenotype of EBNA1 and usually LMP1, LMP2A and LMP2B without detectable EBNA2 expression, as in NPC. These finding have been widely replicated. In multiple specimens of HD from case studies, molecular evidence of clonal EBV genomes with specifically restricted expression of latent viral proteins in the RS cells was found in 30-50% of cases. EBV genome status appears to be uniform in involved nodes within patients and over time in those patients studied longitudinally (Delsol *et al.*, 1992; Brousset *et al.*, 1994). The consistency of the finding of clonal EBV and the expression of LMP1 in about half of HD cases argues strongly against a passenger role for EBV in these cases. Seroepidemiology findings in multiple case studies show that patients with HD can be distinguished by an altered antibody profile to EBV. Thus, the available evidence stongly implicates EBV as a factor in the pathogenesis of EBV-positive HD.

The risk of HD after diagnosis of IM has been evaluated and this study revealed that overall there was a threefold increase in the risk of developing HD. Also, essentially all HIV-1 infected patients with HD have a higher rate of EBV positivity. Generally these patients present with advanced HD and show a relatively poor prognosis (Moran *et al.*, 1992; Tirelli *et al.*, 1995).

1.8.4 Post-transplant lymphoproliferative disorders

Post-transplant lymphoproliferative disorders (PTLD) are a major complication in allograft recipients, occurring in 1-20% of patients. The incidence tends to be lowest for renal transplant patients and highest for lung transplant patients which may reflect the amount of immunosuppressive therapy associated with the latter (Nalesnik and Starzl, 1994; Montone *et al.*, 1996). In PTLD the tumours proliferate unchecked due to the absence of adequate T-cell tumour suppression. The tumours can be polyclonal or monoclonal as determined by analysis of EBV terminal repeats or cellular gene rearrangement status (Joske and Knecht, 1993). It is believed that the pathogenesis of the condition starts with EBV driven polyclonal B-cell proliferation, eventually leading

to fully developed malignant lymphoma. Typically B-cells in these lymphoproliferations express a broad spectrum of virus-encoded latent proteins, including EBNA1, EBNA2 and LMP1. This type-III form of latency is similar to that found in LCLs in vitro and accordingly these cells usually display an LCL pattern of cellular gene expression, including lymphocyte activation and adhesion molecules (Young *et al.*, 1989; Thomas *et al.*, 1990). However, considerable variability has been found in EBV gene expression in and between lesions with type-I and type-II latency also observed (Delecluse *et al.*, 1995).

1.8.5 AIDS-related lymphomas

In the US lymphomas are 60 times more frequent in AIDS patients than in the general population (Beral *et al.*, 1991). Non-Hodgkin's lymphomas are very common in HIV infected individuals, primarily at extranodal sites, particularly common are primary central nervous system lymphomas (Krogh-Jensen *et al.*, 1994). Morphologically, AIDS-related non-Hodgkin's lymphomas fall into two broad groups; diffuse large B-cell non-Hodgkin's lymphomas, which often show a prominent immunoblastic component and Burkitt's lymphoma and Burkitt's-like lymphoma. Superficially the pathogenesis is the same as PTLD; EBV-immortalised B lymphocytes proliferate unchecked due to decimated T-cell numbers resulting in oligo- or monoclonal B-cell proliferations (Joske and Knecht, 1993). The two types of AIDS-related non-Hodgkin's lymphomas show striking differences in their relationship to EBV, suggesting different pathogenic mechanisms. Most diffuse large B-cell non-Hodgkin's lymphomas and all AIDS-related central nervous system lymphomas are EBV-positive (MacMahon *et al.*, 1991). Diffuse large B-cell lymphomas have been reported to occur relatively late in AIDS patients (Gaidano and Dalla-Favera, 1995) and more advanced depression of the immune system is a risk factor for their development (Pedersen *et al.*, 1991). Most AIDS-related non-Hodgkin's lymphomas appear to be monoclonal both with respect to their antigen receptor genes and to the EBV episomes, however, there may be rare polyclonal cases (Ballerini *et al.*, 1993; Delecluse *et al.*, 1993).

HIV appears to contribute to the pathogenesis of some EBV-associated AIDS-related non-Hodgkin's lymphomas by inducing severe immunosuppression, leading to a loss of EBV-specific T-cell immunity (MacMahon *et al.*, 1991). As EBV-positive, AIDS-related B-cell lymphomas consistently lack the HIV genome, a direct contribution of HIV to tumorigenesis beyond suppression of the immune system is unlikely (Knowles, 1993). The relative risk for AIDS-related non-Hodgkin's lymphomas increases with duration of HIV infection and to a certain extent with immune suppression (Munoz *et al.*, 1993). BL is up to one thousand fold more frequent in HIV-positive individuals than in the general US population (Beral *et al.*, 1991) and the tumour is EBV-positive in about 20% of cases (Beral *et al.*, 1991). AIDS related BL, both EBV-positive and EBV-negative, have been consistently shown to harbour the characteristic c-myc translocation. These translocations have been detected in a minority of diffuse large B-cell lymphomas and cases with morphological features between large B-cell lymphomas and BL (Ballerini *et al.*, 1993; Delecuse *et al.*, 1993; Bhatia *et al.*, 1994). Other genetic changes implicated in the pathogenesis of AIDS-related non-Hodgkin's lymphoma whether EBV-associated or not, include p53, N-ras and K-ras point mutations and deletions in the long arm of chromosome 6 (Gaidano and Dalla-Favera, 1995). HIV-positive individuals who develop HD are more likely to have advanced extra-nodal disease, not to respond to therapy and to die of opportunistic infections than those with HD alone (Ames, *et al.*, 1991).

The detection of EBV in T-cell lymphoma opposes the well established process of B-cell lymphotrophisim of the virus in vitro. The interpretation of the detection of EBV in T-cell non-Hodgkin's lymphomas and an assessment of the role of the virus in the pathogenesis of T-cell lymphoma are complicated by two factors. Firstly, if EBV infection of certain T-cells in vitro leads to predominantly lytic infection, EBV infection of T-cells may be accidental rather than part of the viral strategy to establish persistent infection. Such infection of cells not adapted to latent infection may contribute to the development of EBV-associated T-cell lymphomas. Secondly, in many cases the virus is detected in only a small proportion of tumour cells (Anagnostopoulos *et al.*, 1996). Although the virus may be present at the onset of the neoplastic process it may

subsequently be lost from the tumour cell. While there is some evidence to suggest this may happen in vitro it has not yet been shown in vivo. The alternative scenario would be a secondary infection of established neoplastic T-cells with the virus, this would exclude the virus from an initial role in neoplasia but would be compatible with a role of the virus in contributing to the disease process. The frequent expression of LMP1 in T-cell lymphomas would seem to argue in favour of such a role (d'Amore *et al.*, 1992). However, the role of EBV in T-cell related malignancies remains uncertain.

1.8.6 Other tumours

EBV has been detected in the vast majority of gastric lymphoepithelial carcinomas and in a high proportion of lymphoepithelial carcinomas of the lung and salivary gland. A smaller proportion of gastric adenocarcinomas is also EBV-associated. EBV DNA has been detected occasionally in epithelial tumours at a wide variety of other anatomical sites. An aetiological role for EBV in lymphoepithelial and adenocarcinomas has not been conclusively established. Smooth-muscle tumours in immunosuppressed individuals uniformly contain EBV, indicating a possible causal role for the virus in this setting (IARC Monographs, 1997).

1.9 Aims

The purpose of this study was to investigate the functions of two important EBV latent proteins. LMP1 is an integral membrane protein with an essential role in the immortalisation of primary B cells. It was proposed to explore the role of LMP1 in interfering with cell cycle control by studying its effects on the expression of a panel of cell cycle-related genes using ribonuclease protection assays. The effect of LMP1 expression was also compared to that of EBNA2 (the other main effector of phenotypic change in EBV-immortalised B cells). EBNA3 comprises a family of three related latent nuclear proteins, EBNA3A, EBNA3B and EBNA3C. EBNA3B is the only member of the family which is not required for immortalisation and it was proposed to investigate its role by screening for interacting proteins using the yeast two hybrid system.

CHAPTER 2

MATERIALS AND METHODS

2.1 BIOLOGICAL MATERIALS

2.1.1 Cell lines

Table 2.1. Cell lines used in this study

Cell Line	EBV Status	Cell Classification	Description
DG75	-	EBV negative BL	Lymphoid B cell line derived from an Israeli Burkitt-like lymphoma case (Ben-Bassat *et al.*, 1977).
DG75 tTA	-	Stable transfectant	EBV negative BL cell line stably transfected with the tetracycline-regulated transactivator only (Floettmann *et al.*, 1996).
DG75tTA EBNA2	-	Stable transfectant	Tetracycline regulated system whereby the expression of EBNA2 can be induced by the removal of tetracycline from the growth media (Floettmann *et al.*, 1996).
DG75tTA LMP1	-	Stable transfectant	Tetracycline regulated system whereby the expression of LMP1 can be induced by the removal of tetracycline from the growth media (Floettmann *et al.*, 1996).
Mutu 1	+	Type I	Early passage BL cell line expressing EBNA1 as the only viral gene (Gregory *et al.*, 1990).
Mutu 3 c95	+	Type III	Stable clone of the early passage BL cell line Mutu 1 which has, upon serial passage in culture, "drifted" to express the full compliment of EBV latent genes (Gregory *et al.*, 1990).
BL41, BL41-B958, IARC 171.	+ + +	Type I Type III LCL	These cells are a matched set. BL41 is an early passage BL cell line expressing EBNA1 as the only viral protein, BL41 B958 is the cell line stably transformed with the EBV virus strain B958 expressing all the EBV latent genes, (Calender *et al.*, 1987) IARC 171 is a spontaneously transformed Lymphoblastoid cell line derived from the same patient (Andersson *et al.*, 1991).
BL72 III IARC 307	+ +	Type III LCL	These cells are a matched pair, BL72 is a group 3 BL cell line expressing all EBV latent genes. IARC 307 is a spontaneously transformed LCL from the same patient (Rowe *et al.*, 1990).

BL74 IARC 290B	+ +	Type I LCL	These cells are a matched pair, BL74 is a group 1 BL cell line expressing only EBNA1. IARC 290B is a spontaneously transformed LCL from the same patient.
Ag876	+	Type III	Type III BL cell line expressing all the EBV latent genes (Dambaugh *et al.*, 1984).
X50-7	+	LCL	Spontaneously transformed LCL (Miller *et al.*, 1984).
Jurkat	-	T cell	Acute T-lymphocytic leukemic cell line (Brattsand, *et al.*, 1990).
C33A, C33A Neo, C33A LMP1.	- - -	Epithelial cell	These are cervical epithelial cell lines. C33A is the parental cell line, C33A Neo is stably transfected with an empty vector, C33A LMP1 is stably transfected with a vector constiutively expressing LMP1 (Miller *et al.*, 1995).

All BL cell lines and LCLs were obtained from Professor Martin Rowe, University of Cardiff, Wales. The epithelial cell lines C33A were a gift from Dr Nancy Rabb-Traub University of North Carolina, USA.

2.1.2 Antibodies

PE2, T2.78, E3C.A10.3 and CS1-4 (antibodies specific for EBNA2, EBNA-3A, EBNA-3C and LMP1, respectively) were gifts from Professor Martin Rowe, University of Cardiff, Wales. The antibodies were supplied as cell culture supernatants and stored at 4°C or -20°C prior to dilution.

Monoclonal Antibody	**Supplier**
Anti-p21/WAF1	Santa Cruz, SC-6246
Anti-p53	Santa Cruz, SC-126
Anti-Rb	Santa Cruz, SC-102
Anti-c-Myc	Calbiochem
Anti-HA 12 CA5	Boehringer Mannheim
Anti-mouse-alkaline phosphatase (AP) conjugate	Promega
Rabbit anti Mouse IgG	Dako
Goat anti-rabbit HRP	Dako

2.1.3 Bacterial strains

E.coli DH5α, genotype: F-, *end* A1, hsdR17 (r_k-,m_k=), *sup*E44, *thi* -1,λ-, *rec* A1, *gyr* A96, *rel* A1,ϕ 80*lac* ZδM15.

2.1.4 Yeast strains (*Saccharomyces cerevisiae*)

Yeast strains were grown in YPD medium at 30°C, while selection for plasmids was carried out by growing yeast in Yeast Nitrogen Base (YNB) medium without amino acids (Difco) which was supplemented with appropriate combinations of the following amino acids where required: leucine (60 μg/ml.), tryptophan (40 μg/ml.), histidine (20 μg/ml.), uracil (20 μg/ml.) denoted +L+T+H+U respectively. YNB plates were supplemented with sugars as indicated: 2% (w/v) glucose or 2% (w/v) galactose + 1% (w/v) raffinose.

EGY48 (*URA3 TRP1 HIS3 6LexA operator-LEU2*) The *LexAop-LEU2* gene is normally not transcribed and the yeast are auxotrophic for leucine.
Y187 (*MATα gal4 gal80 his3 trp1-901 ade2-101 uar3-52 leu2-3,-112 URA3 GAL→lacZ met-*) was made by crossing Y153 and GGY171 (Fields and Song, 1989).

2.1.5 Plasmids

pTAg Cloning vector from R&D Systems

2.1.5.1 YTHS-A plasmids

All plasmids used in YTHS-A were gifts from Dept. of Biochemistry, TCD and are summarised in Table 1.
pEG202 A yeast - E. coli shuttle vector, pEG202 is a derivative of 202 that contains an expanded polylinker region. Bait proteins expressed from this plasmid contain aa

1 to 202 of LexA, which include the DNA binding and dimerisation domains (see Chapter 4, Figure 4.4).

pNLEX A derivative of pEG202 in which an additional nuclear localisation signal has been inserted in to the unique EcoR1 site. This destroys the EcoR1 site at the N terminus but retains the site at the C terminus. Therefore the EcoR1 site in pNLEX is also unique.

2.1.5.2 YTHS-B plasmids

pAS-1 CHX (pAS-2) bait plasmids (see chapter 4, Figure 4.10) cDNA library DNA and all control plasmids used in YTHS-B were gifts from Dr Stephen Elledge, Bayor College of Medicine, Texas. Plasmids used in verification of specificity experiments were gifts from Dr Geraldine Butler, Department of Biochemistry, UCD.

2.1.6 Oligonucleotides

Genosys Biotechnologies Europe Ltd.

EBNA-3A

Forward primer 5' ATC GGG CCA TGA TCA AAC TGG ACA AGG A 3'

Reverse primer 5' TGT TAT AAC GTG ATC AAA GGC CTG CCC C 3'

EBNA-3B

Forward primer 5' CG CGG ATC CTG AAG AAG CGT GGC TCA G 3'

Reverse primer (1575 bp) 5' CG CGG ATC CAG TAG GGT TGC CAT AAC CC 3'

Reverse primer (837 bp) 5' CG CGG ATC CGA ACT CGG TTT TTC GTG CC 3'

EBNA-3C

Forward primer 5' CGC GGC TCC TGG AAT CAT TTG AAG GAC AGG 3'

Reverse primer CGC GGA TCC ATC GAC GAT GGA TCT TCG G 3'

BCO1 5' CCA GCC TCT TGC TGA GTG GAG ATG 3'

BCO2 5' GAC AAG CCG ACA ACC TTG ATT GGA G 3'

pTAg 5' TTC AGT ATC TAC GAT TC 3'

2.1.7 Commmercial kits and restriction enzymes

All restriction enzymes were supplied by Boehringer Mannheim.

Klenow enzyme (DNA Polmerase I large fragment) was supplied by Biolabs (#210S)

Enhanced Chemiluminesence substrate (ECL)Amersham

Riboquant mutiprobe RPA :	Pharmingen
In Vitro Transcription kit (#45004K)	
Multiprobe Template set HCC-2 (#54091P)	
RPA kit (#45014K)	
Qiagen Tip -100	Qiagen
RPA kit	Pharmingen
Taq DNA polymerase	Perkin Elmer and Boehr. Mannheim.
The LigATor	R&D Systems
T7 Sequencing kit	Pharmacia
Capture-Tec pHook1 System	Invitrogen
Luciferase Assay System	Promega (#E1500)

2.2 CHEMICAL MATERIALS

Protein prestained markers	NEB
^{35}S labeled dATP	Amersham
$\alpha^{32}P$ labeled UTP	Amersham
dNTPs	Pharmacia Biotech
RNase A	Pharmacia Biotech
Marvel	Premier Beverages
Chloroform	ROMIL
Isopropanol	ROMIL
Dimethyl formamide	Riedel-de-Haën

Sigma-Aldrick-Fluka Chemical Co.:

Urea, Dithiothreitol (DTT), Coomassie Blue R, BCIP/NBT, Tetracycline, Liquid Phenol, Nitocellulose, Ampicillin, Potassium acetate, Tween-20, Bovine serum albumin (BSA), Sodium azide, Sigmacote, Ammonium phosphate, Mineral oil, α-Thiol-glycerol (ATG), PMSF, MOPS, BCS, Formaldehyde, Diethylenepyrocarbonate (DEPC), Salmon Sperm DNA Type III sodium salt (D1626), Polyethylene Glycol (PEG), Cycloheximide, 3-AT, β-mercaptoethanol, all

amino acid supplements (see Appendix), actinomycin D, Micophenolic acid, Xanthine, aqueous mountant.

Merck:

Boric acid, Ammonium persulphate, Sodium acetate, Magnesium chloride, Glucose, Sodium chloride, Potassium chloride, Sodium hydroxide, Sodium dodecylsulphate, Calcium chloride, Glycine, Methanol.

BDH:

TEMED, Bromophenol blue, Potassium dihydrogen phosphate, Potassium hydrogen phosphate, Sodium phosphate, Glycerol, Tris(hydroxymethyl)methylamine, EDTA, Magnesium sulphate, Ethidium bromide, Isoamyl alcohol, Hydrochloric acid, Acetic acid, Methanol, Isopropanol.

Boehringer Mannheim:

Agarose, Low melt agarose, IPTG, Hygromycin B, Geneticin (G418), Leupeptin.

Oxoid:

Agar technical, Bacto-Tryptone, Yeast extract.

Difco:

YNB without amino acids

KODAK:

X-ray film, X-ray film developer, X-ray film fixer.

National diagnostics:

Acrylagel, Bis-acrylagel.

Gibco-BRL:

RPMI 1640, Foetal calf serum, Pencillin, Streptomycin, L-Glutamine, Hepes, Sodium Pyruvate, 1Kb DNA ladder, β-galactosidase (X-gal).

Promega:
100bp DNA ladder.

Calbiochem:
Staurosporine, SB203580 p38 inhibitor.

2.3 DNA MANIPULATION

Preparation of all solutions used in chapter two are outlined in the Appendix .

2.3.1 Storage of DNA samples

DNA samples were stored in TE buffer pH 8.0 at 4^{o}C. EDTA was used to chelate heavy metal ions that are needed for DNase activity while storage at pH 8.0 minimises deamidation. DNA was also stored in sterile distilled H_2O (dH_2O).

2.3.2 Equilibration of phenol

As DNA partitions into the organic phase at <pH 7.8, phenol was prepared by equilibration to pH 8.0 with TrisCl pH 8.0 as follows: Solid phenol was melted at 68^{o}C, hydroxyquinoline was added to a final concentration of 0.1% (w/v) (acts as an antioxidant, a chelator of metal ions, and an RNase inhibitor). An equal volume of buffer (0.5 M TrisCl pH 8) was then added to the liquefied phenol and stirred for 15 min. After allowing the two phases to equilibrate, as much as possible of the upper aqueous phase was removed. The extraction was repeated using equal volumes of 0.1 M TrisCl pH 8 until the pH of the phenol was > 7.8. An equal volume of TrisCl pH 8 and 0.2% (w/v) β-mercaptoethanol were then added to the phenol, which was stored at 4^{o}C in the dark until required.

2.3.3 Phenol/chloroform extraction and ethanol precipitation

Phenol/chloroform extraction and ethanol precipitation was carried out to purify and concentrate nucleic acid samples as follows: An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the DNA solution,

vortexed to an emulsion and centrifuged for 10 min at 12,000 x g. The upper aqueous phase was removed, taking care not to take any material from the interphase, and this was placed into a fresh autoclaved eppendorf. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the aqueous phase, vortexed as before and centrifuged for 5 min at 12,000 x g. Again the upper aqueous phase was removed to a fresh tube. One tenth volume of 3 M sodium acetate (pH 5.2) was added to the solution of DNA, mixed and then 2 and a half volumes of 100% (v/v) ethanol was added. This mixture was vortexed and incubated at room temperature for 5 min (when dealing with very small quantities of DNA, samples were precipitated in ethanol at –20°C overnight). DNA samples were then centrifuged for 30 min at 12,000 x g at 4°C, the supernatant was removed and pellets were washed with 1 ml 70% (v/v) ethanol to remove excess salts. The tube was centrifuged for 5 min at 10,000 x g, the supernatant was removed and pellets were air dried for 10-15 min. Pellets were resuspended in an appropriate volume of sterile Tris-EDTA (TE) (pH 8.0) or sterile dH_2O.

2.3.4 Restriction digestion of DNA

Restriction enzymes bind specifically to and cleave double-stranded DNA at specific sites within or adjacent to a particular sequence known as the recognition site. The restriction enzymes used were supplied with incubation buffers at a concentration of 10X (working concentration 1X). DNA was digested with restriction endonucleases for identification purposes or to linearise or cut fragments from a plasmid. DNA digests were performed by adding the following, usually to a final volume of 20µl.

200 ng - 1 µg of DNA (Final concentration of <300 ng/µl)
1 µl of enzyme/µg of DNA solution (~10 U).
10 X buffer to a final concentration of 1X
dH_2O to the final volume required

The reaction was gently mixed, centrifuged, then incubated for 2 h at the optimum enzyme temperature (between 25°C and 50°C, usually 37°C).

2.3.5 Cohesive Ligation of DNA

Two fragments of DNA may be ligated in one of 2 ways. The majority of restriction endonucleases digest DNA leaving either a 5' or a 3' overhang, in which two compatible cohesive ends may be ligated by cohesive ligation. Alternatively, blunt-ended ligation may be required. Cohesive-end ligations of equimolar amounts of vector and insert DNA (<1 μg) were generally carried out overnight at 16°C in a commercial ligation buffer (5 mM ATP) with 10 units of T4 ligase/ml. As small a volume as possible is recommeded for ligation reactions and usually the total volume of reactions was 10 μl. After ligation, the samples were heated to 10 min at 70°C to inactivate the ligase (this appears to improve transformation efficiencies), and stored on ice until required.

2.3.6 Blunt-ended ligation of DNA

Blunt-ended ligations are usually less desirable than cohesive-end ligations due to their much lower efficiency. Nonetheless, blunt-ended ligations were required in cases where restriction enzymes generated blunt ended DNA molecules. Also, if two compatible cohesive DNA ends cannot be generated, it is sometimes necessary to fill in the unmatched bases of the 5' or 3' overhangs and carry out a blunt-ended ligation reaction. The large fragment of *E.coli* DNA polymerase I (Klenow, Biolabs) was used for this purpose in the following reaction: DNA was resuspended at a concentration of 50 μg/ml in 1X Eco Pol buffer (supplied with the Klenow), dNTPs were added to a final concentration of 33 μM each, 1 μl of Klenow was added and the reaction was placed at 25 °C for 15 min. The enzyme was inactivated by heating to 70°C for 10 min. This DNA was then purified by phenol/chloroform extraction and concentrated by ethanol precipitation (2.3.3). Ligation reactions were then performed as for cohesive-end reactions (2.3.5)

2.3.7 BamH1 linkers

Synthetic linkers (8-12 bp) containing a restriction site for an enzyme which generates cohesive ends in DNA molecules may be ligated to the ends of DNA fragments in order to improve the efficiency of blunt-ended ligations. Thus, a high

concentration of BamH1 linker termini (4-20 μm) was ligated to the relevant insert DNA at a 2-3 : 1 concentration ratio over target DNA. The resulting ligations were then digested with BamH1 and purified by phenol/chloroform extraction and ethanol precipitation. Due to the very high concentration of excess linker molecules, DNA was further purified by extraction from low melt agarose (2.3.7). Ligations were then carried out as for cohesive-end reactions (2.3.5).

2.3.8 Dephosphorylation of linearised plasmid DNA

During ligation, T_4 DNA ligase will catalyse the formation of a phosphodiester bond between adjacent nucleotides only if one contains a 5' phosphate group and the other contains a 3' hydroxyl group. Recircularisation of plasmid DNA can therefore be minimized by removing the 5' phosphate groups after treatment with calf intestinal alkaline phosphatase enzyme (CIP). Digested DNAs (<100 ng/μl) were dephosporlylated using CIP in a 100 μl volume (CIP was added 1 unit/100 pmoles for cohesive termini and 1 unit/2 pmole for blunt termini). The solution was vortexed, centrifuged briefly and incubated for 30 min at 37°C. This was followed by an enzyme denaturation step achieved by heating to 75°C for 10 min. DNA was then purified by phenol/chloroform extraction and ethanol precipitation (2.3.3).

2.3.9 Preparation of competent cells

The calcium chloride ($CaCl_2$) method was employed to prepare competent bacterial cells for transformation of DNA. An *E.coli* strain (DH5α) was streaked from a frozen glycerol stock on to an LB agar plate and incubated at 37°C overnight. An isolated colony was then picked using a sterile inoculating loop and used to inoculate 5 ml of SOB (appendix A) broth. This culture was incubated in a shaking incubator at 200 rpm overnight at 37°C. An aliquot of this starter culture (2 ml) was then used to inoculate 100 ml of sterile SOB in a conical flask and incubated at 37°C with shaking to an $O.D._{.640\ nm}$ of between 0.4 and 0.8 (approximately 2 h). The cells were then transferred to two sterile 50 ml falcon tubes and incubated on ice for 10 min followed by centrifugation at 4,000 x g for 10 min at 4°C. Cell pellets were gently resuspended in 25 ml of 100 mM ice-cold $CaCl_2$, and incubated on ice for a further

20 min. Centrifugation was carried out as before (4,000 x g at 4°C for 10 min) followed by removal of the supernatant. Finally cells were resuspended in a 1% (w/v) volume of $CaCl_2$. Competent cells were stored on ice and used within 24 hr.

2.3.10 Transformations

Two hundred microliters of competent cells were added to a pre-chilled microcentifuge tube containing 10 µl of DNA at a concentration of ~100 ng/10 µl. The contents were mixed gently and incubated on ice for 30 min, during which time an aliquot of SOC (appendix A) was pre-heated at 42°C. At the end of the incubation on ice, cells were heat-shocked at 42°C for 90 s followed by incubation on ice for a further 2 min. One mililiter of preheated SOC was then added to the cells which were incubated at 37°C in a shaking incubator for 1 h 10 min. The cells were concentrated by centrifugation after which ~800 µl of supernatant was removed and discarded. The cells were resuspended in the remaining supernatant and plated out with the appropriate controls on LB plates containing ampicillin and incubated overnight at 37°C. Only bacteria which took up ampicillin-resistant plasmid DNA grew on LB Amp plates. Recombinant colonies were thus used to inoculate 5 ml aliquots of LB broth containing ampicillin which were then incubated overnight at 37°C. DNA minipreparations were subsequently prepared using fresh cultures as described in section 2.3.10.

2.3.11 Small scale preparation of plasmid DNA (miniprep)

This method is a modification of a protocol from Maniatis et al, 1978. A single bacterial colony was used to inoculate 5 ml of LB medium (with appropriate antibiotic) and incubated overnight at 37°C. An aliquot (1.5 ml) of this culture was added to a sterile microfuge tube and centrifuged for 30 s at room temperature, the remainder was stored at 4°C. Supernatant medium was removed from the tube, leaving the pellet as dry as possible. The pellet was then resuspended thoroughly in 100 µl of ice-cold solution I by vigorous vortexing. To this, 200 µl of freshly prepared solution II was added and the tube contents were mixed by inverting the tube rapidly 5-6 times. Ice-cold solution III (150 µl) was added and the tubes were

vortexed gently for 10 s. The lysate was centrifuged for 5 min at 12,000 x g and the supernatant was transferred to a fresh tube, taking care not to carry over any of the white precipitate. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed by vortexing and centrifuged for 5 min at 12,000 x g. The upper aqueous phase was removed to a fresh tube, to which 2 volumes of 100% (v/v) ethanol were added, the solution was vortexed and centrifuged for 5 min at 12,000 x g. After discarding the supernatant, the pellet was washed with 200 µl 70% (v/v) ethanol, centrifuged as before and the supernatant was removed. The pellet was air-dried for 10-15 min. After resuspending in 50 µl of TE (pH 8.0), 1 µl of DNase-free RNase A (20 µg/ml) was added, tubes were vortexed, incubated at 37°C for 1 h, then stored at 4°C. Glycerol stocks of all bacterial cultures were prepared at this stage by the addition of 0.5 ml of a 50% (v/v) glycerol solution to 0.5 ml of the overnight bacterial culture of interest and storing at -80°C.

2.3.12 Qiagen™ plasmid DNA purification protocol

Plasmid DNA was purified using the QIAGEN-tip 100 isolation system from Promega. All buffers used are described in appendix A. A glycerol stock of the bacteria of interest was streaked out on LB ampicillin agar and incubated overnight at 37°C. An isolated colony from this plate was used to inoculate a 5 ml LB ampicillin starter culture and incubated in a shaking incubator (~300 rpm) at 37°C for 8 h. One millilitre of the starter culture was used to inoculate 25 ml of LB ampicillin in a 250 ml sterile flask and incubated overnight in a shaking incubator at 37°C. The following day, cultures were harvested at an optimal $O.D._{600\ nm}$ of between 1 and 1.5. The following centrifugation steps were carried out using a JA-20 rotor in a Beckman centrifuge. The bacteria culture was transferred to a centrifuge tube and centrifuged by spinning at 6,000 x g for 15 min at 4°C. The supernatant was removed and the pellet was dried by inverting the tube on tissue paper and allowing the supernatent to drain off. The bacterial pellet was resuspended completely in 4 ml of cold Buffer P1 containing RNase, 4 ml of freshly prepared Buffer P2 was added and tubes were incubated at room temperature for 5 min. Following incubation, 10 ml of prechilled Buffer P3 was added, immediately mixed by gentle inversion of the

tube (5-6 times). Tubes were then incubated on ice for 20 min before centrifugation for 1 hr at 20,000 x g at 4°C.

The Qiagen-tip 100 was equilibrated by applying 4 ml of QBT buffer and allowing the column to empty by gravity. The column does not dry out at this stage as the flow of buffer will stop when the buffer reaches the upper filter. After the centifugation step the supernatant was removed immediately from the tube without disturbing the pelleted material and applied to the column by filtering through 1MM filter paper. The QIAGEN-tip was washed with 2 x 10 ml of Buffer QC. DNA was then eluted with 5 ml of Buffer QF. DNA was precipitated by adding 0.7 volumes of room-temperature isopropanol and centrifuging immediately at 15,000 x g for 30 min at 4°C. Supernatant was then carefully removed and the DNA pellet was washed with 70% (v/v) ethanol, allowed to air dry for 5 min and re-dissolved in a suitable volume of TE or dH_2O. DNA was then quantified by spectrophotometric analysis as described in section 2.3.15.

2.3.13 Agarose gel electrophoresis of DNA

Electrophoresis through agarose gels is the standard method used to separate, identify and purify DNA fragments. The technique is simple, rapid to perform and can be used for the isolation of DNA fragments.

An appropriate quantity of agarose or low melt agarose was added to 100 ml 1X TBE /TAE buffer based on the percentage agarose gel required. Increasing the percentage agarose (1.8-2.0%) in the gel was generally used to improve resolution of smaller DNA fragments while separation of larger DNA molecules was observed more readily on low percentage gels (0.6-0.8%). The agarose was completely dissolved by boiling and after sufficient cooling (~60°C) the gel was cast into the Hybaid horizontal gel electrophoresis system and a comb was inserted for formation of sample wells. The gel was allowed to set before filling the chamber with 1X TBE/TAE and removing the comb. Sample buffer containing bromophenol blue as a tracking marker was added to each sample before loading up to a maximum volume of 20 µl per well. DNA sample buffer was also added to 500 ng of a 1 Kb or 100 bp DNA ladder which was loaded as a size marker. The gel was run at constant voltage (5 V/cm), usually at ~100 V, for 1-2 h. When complete, the gel was stained in

ethidium bromide (0.5 mg/ml) for 30 min, placed in distilled water to destain for 15 min and viewed under UV illumination.

2.3.14 Isolation of DNA from agarose gels

Low melting point agarose gels were prepared in 1X TAE buffer (gel isolation is not carried out in TBE buffer as borate ions are difficult to remove from the resultant DNA solution). Ethidium bromide was added to the samples before electrophoresis so as to minimise manipulations with the fragile low melting point agarose gels. After electrophoresis, the gels were viewed under 70% UV illumination. The time of exposure to UV light was kept to a minimum, as overexposure to UV would cause damage to the DNA. The DNA band of interest was excised from the gel using a clean scalpel, excess agarose was cut away to minimise the size of the gel slice which was then placed in a sterile microfuge tube.

2.3.15 Purification of DNA by Gene Clean Method

This method was used to purify DNA from low-melt agarose gels. Under UV illumination, the appropriate band was excised from the gel using a clean, sharp scalpel and placed in an eppendorf. After estimating the weight of the excised piece of gel, 2-3 volumes of NaI was added to the agarose. This was then incubated at 55°C or until the agarose had dissolved. Approximately, 2 µl of silica 325 mesh glass beads were then added and the tube contents were mixed by vortexing. The mixture was then left at room temperature for 5 – 10 min, before spinning for 20 s at 12000 x g. The supernatant was removed and discarded. The pellet was resuspended in 200µl wash solution by vortexing and then pelleted at 12000 x g as before. This wash step was repeated twice to fully remove residual agarose and salt contaminants. The DNA pellet was resuspended in ~10 µl TE or sterile dH_2O by vortexing. Finally, DNA was eluted from glass beads by incubation at 55°C for 10 min, glass beads were then pelleted by spinning at 12000 x g for 10 min. The supernatant was removed to a fresh tube and retained for further analysis. The purified DNA was stored at 4 °C..

2.3.16 Spectrophotometric analysis of nucleic acids

DNA and RNA concentration was determined by measuring the absorbance at 260 nm, which is the wavelength at which nucleic acids absorb maximally (λ_{max}). A 50 µg/ml preparation of pure DNA has an absorbance of 1 unit at 260 nm while 40 µg/ml of pure RNA also has an absorbance reading of 1 at this wavelength. The purity of an RNA or DNA preparation was determined by reading absorbance at 260 nm, the λ_{max} for nucleic acids and at 280 nm, the λ_{max} for proteins and obtaining the ratio for these absorbances. Pure DNA and RNA have A_{260}/A_{280} ratios of 1.8 and 2.0 respectively. Lower ratios indicate the presence of protein while higher ratios often indicate residues of organic reagents.

2.3.17 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) involves the amplification of specific DNA sequences using DNA primers which anneal to the DNA of interest. The primers are designed so that one anneals to the forward DNA strand and the other anneals to the reverse strand thus allowing polymerisation of both strands by the enzyme Taq DNA polymerase. This results in exponential amplification of the sequence of interest. PCR protocols varied with respect to the DNA amplified.

PCR reaction mix

	Volume	Final Concentration
Template DNA (p7CMVE4)	1 µl	200 ng or 10 ng
10X buffer	10 µl	1X
*dNTPs	2 µl	200 µM
Forward primer (0.1 µg/µl)	1 µl	100 ng
Reverse primer (0.1 µg/µl)	1 µl	100 ng
dH_2O	84.5 µl	-
Taq DNA Polymerase	0.5 µl	2.5 U
Final volume	100 µl	

*dNTPs: 10 mM dATP, dTTP, dCTP, dGTP. Final concentration, 0.2 mM each. Reactions were overlaid with 100 µl mineral oil before placing in the minicycler (Hybid)

Cycling parameters

94°C	1 min	1 cycle
94°C	1 min	
**57°C	1 min	25 cycles
72°C	1.30 min	
72°C	5 min	1 cycle

Holding temperature : 4°C.

**This temperature was based on the Tm (melting temperature) of each primer.

All PCR products where visualised on agarose gels as outlined in section 2.3.12

2.3.18 Cloning of PCR products in pTAg

The reamplified PCR products were cloned in to the pTAg cloning vector using the LigATor rapid cloning system from R&D systems. In order to reduce the risk of removing the A overhang by nuclease contamination unpurified PCR products were used for cloning. The use of freshly amplified PCR fragments yielded best results as storage of PCR products can lead to loss of the A overhang preventing ligation to the pTAg vector. Prior to ligation residual DNA polymerase activity was removed to avoid false positives by the addition of an equal volume of chloroform:isoamyl alcohol (24:1) to the PCR reaction and vortexing for 1 min. The tube was then microcentrifuged for 1 min at room temperature at 12,000 x g. The upper aqueous phase was transferred to a fresh tube, 2 µl of this aqueous phase was used in the ligation reaction. The maximum volume used was 2 µl as the salts may inhibit the ligation reaction.

2.3.18.1 pTAg Ligation reaction

The following reagents were combined in a 1.5 ml microcentifuge tube:

10X Ligase buffer	1 µl
100 mM DTT	0.5 µl
10 mM ATP	0.5 µl
50 ng/µl pTAg vector	1µl
Amplified fragment	2 µl
Nuclease-free water	4.5 µl

The tube was vortexed briefly to mix and then microcentrifuged to collect the contents. T4 DNA ligase (0.5 µl) was added using a fresh tip and mixed gently without vortexing. The ligation reaction was incubated over night at 16°C, then placed on ice until required.

2.3.18.2 Transformation reaction

Competent cells used for the transformation reaction were provided with the LigATor Kit. One 1.5 ml tube contained 40 µl of cells which was sufficient for two transformation reactions. Cells were thawed on ice, 20 µl of which were gently pipetted into a prechilled sterile 1.5 ml microcentifuge tube. One microlitre of the ligation reaction was added to the cells and tapped gently to mix. The cells were then incubated on ice for 30 min. SOC media was also provided in the LigATor kit and it was thawed at room temperature. After 30 min on ice the cells were heat shocked at 42°C for exactly 30 s without shaking or mixing. The transformation reactions were then incubated on ice for 2 min, 80 µl of SOC media was added to each tube which were placed in a shaking incubator at 37°C for 1 hr. Prepared LB agar plates containing IPTG/X-Gal (see appendix A) were placed at 37°C for 30 min to equilibrate. Spread plates were prepared using 50 µl of the transformation reaction. The plates were left at room temperature to allow absorption of liquid and the incubated at 37°C overnight. pTAg contains a *LacZ*α peptide sequence which when functionally produced complements the N-terminal truncated *LacZ* peptide synthesised in the competent cells provided in the LigATor Kit. The resulting

enzyme β-galactosidase, cleaves X-gal to give blue colonies. IPTG depresses the expression of the *LacZα* gene in cells containing pTAg. When an insert was cloned into the cut pTAg vector the *LacZα* peptide sequence is interrupted. This interfered with the function of the peptide and white colonies were produced on the plates. This formed the basis for the selection of colonies containing inserts. Transformed (white) colonies were inoculated into 5 ml of LB amp broth and incubated in a shaking incubator overnight at 37°C. DNA minipreparations were prepared from the resulting cultures as described in section 2.3.10. The presence of inserts and their orientation was determined by restriction analysis (as described in section 2.3.4).

2.3.19 Sequencing reactions

A T7 Sequencing ™ Kit from Pharmacia Biotech was used, which is based on the dideoxy method of sequencing (Sanger *et al.,* 1977). The major steps involved in using T7 DNA polymerase to sequence DNA using a radioactive label were as follows; isolation of template DNA as described above, annealing of primer, labelling reaction, termination reaction, electrophoresis and autoradiography.

2.3.19.1 Annealing of primer to double stranded template

The concentration of DNA was adjusted to contain 1.5-2.0 μg of DNA in 32 μl of water (32 μl of miniprep DNA was used in each sequencing reaction). To denature the template DNA 8 μl of NaOH was added, the tube was vortexed and centrifuged briefly to collect drops and incubated at room temperature for 10 min. To precipitate the DNA 7 μl of 3 M sodium acetate (pH 4.8), 4 μl of dH_2O and 120 μl of 100% (v/v) ethanol

were added to the denatured template, mixed gently and placed at -20 °C overnight. The precipitated DNA was collected by spinning at 13,000 x g for 15 min the resulting pellet was washed in 70% (v/v) ice cold ethanol the tube was then centrifuged for 10 min the supernatant was removed the pellet was air dried and resuspended in 10 μl of dH_2O. Two microlitres of undiluted universal primer and 2 μl of annealing buffer was added to the template DNA vortexed and centrifuged

briefly followed by incubation at 65°C for 5 min. The tube was then transferred to 37°C for 10 min followed by room temperature for 5 min and then used directly for labelling reactions.

2.3.19.2 Labelling reaction

The following were added to the annealed template/primer mix,

Labelling mix (dATP)	3 µl
Labelled dATP (^{35}S)	1 µl
T7 DNA polymerase	2 µl

These were mixed gently by pipetting and incubated at room temperature for 5 min.

2.3.19.3 Termination reaction

Four tubes were labelled A,C,G,T for each DNA template, 2.5 µl of each of the "read short mixes" were added to their corresponding tubes and incubated for 5 min at 37°C. To each of the 4 pre-warmed sequencing mixes, 4.5 µl of the labelling reaction was added, mixed by gentle pipetting, and incubated at 37°C for 5 min. Five microlitres of stop solution was added and mixed gently. Four microlitres of each reaction was added to a fresh tube, incubated at 75-80°C for 2 min and immediately loaded on the sequencing gel. The remainder of the unheated reactions was stored at -20°C. Electrophoresis and autoradiography was carried out as described in section 2.7.4.

2.4 RNA ANALYSIS

2.4.1 RNase free environment

RNA is easily degraded by ubiquitous RNase enzymes and thus stringent measures were employed to avoid this potential hazard. All glassware and metal spatulas were baked prior to use at 180°C for 8 h in order to inactivate any RNase activity. Sterile disposable plasticware is generally considered Rnase-free and thus did not require treatment. RNases are resistant to autoclaving but they can be deactivated by the

chemical diethylpyro-carbonate (DEPC) when it is added to solutions at a final concentration of 0.1% (v/v), incubated at room temperature for 18 h and autoclaved. Solutions which contain amines such as Tris cannot be DEPC-treated as the DEPC is inactivated by these chemicals. Solutions containing these chemicals were prepared using DEPC treated H_2O followed by autoclaving. Hands are a major source of RNase contamination thus gloves were used at all times and changed frequently.

2.4.2 RNA extraction from cultured cells

Prior to RNA isolation cells were examined by phase contrast microscopy to determine the condition of the cells. A viable cell count was performed as described in section 2.6.4. RNA was extracted from cultured cells using the commercial reagent RNA ISOLATOR™. Cells grown in suspension were pelleted and then lysed in RNA ISOLATOR™ by repetitive pipetting. One millilitre of RNA ISOLATOR™ was used per 1 x 10^7 cultured cells. The homogenised sample was incubated at room temperature for 5 min to allow complete dissociation of nuclear protein complexes, (the procedure may be stopped at this point by storing samples at -70°C). Phase separation was achieved by adding 0.2 ml of chloroform per 1 ml of RNA ISOLATOR. The samples were covered and shaken gently but thoroughly for 15 s or until completely emulsified. Samples were incubated at room temperature for 15 min. The resulting mixture was centrifuged at 12,000 x g for 15 min at 4°C. Following centrifugation the mixture separated into a lower red phenol-chloroform phase, an interphase and a colourless upper aqueous phase. The aqueous phase, which contains the RNA, was removed to a fresh tube and RNA was precipitated by adding 0.5 ml of isopropanol per ml of RNA ISOLATOR used initially. The samples were stored for 10 min at room temperature, then centirfuged at 12,000 x g for 10 min at 4°C. The resulting RNA pellet was washed using 1 ml of 75% (v/v) ethanol by inverting the tube 5 times. The pellets were then recentrifuged at 10,000 x g for 5 min at 4°C and the 75% (v/v) ethanol was removed. Pellets were air dried and dissolved in DEPC treated upH_2O. The resulting RNA preparation was heated to 60°C and mixed gently to ensure a homogeneous solution prior to aliquoting. An aliquot was removed for spectrophotometric and gel electrophoretic analysis.

2.4.3 RNA analysis by gel electrophoresis

In order to check the integrity of RNA, isolated samples were run on 1.6% (w/v) agarose gels. These gels were prepared as outlined in section 2.3.12. The RNA samples (5 µl) were prepared for electrophoresis by adding 15 µl of RNA sample buffer and 3 µl of RNA loading buffer. The samples were heated to 65°C for 10 min prior to loading on the gel, which was run in 1X TAE as described in section 2.3.12. As ethidium bromide is included in the RNA loading buffer the gels did not require further staining and could be visualised directly on a UV transilluminator. The presence of two strongly staining bands, representing the 28 S and the 18 S ribosomal RNAs, indicated intact RNA. Degradation is observed by a smear running down the length of the gel.

2.5 PREPARATION OF CELL PROTEIN

Prior to protein isolation cells were examined by phase contrast microscopy to determine the condition of the cell cultures. Viable cell counts were then performed as described in section 2.6.3. All buffers required are outlined in Appendix.

2.5.1 Preparation of total cellular proteins

This method was employed to isolate total cellular protein including nuclear proteins from cultured mammalian cells. Approximately 6 x 10^7 cells from a cell line were used in each protein preparation. Cells were pelleted at 1000 x g for 5 min and washed with 10 ml of ice-cold PBS. The cells were then spun at 3,000 x g and all the supernatant was removed. The volume of the pellet was estimated and the cells were dispersed in five volumes of ice-cold suspension buffer containing freshly-added anti-proteolytic enzymes (see Appendix). This step was carried out rapidly to avoid proteolytic degradation. An equal volume of 2X SDS gel loading buffer was added, immediately after the suspension buffer, at which stage the sample becomes extremely viscous. Samples were then placed in a boiling water bath for 10 min. When required, the DNA in each sample was sheared by sonication for 1 min on full power. The resulting lysates were transferred to a microcentifuge tube and centrifuged at 10,000 x g for 10 min at room temperature. Supernatants were

aliquoted and stored at -20°C. Samples were analysed by SDS PAGE, loading approximately 6 x 10^5 cells per lane as described in section 2.6.5.

2.5.2 Protein electrophoresis, preparation of SDS-PAGE gels

A two phase SDS-PAGE system was used to analyse proteins with a 5% stacking gel and a 10% resolving gel as outlined below.

Resolving Gel (10 ml)

	10 % resolving gel (ml)	15 % resolving gel (ml)
acrylagel	3.33	5.00
bis-acrylagel	1.35	2.03
1.5 M Tris (pH 8.8)	2.50	2.50
distilled water	2.62	0.265
10% (v/v) SDS	0.10	0.10
10% (v/v) APS	0.10	0.10
TEMED	0.01	0.01

Stacking gel (2.5 ml)

	5% stacking gel(ml)
acrylamide	0.42
bis-acrylagel	0.168
1 M Tris (pH 6.8)	0.312
upH_2O	1.55
10% (v/v) SDS	0.025
10% (v/v) APS	0.025
TEMED	0.0025

2.5.3 Polyacrylamide gel electrophoresis (PAGE)

An ATTO protein gel electrophoresis system was used in this study. Glass plates were washed with detergent, rinsed first with tap water and then with dH_2O and finally wiped in one direction with tissue soaked with 100% (v/v) ethanol. The gasket was placed about the ridged plate before assembling plates and securing with

clamps. The resolving gel was then poured to within 2 cm of the top of the larger plate, overlaid with 100% (v/v) ethanol and allowed to polymerise for 45 min-1 hr. All traces of ethanol were removed by several washes with dH_2O and the stacking gel was poured. A clean comb was inserted and the gel was again allowed to set. The electrophoresis tank was filled with 1X Tris glycine running buffer to the level of the horizontal rubber gasket. After polymerisation the gaskets clamp stands and comb were removed. Unpolymerised gel was removed by gently rinsing the wells with dH_2O, the wells were then straightened using a loading tip. The prepoured gels were lowered into the buffer at an angle to exclude air bubbles from the gel buffer interface. The gel plates were fixed firmly in place with the notched plate innermost. The chamber formed by the inner plates was filled with 1X running buffer, samples were loaded and the electrodes were attached. The gels were run at 30 mA for approximately 1 hr. When complete the plates were removed, separated and the gel was either placed in transfer buffer prior to Western blotting or stained in Coomassie blue for 30 min, with agitation. The gel was then placed in several changes of destain (see appendix A) with constant agitation, until all background staining was removed.

2.5.4 Western blot analysis

An SDS-PAGE gel was run as described above with pre-stained markers (New England Biolabs). Two pieces of 3MM filter paper were cut to the size of the gel as was the nitrocellulose membrane. The sponges from the transfer apparatus along with 2 pieces of 3 MM filter paper and the SDS gel were soaked in transfer buffer. One sponge was placed on each side of the transfer apparatus and 1 piece of filter paper in turn, on each of these. The gel was placed on one piece of filter paper and the nitrocellulose membrane which had been briefly soaked in transfer buffer was placed directly onto the gel, ensuring that no bubbles were trapped between any of the layers. The second piece of filter paper and sponge were then placed on top of the membrane, the transfer apparatus was assembled and placed in the blotting apparatus with the gel on the side of the negative (black) electrode and the nitocellulose on the positive (red) side. The voltage was set at 80 volts for 2 hr. After transfer, the apparatus was disassembled and the membrane was washed briefly in TBS to remove any traces of gel, followed by blocking buffer for 1 hr. The membrane was then incubated with primary antibody at 4°C overnight. Sodium

azide was added to each antibody solution to a final concentration of 0.02% (w/v) as a preservative thus permitting reuse of the antibody.

2.5.4.1 Alkaline phosphatase detection

This method of detection was used in all immunoblotting experiments, with the exception of detection of p21/WAF1 expression, which was detected using ECL. EBNA-3A expression was detected using either method, but the ECL protocol was more optimal. After overnight incubation, the membrane was washed twice in TBST (0.1%(v/v) Tween-20) for 10 min and once in blocking buffer for 15 min. The filter was then incubated in the secondary antibody, a mouse anti-human alkaline phosphatase conjugated antibody (Promega) diluted 1/5000 in 5% blotto, for 1-2 h at room temperature, followed by washing three times with TBST for 10 min each. All the above incubations were carried out with agitation. Membranes were then placed in a clean container and covered with BCIP/NBT substrate. The container was placed in the dark at room temperature without agitation for 30 min or longer if required. The filter was then rinsed in distilled water to stop the reaction, photographed then wrapped in cling film to store.

2.5.4.2 ECL detection

This method was used in the detection of expression of p21/WAF1. After overnight incubation, primary antibody was removed and saved for re-use (up to 3 times). The membrane was rinsed x 2, then washed for 3 x 10 min in TBST after which rabbit, anti-mouse IgG (DAKO, 1:2000) was added for 60 min. The membrane was washed as before and incubated in goat, anti-rabbit antibodies (DAKO, 1:2000) for 60 min. The final washing step was as follows: rinsed x 2, 1 x 10 min followed by 3 x 5 min in TBST, with a final 5 min wash in TBS to remove tween. Freshly-prepared detection reagent (Amersham) was added such that the entire surface of the membrane was covered and left for precisely 60 s. It was important to work quickly from this point. The membrane was then carefully lifted and as much as possible of the detection solution was allowed to drain from the membrane, which was then wrapped in cling-film and exposed to X-ray film in the dark. Initial exposures were for one minute, after which the exposure time was reduced or increased accordingly

having visualised the result by developing the film. p21 was generally detectable within one minute of exposure, but exposure times may be extended if required, as the reaction intensity continues to increase up to approximately 20 min.

2.6 CELL CULTURE METHODS

All cell culture techniques were performed in a sterile environment using a Holten laminar air flow cabinet. Cells were visualised with an Olympus CK2 inverted phase contrast microscope.

2.6.1 Culture of cells in suspension

All media compositions and media supplements are given in appendix A. The cell lines DG75, DG75 tTA, DG75 tTA EBNA 2, DG75 tTA LMP1, Mutu 1, Mutu 3 c95, X50-7, BL41, BL41.B958, IARC.171, IARC 307, IARC 290B, Ag876 III, BL72 III, and BL74 were maintained in supplemented RPMI 1640 (see Appendix). Additional supplements were added to some culture media see section 2.6.2. Cultures were seeded at a density of 2 $x10^5$ to 5 x 10^5 cells per ml in 25 cm^2 flasks and expanded in 75 cm^2 flasks. Cells were sub-cultured two or three times per week by harvesting into a sterile centrifuge tube and centrifuging at 1000 x g for 5 min at room temperature. The cell pellet was resuspended gently in an appropriate volume of fresh media and replaced into the tissue culture flask. All cell lines were incubated in a humid 5% CO_2 atmosphere at 37°C in a Heraesus cell culture incubator.

2.6.2 Media supplements

Supplements were added to the growth media of certain cell lines to (a) improve cellular proliferation or (b) to select cells containing transfected plasmids (all media supplements are outlined in appendix A). L-cysteine is required for the survival and proliferation of most group 1 BL cell lines. However L-cysteine is rapidly oxidated under normal culture conditions. To improve proliferation of the group 1 Burkitt lymphoma cell line Mutu 1 α-thioglycerol was added to growth media as a stable substitute for L-cysteine. The α-thioglycerol was dissolved in bathocuproine

disulfonic acid (BCS) which effectively prevents autoxidation of thiols in liquid solutions. Sodium pyruvate was also added to protect against H_2O_2 which may be generated. HEPES was added to maintain an alkaline pH of 7.4.

The cell lines DG75tTA-EBNA2 and DG75tTA-LMP1 are tetracycline responsive cell lines in which the gene of interest is cloned downstream of a promoter containing a binding site for a hybrid tetracycline regulated transactivator (tTA) which is constitutively expressed from a second co-transfected plasmid. Tetracycline binds to the tTA and prevents it binding to the promoter which remains silent, but upon removal of tetracycline from the growth medium the tTA binds the promoter sequence and activates transcription. These cell lines were maintained in supplemented RPMI containing 1 μg/ml of tetracycline. Every three weeks the transfected cells were reselected by the addition of 500 μg/ml of hygromycin B to DG75 tTA, 500 μg/ml of hygromycin B and 1,000 μg/ml of geneticin (G418) to DG75 tTA EBNA2 and 800 μg/ml of hygromycin and 2,000 μg/ml of geneticin (G418) to DG75 tTA LMP1. The stably transfected cell lines C33A Neo and C33A LMP1 were maintained in supplemented high-glucose DMEM containing 600 μg/ml of geneticin. The parental cell line C33A was maintained in supplemented high glucose DMEM.

2.6.3 Cell counts

Cell counts were performed using an improved Neubauer haemocytometer slide. Trypan blue exclusion dye was routinely used to determine cell viability. Ten microlitres of trypan blue was added to 90 μl of a cell suspension and mixed. A sample of this mixture was added to the counting chamber of the haemocytometer and cells were visualised by light microscopy. Viable cells excluded the dye and remained clear while dead cells stained blue. Cell numbers were ascertained by multiplying the average cell count based on 4 individual counts by the dilution factor (1.1) and by taking into account the volume of the haemocytometer chamber ($1x\ 10^{-4}$ ml). Thus, counts were expressed as number of cells per ml.

2.6.4 Cell storage and recovery

Cell stocks were prepared for long term storage as follows: 1 x 10^7 cells in exponential phase were pelleted and resuspended in 800 µl of supplemented RPMI to which 100 µl of FCS was added, then placed on ice for 10 min. DMSO was added to a final concentration of 10% (v/v), mixed gently and transferred to a sterile cryotube. The cryotubes were slowly lowered into the gas phase of liquid nitrogen and immersed in liquid nitrogen in a cryofreezer (Cooper Cryoservices Ltd). Cells were recovered from liquid nitrogen by thawing rapidly at 37°C and transferring to a sterile centrifuge tube containing 5 ml of prewarmed supplemented media. The cells were centrifuged at 1000 x g for 5 min, the pellet was resuspended in 5-10 ml of fresh supplemented medium, transferred to a culture flask and incubated at 37°C in 5 % CO_2.

2.6.5 Induction of gene expression using the tetracycline-regulated system

After counting cells, an appropriate volume was pelleted at 1000 x g for 5 min and washed x 3 in sterile PBS. Cells were then incubated in supplemented RPMI for 1 h at 37°C. Cells were then washed again once in sterile PBS and seeded at approximately 2 x 10^5 cells per ml in the presence or absence of tetracycline. Uninduced cells (Tet+) were washed as per induced cells (Tet-) but were constantly maintained in the presence of tetracylcine.

2.6.6 Transient transfections

In all cases transiently transfected cells were incubated for 48 h at 37°C in a 5% CO2 incubator, before harvesting. Total DNA for transfection was normally co-precipitated in 100% ethanol the day before transfection, then washed in 70% ethanol and resuspended in a final volume of 30 µl T.E pH 7.4 (this pH is very important), using the same total quantity of DNA per transfection.

2.6.6.1 Electroporation of B lymphocytes

Cells were passaged 2 days before transfection. On the day of transfection, cells should be at about 5-7 x 10^5 /ml (definitely less than 10^6). For each transfection a 60 mm culture dish with 5 ml complete medium (RPMI 1640/glutamine/10 %FCS) was preincubated at 37°C. 10^7 cells per transfection were spun at 1000 x g for 5 min and washed in a small (one fifth) volume of cold complete RPMI. Meanwhile, DNA was added to labelled cuvettes (Biorad, 0.4 mm) and placed on ice. Cells were resuspended in cold complete RPMI at 250 µl medium per 10^7 cells, and added to cuvettes. Each cell/DNA mix was pulsed at 270V/960µF (with capacitance extender), and time was recorded in millisecs, and returned to ice immediately. Cells must not stay longer than 10 min on ice before being transferred to media. Contents of cuvettes were transferred to culture dishes using using a micropipette and yellow tip, treating cells gently. Cuvettes were washed with media from the culture dish, and placed at 37°C in a 5% CO_2 incubator for the required amount of time.

2.6.6.2 DEAE Dextran-mediated transfection (modified protocol)

Details of all solutions required for this protocol are given in the Appendix section. The day before transfection, cells were seeded at 5 x 10^5 cells/ml. After 24 h in culture, cells were counted again – it was essential for cell numbers to have almost doubled before beginning the transfection, thus ensuring that cell growth is in logarithmic phase and that cells are at their optimum for the uptake of DNA during transfection. Thus, 5 x 10^5 cells per transfection were pelleted at 900 rpm for 5 min. All traces of media were removed and cells were washed twice in TBS. All traces of TBS were carefully removed using a pipette tip. During the washing steps DNA for transfections was prepared in 20 ml sterilins. A total of 5 µg DNA per transfection was found to be optimal. The DNA for each transfection was made up to 50 µl with TE. Cells for each transfection were resuspended in 250 µl TBS and added to the DNA mix. Using gentle swirling, the DNA and cells were mixed. 300 µl of DEAE Dextran (1 mg/ml) was then added to each sterilin, which was gently swirled again to mix. The transfection cocktails were incubated at room temperature for 30 min with gentle swirling every 5-10 min to allow homogenisation. TBS (10 ml) was added to each transfection. Mixes were then spun at 900 rpm for 5 min and supernatant was removed taking care not to dislodge any cells from the pellet. Each pellet was

resuspended in 1 ml fresh complete medium. A further 9 ml complete medium was added to each sterilin and transfected cells were then transferred to 25 cm^2 cell culture flasks for incubation.

2.6.7 Stable transfections

2.6.7.1 Preparation of drug curve for G418

DG75 cells were grown in RPMI1640 with 10% FCS until just sub-confluent. Cells were then plated in 24-well or 96-well culture plates at a concentration of ~1 x 10^3 cells/ml and grown overnight before addition of drugs. As cells can divide once or twice in selective media that can kill them, it was important to seed cells at low density to ensure that cells did not reach confluency before selection could take effect. After 24 h, G418 was added in various quantities of geneticin (G418) as follows: 0, 500, 1000, 1500, 2000, 2250, 2500, 3000 μg/ml and cells were grown for 10-14 days. All cells were maintained in 500 μg/ml hygromycin B to select for the tTA plasmid and 1 μg/ml tetracycline throughout. Media was changed every 4 days during this time and cells were observed for decline of cell numbers under the inverted microscope after one week and every day thereafter. At the end of 10-14 days incubation, trypan blue exclusion was used to evaluate cellular viability in wells which still contained cells. The concentration of G418 which just killed all the cells was deemed suitable for use in subsequent selection procedures.

2.6.7.2 Stable transfection and selection of pJef-3A

DG75tTA cells were passaged the day before transfection such that cells were at approximately 50% confluency at the time of transfection. Transfections were carried out in duplicate on each ocassion. Cells (1 x 10^7) were transfected with 5 μg pJef-3A by electroporation as per section 2.6.5.1. and were allowed to recover for 24 h in 9 ml media in the presence of tetracycline. The following day 10 ml of fresh media containing drugs was added to give a final concentration of hygromycin B at 800 μg/ml, G418 at 2 mg/ml and tetracycline at 1 μg/ml. Cells were then plated out

in 96-well plates at 200 µl per well. Tetracycline is stable for 5 days, therefore media was changed every 4 days.

2.6.7.3 Immunocytochemistry (fixation and staining of tissue culture cells)

Expression of EBNA-3A in DG75tTA cells was monitored in clones using immunocytochemistry following stable transfection experiments. Prior to starting the procedure a bath of methanol and acetone were placed at -20°C for 2 h. A cell suspension was prepared of 1 x 10^6 cells/ml in supplemented RPMI, 50 µl of this suspension was placed in a 1.5 ml microcentifuge tube which had a hole pierced in the bottom (20G needle). This tube was then centrifuged in a cytospin onto a clean microscope slide at 750 x g for 5 min. The slide was allowed to air dry for 5 min then placed in ice-cold methanol for 5 min. The slide was then air-dried briefly and placed into an ice-cold acetone bath for 1 min 45 s. This procedure served to fix and permeate the cells. Slides were left to air dry overnight. Primary antibody (T2.78 anti-EBNA3A) was diluted 1/50 in PBS with 5% (v/v) FCS, 30 µl of the dilution was placed on the cell smear for 30 min in a humid chamber at 4°C. The slide was washed (3 x 3 min) with PBS, taking care to ensure that the slide did not dry out. Secondary Ab (Anti-mouse AP, Promega) was again diluted 1/50 in PBS containing 5% (v/v) FCS, 100µl was placed on the smear and incubated for 30 min in a humid chamber at 4°C. PBS washing was repeated as before and the slide was allowed to air dry. Finally, 100 µl of substrate (BCIP NBT) was added to the smear and colour was allowed to develop for a minimum of 30 min. When colour was apparent the slide was washed with water and viewed under microscope. When mounting of slides was required, it was important not to let slides dry out. A small drop of aqueous mountant was added to the slide and a coverslip was placed over the slide without trapping air bubbles.

2.6.8 Luciferase assay

Cells for luciferase assay were normally harvested 48 h post-transient transfection. After counting cells, an appropriate volume of cells was pelleted at 1000 x g and washed twice in sterile PBS. Reporter lysis buffer (1X; Luciferase Assay System,

Promega) was added to cells at 20 µl per 10^6 cells. Lysates were then transferred to microcentrifuge tubes and placed on ice. Tubes were vortexed for 10-15 s, centrifuged at 12000x g for 2 min and each supernatant was transferred to a fresh tube. Samples were stored at –80°C until required, when 20 µl was taken for assay. Samples are stable in lysis buffer over several freeze-thaw cycles. At the time of assay, it was important to allow sufficient time for detection reagent to come to room temperature. In order to obtain a background reading, luciferase activity was assayed in initially in the absence of detection reagent. Subsequently, 100 µl detection reagent was added for assay. Luciferase activity levels were adjusted for transfection efficiencies, estimated using β-galactosidase activites from lacZ reporter construct which was co-transfected with all transfections (2.6.6).

2.6.9 β-galactosidase assay

Cells for assay were normally harvested 48 h post-transient transfection. After performing a cell count, an appropriate volume of cells was pelleted at 1000 x g and washed once in sterile PBS. Cells were resuspended in Reporter lysis buffer (1X; Luciferase Assay System, Promega) at 200 µl per 10^6 cells and incubated at room temperature for 10-15 min. Cells were then centrifuged at 12000 x g for 10 min, each supernatant was transferred to a fresh tube and stored at –80°C until required. Mock transfected cells were included as control. Cell extract (30 µl) was added to 3 µl 100X Mg solution, 66 µl ONPG and 201 µl 0.1 M sodium phosphate (see Appendix A) and incubated at 37°C for 30 min or until a faint yellow colour had developed. A reaction tube was included in which ONPG substrate was omitted to be used to obtain a background reading. Reactions were inhibited, when required, by adding 500 µl 1 M Na_2CO_3. Optical densities were read at 420nm over a linear range of 0.2 – 0.8.

2.7 RNASE PROTECTION ASSAY

The ribonuclease protection assay (RPA) is a highly sensitive and specific method for the detection and quantitation of mRNA species. The RiboQuant® RNase

protection assay system (PharMingen) was employed during this study. The procedure is outlined below.

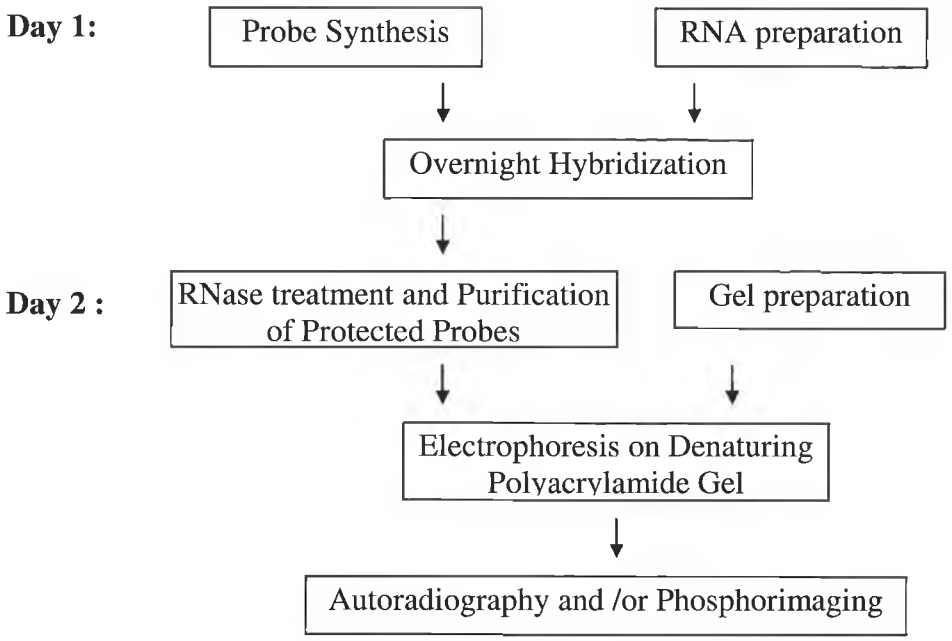


Figure 2.1 Overview of the ribonuclease protection assay protocol.

2.7.1 Probe Synthesis

The [α-^{32}P]UTP, GACU nucleotide pool, DTT, 5X transcription buffer and the template DNA set was brought to room temperature prior to setting up the reactions. The following were added to a 1.5 ml microcentifuge tube for each probe synthesis:

RNasin	1 µl
GACU pool	1 µl
DTT	2 µl
5X transcription buffer	4 µl
Template DNA (HCC-2)	1 µl
[α-^{32}P]UTP (10 µCi/ µl)	5 µl
T7 RNA polymerase	1 µl

The contents of the tube were mixed by gentle pipetting and centrifuged briefly followed by incubation at 37°C for 1 hour. The reaction was terminated by adding 2

µl of RNase free DNase, mixing gently and incubating at 37°C for 30 min. The following reagents were then added to the reactions:

EDTA 20 mM	26 µl
Tris-saturated phenol	26 µl
Chloroform:isoamyl alcohol (50:1)	25 µl
Yeast tRNA	2 µl

The contents were vortexed to an emulsion and centrifuged for 5 min at room temperature. The upper aqueous phase was transferred to a fresh tube containing 50 µl of chloroform:isoamyl alcohol (50:1), the tube was vortexed and microcentrifuged (top speed) for 2 min at room temperature. The upper aqueous phase was transferred to a sterile 1.5 ml tube to which 50 µl of 4M ammonium acetate and 250 µl of ice cold 100% (v/v) ethanol was added. The tube was inverted to mix and incubated at -70°C for 30 min followed by centrifugation at 4°C for 15 min. The supernatant was removed and the pellet was washed with 100 µl of ice cold 90% (v/v) ethanol after which the supernatant was removed and the pellet was air-dried for 5-10 min. The pellet was solubilised by the addition of 50 µl of hybridization buffer and gentle vortexing and contents were collected by brief centrifugation. Duplicate 1 µl samples of the labelled probe were quantified in a scintillation counter. A maximum yield of ~3 x 10^6 Cherenkov counts/µl with an acceptable lower limit of ~3 x 10^5 Cherenkov counts/µl was expected. The probe was diluted to approximately 3.9 x 10^5 counts/µl, which is recommmended for this particular probe, and stored at -20°C until required. Generally probes can only be used for two successive overnight hybridizations when labeled with [α-^{32}P]UTP.

2.7.2 RNA preparation and hybridization

RNA was prepared using the RNA isolation method outlined in section 2.4.2 and 20 µg of total RNA was precipitated as follows for each probe hybridization. Each RNA sample was made up to 50 µl with DEPC-treated upH_2O to which 50 µl of 4 M ammonium acetate and 250 µl of ice cold 100% (v/v) ethanol were added. The samples were mixed by inverting and stored at -70°C for 1 h or at -20°C overnight.

The precipitated RNA was collected by centrifugation at 12,000 x g for 30 min at 4°C and the pellet was washed with 90% (v/v) ice cold ethanol. After careful removal of the supernatant and subsequent air-drying, the pellet was resuspended in 8 µl of hybridization buffer by gentle vortexing for 3-4 min followed by a brief centrifugation. Two microlitres of the probe was then added to each RNA sample and mixed by pipetting. A drop of mineral oil was added to each sample and the tubes were centrifuged briefly in the microfuge. Samples were placed in a heating block preheated to 90°C, which was immediately turned down to 56°C, allowing the temperature to ramp down slowly, and incubated for 12-16 hr. The heating block was then turned down to 37°C prior to RNase treatment. Again the temperature was allowed to ramp down slowly and then was held at 37°C for 15 min.

2.7.3 RNase treatments

An RNase reaction solution was prepared by adding 2.5 ml of RNase buffer to 6 µl of RNase A + T1 mix, per 20 RNA samples (RNase A 80 ng/µl; RNase T1 250 U/µl). The RNA samples were removed from the heating block and 100 µl of the RNase cocktail was added underneath the oil into the aqueous layer (bubble). The tubes were microcentrifuged for 10 s and incubated for 45 min at 30°C. Before the RNase treatment was completed a Proteinase K mixture was prepared (per 20 samples) as follows:

Proteinase K buffer (1 X)	390 µl
Proteinase K (10 mg/ml)	30 µl
Yeast tRNA (2 mg/ml)	30 µl

An aliquot of 18 µl was added to a sterile 1.5 ml microcentifuge tube for each sample. The RNase digests were extracted from underneath the oil and transferred to the tube containing the proteinase K mixture (avoiding transfer of oil). The RNase/Proteinase K mixture was vortexed briefly, microfuged quickly and incubated for 15 min at 37°C. Tris-saturated phenol (65 µl) and 65 µl of chloroform:isoamyl alcohol (50:1) were added to the samples, vortexed to an emulsion then centrifuged for 5 min at room temperature. The upper aqueous phase was extracted, avoiding the interphase, and transferred to a fresh tube to which 120 µl of 4M ammonium acetate

and 650 µl of ice cold 100% (v/v) ethanol was added. The tubes were then mixed by inversion and were subsequently incubated at -70°C for 30 min. Samples were centrifuged for 5 min at 4°C, the pellet was then washed with ice cold 90% (v/v) ethanol, the supernatant was removed and the pellet was allowed to air-dry for 5-10 min. Pellets were resuspended in 5 µl of 1X loading buffer (provided in kit). Prior to loading onto the gel the samples were heated to 90°C for 3 min and chilled immediately in an ice bath.

2.7.4 Electrophoresis

Five percent (w/v) gels were prepared according to the formula given in Appendix. The gel apparatus was assembled and gel was cast and generally allowed to set overnight (2 h is sufficient). The gel was pre-run for 45 min at 50W in 1X TBE buffer (in upH_2O), samples were loaded and electrophoresis was allowed to proceed for 2-2.5 h at 50W. After disassembling the apparatus, the gel was lifted from the glass plates using a 3MM sheet of Whatmann cut to size and covered with cling film. The gel was allowed to dry in the gel-drier for 2 h at 80°C, then was placed in a cassette which contained an intensifying screens on each side (optional). The gel was subsequently exposed to X-ray film overnight at –70°C (or longer if required) before developing.

2.8 YEAST TWO HYBRID METHODS

Methods used in YTH-B were similar to those described for YTH-A except where stated.

2.8.1 Yeast transformation protocol (modified from Gietz et al., 1992)

A single yeast colony was inoculated in 5 ml YPD broth and incubated at 30°C overnight with shaking. This starter culture was diluted in a final volume of 25 ml YPD to give an OD_{600} of 0.2 (approximately a 1 in 10 dilution) and was then grown to an OD_{600} of betweeen 0.6 and 0.8. Cultures were spun at 3000 x g for 5 min and pellets were resuspended in 125 µl 0.1 M lithium acetate. Equilibration was carried out in a 30°C waterbath for 15 min. Aliquots of cells (50 µl) were dispensed into 1.5

ml sterile eppendorf tubes and 2 µl (1 to 10 µg) DNA plus 5 µl (20 µg) carrier DNA was added (total volume of added DNA should be no more than 15 µl). The contents of each tube were mixed by gently pipetting up and down. The following were then added and the contents of each tube was mixed by gently flicking the tubes: 240 µl 50% PEG (filter sterilised), 30 µl 10X TE buffer, 30 µl 1M lithium acetate, 5 µl carrier DNA. Tubes were incubated in a 30°C waterbath for 30 min, followed by a 20 min incubation at 42°C. Tubes were centrifuged at 3000 x g for 5 min, supernatant was removed and cells were resuspended in 100 µl sterile distilled water. Each 100 µl was spread onto a single YNB plate containing the appropriate concentrations of amino acids and incubated at 30°C usually for up to 4 days. (Colonies may take 2 to 4 days to grow to a diameter of 1-2 mm.)

2.8.2 Transcription Activation Assays

2.8.2.1 X-gal filter lift assays

Five individual colonies from each plate were patched onto the appropriate nutrient media and incubated at 30°C for 4 days eg. in YTHS-A, Glu -U-H+T+L and G/R -U-H+T+L plates were used to confirm that baits did not transactivate the lacZ promoter. Colonies were lifted by overlaying patches on each plate with nitrocellulose filters and allowing them to become wet through. Filters were subsequently removed, air-dried for 5 min, and then chilled, colony side up, at -70°C. Whatman 3MM filter paper was placed in petri-dishes each containing 3 ml 1X Z buffer with 1 mg/ml X-Gal, and allowed to soak through. Filters were then placed colony side up on the Whatman paper, incubated at 30°C and monitored for colour changes at 30 min, 60 min and overnight.

2.8.2.2 Transactivation of nutrient gene promoters

To test for ability to grow in the absence of a particular nutrient, 5 individual colonies from each plate (see transformations above) were patched onto plates lacking the appropriate nutrient and incubated at 30°C for 4 days. For example, to

test for Leu-positive least (YTHS-A), colonies were patched onto Glu-U-H+T-L and G/R-U-H+T-L plates.

2.8.3 Repression assay

To ensure that baits entered the yeast nucleus and bind to LexA operators, using the Ura+ reporter plasmid, pJK101 (see table 1). This plasmid expresses high levels of β-galactosidase when gown on galactose-containing medium. pJK101 also has LexA operators positioned between the TATA box and upstream activating site (UAS). Transcriptionally inert LexA fusions that bind to the operator in pJK101 repress expression of β-galactosidase from 2 to 20 fold in the presence of galactose. Plasmid pRFHM-1 was used as a positive control for nuclear localisation.

A rapid, quantitative assay for B-galactosidase activity in liquid cultures was employed (adapted from Bartel *et al*, 1993), in which yeast cells are permeabilised and the chromatogenic substrate o-nitrophenyl-B-d-galactoside (ONPG) is added in excess. After incubation at 30°C, the reaction was stopped by raising the pH to 11, inactivating β-galactosidase. Product formation was determined by spectrophotometry.

2.8.3.1 Preparation of cells for β-galactosidase assay

Yeast transformations were performed with the following plasmids using the protocol detailed in section 2.8.1.

pJK101 (negative control)	Glu -U+H+T+L
pJK101 + pRFHM-1 (positive control)	Glu -U-H+T+L
pJK101 + pEG-3B-525	Glu -U-H+T+L
pJK101 + pEG-3B-311	Glu -U-H+T+L
pJK101 + pLex-3B-525	Glu -U-H+T+L
pJK101 + pLex-3B-311	Glu -U-H+T+L

A 2 ml overnight culture of each transformation reaction was prepared by inoculating colonies in YNB liquid medium as follows and incubating at 30°C with shaking:

Pos control - G/R -U+H+T+L

All others - G/R -U-H+T+L

Overnight cultures were diluted by adding 2 mls of the above liquid media and grown for 2 to 3 h to an OD_{600} of 0.5. Cells were centrifuged at 2500 x g in a tabletop centrifuge, resuspended in an equal volume of Z buffer and placed on ice. OD_{600} for each sample was determined (cells in mid-log phase required no dilution to obtain an accurate OD reading; however, readings > 0.7 are inaccurate). Two reactions for each sample were prepared as follows, with mixing:

a. 100 µl cells + 900 µl Z buffer, b. 50 µl cells + 950 µl Z buffer.

One drop of 0.1% SDS and 2 drops chloroform were added to each tube using a Pasteur pipette to permeabilise the cells. Samples were then vortexed for 10 - 15 s and equilibrated for 15 min in a 30°C waterbath.

2.8.3.2 Assay for β-galactosidase activity

ONPG substrate (0.2 ml of a 4 mg/ml ONPG stock in 0.1 M KPO_4, pH 7.0, filter sterilised and stored at –20°C) was added to each sample which was then vortexed for 5 s. Tubes were immediately placed in a 30°C waterbath and timing was begun. When a medium yellow colour had developed, the reaction was stopped by adding 0.5 ml 1 M Na_2CO_3 and the time was noted. (For accuracy, the OD_{420} should be 0.3 to 0.7) Cells were centrifuged for 5 mins. at 2500 rpm in a tabletop centrifuge. OD_{420} and OD_{550} of each supernatant were determined (if the cell-debris has been well pelleted, the OD_{550} - which measures light scattering by cell debris - is usually zero and therefore is not necessary to read). Units of activity were calculated using the following equation:

$$U = \frac{1000 \times \{OD_{420} - (1.75 \times OD_{550})\}}{(t) \times (v) \times (OD_{600})}$$

where t = time of reaction (min)

v = volume of culture used in assay (ml)

OD_{600} = cell density at the start of the assay

OD_{420} = combination of absorbance by o-nitrophenol and light scattering by cell debris.

OD_{550} = light scattering by cell debris.

2.8.4 Transformation of library for interactor hunt (YTHS-B)

2.8.4.1 Library transformation (YTHS-B)

The cDNA library in pACT was transformed into yeast strain Y187 using a high efficiency protocol (modified from Gietz *et al*, 1992). Transformation was carried out by adapting the basic protocol according to section 2.8.1 to large-scale cultures. In the case of sequential transformation of bait and library DNA, the transformants can be grown in selective media or in YPD before the second transformation; the cells loose plasmids at a low rate such that selective pressure is not absolutely required at this step. Maintaining transformed cells in selective minimal media gave poor growth rate in bulk (400 ml) cultures. Use of YPD complete media speeds up growth, but resulted in loss of bait plasmid unless cells were first maintained for a day or so in selective broth in a smaller culture (50 ml). Also high quality carrier DNA was found to be critical. Sheared salmon sperm DNA (sssDNA) was prepared using the optimised protocol below (Gietz *et al*, 1992) as good quality is especially important for transformation of library DNA. The sssDNA quality was assessed by agarose gel electrophoresis on a 0.6% gel, which should give a smear of DNA ranging 2-15 kb, with an average size of about 7 kb. Oversonication leading to an average size of about 2 kb will usually cause a reduction in transformation efficiency. In addition, 10% DMSO has been found to increase efficiencies by 3-5 fold. It is important that LiAc and PEG solutions are freshly prepared. Also heat shock should be timed to precisely 15 min.

2.8.4.2 Preparation of carrier DNA

Salmon sperm DNA (Sigma grade III sodium salt) was dissolved in T.E buffer pH 7.5 at a concentration of 10 mg/ml by stirring at room temperature overnight. The DNA was sheared by sonicating at 75 % power for 2 x 30 s pulses and aliquoted into 0.5 ml volumes in eppendorfs. Sheared salmon sperm DNA (sssDNA) was then extracted with an equal volume of phenol followed by phenol/chloroform extraction and finally extracted with chloroform as described previously in DNA preparation methods. The DNA was then precipitated by adding one tenth volume 3 M sodium acetate and 2.5 volumes 100 % ethanol. Tubes were mixed by inversion and spun

immediately at 12000 x g for 15 min. The DNA pellets were then washed in 70% ethanol, dried briefly at room temperature and resuspended in sterile T.E. pH 7.5 at 5-10 mg/ml. SssDNA was denatured by boiling for 20 min and stored at –20 ^{0}C. Directly before use sssDNA was reboiled for 5-10 min then chilled on ice.

2.8.5 Harvesting transformants

Plates were placed at 4°C for about 4 h to harden the agar. Approximately 3 ml TE buffer (pH 8.0) was added to each plate and left for 1-2 min. Colonies were collected by careful scraping and gentle pipetting as it was important to avoid scraping any agar. Cells were washed twice with 3 volumes of TE buffer by pelleting each time at 2000 x g for 5 min in 20 ml sterile universal containers. The pellet was resuspended in glycerol solution to give a final volume of 2 ml. Solutions were then mixed by vortexing at low speed and frozen at -80°C in 1 ml aliquots.

2.8.6 Determination of plating efficiency

An aliquot of library transformants was thawed and 100 µl was diluted to1 ml in YNB G/R -U-H-T+L broth. Cells were then incubated at 30°C with shaking to induce the GAL1 promoter on the library. Normally, there is almost no increase in cell number during this time and any increase can be neglected when calculating the number of CFUs or transformants to plate onto Leu- selection plates. Serial dilutions were then prepared using the same broth and 100 µl of each dilution was spread on YNB G/R -U-H-T+L plates and incubated for 4 days at 30°C. Colonies were counted and used to estimate plating efficiency in colony forming units (CFUs) per unit volume of frozen cells. This should be in the order of 10^{8} CFUs/100 µl.

2.8.7 Selection of interactors

Synthesis of activation-tagged cDNA-encoded proteins was induced by thawing an aliquot of stored transformants and diluting 10-fold in YNB G/R-U-H-T+L broth. Cultures were incubated with shaking for 4 h to induce the GAL12 promoter. Cultures were incubated with shaking for 4 h to induce the GAL1 promoter. Cells

were pelleted at 3000 x g for 4 min and resuspended in sterile distilled water. Cultures were plated onto YNB G/R -U-H-T-L using 10^6 CFUs (determined from plating efficiency) per 100 mm plate. Plates were incubated at 30°C for 2-5 days, as some putative interactors appear sooner than others.

2.8.8 DNA isolation for recovering plasmids from yeast cells

A 2 ml culture of yeast carrying the plasmid of interest was grown in selective medium (YNB-L) to stationary phase (about 3 days). A 1.5 ml aliquot of culture was transferred to an eppendorf and centrifuged for 30 s at 4000 x g, washed in 200 µl sterile distilled water at room temperature and cells were again pelleted at 4000 x g. Supernatant was then aspirated off and cells were resuspended in 200 µl breaking buffer by pipetting up and down. A volume of acid-washed glass beads equivalent to about 200 µl was added, followed by 200 µl of phenol/chloroform/isoamylalcohol at a ratio of 25:24:1. Eppendorfs were vortexed at maximum for 2 min and then spun at 10,000 x g for 5 min at room temperature. Supernatant was removed and 2.5 volumes ethanol was added, mixed and incubated on ice for 10 min (or –20°C for 1 h). Supernatant was again removed and the pellet was washed with 200 µl 70% ethanol. After air-drying for about 15 min at room temperature, the pellet was resuspended in 5 µl sterile distilled water.

2.8.9 Preparation of yeast protein lysates for SDS-PAGE

Using colonies from the transactivation assay transformations, overnight cultures of the 4 baits were prepared by inoculating in 2 ml. YNB liquid medium containing Glu -U-H+T+L and incubating at 30°C with shaking. Controls were similarly prepared as follows: EGY48 in 2 mls. YPD (negative control)

pEG202 in 2 mls. YNB/Glu +U-H+T+L (positive control)

Cultures were diluted 1 in 2 by adding 2 ml. YPD and grown for 2 - 3 hours at 30°C with shaking to an OD_{600} of approx. 0.5. One mililitre of each culture was centrifuged at 4000 x g for 15 min and each pellet was resuspended in 2X FSB and frozen at –70°C for a minimum of 15 min. Just before loading, samples were boiled

for 5 min, spun at 12000 x g for 30 s and 20 µl of supernatant was used to load the gel.

2.8.10 Western immunoblotting

SDS PAGE and immunoblotting was performed according to standard methods section 2.5.2–2.5.4 using 10% acrylamide minigels.

YTHS-A: Proteins were transferred to PVDF membranes by either semi-dry or wet-blot techniques. Proteins were transferred to PVDF membrabes (as recommended). LexA-EBNA-3B fusion proteins were detected by incubation with anti-LexA (1:1000) overnight at 4°C with secondary antibody, Protein A peroxidase (1:1000), incubation period of 1 h at room temperature with shaking. Detection of proteins was performed using an ECL detection reagent (Amersham).

YTHS-B: Nitrocellulose membranes were used in association with anti-HA primary antibody (BM). An anti-mouse alkaline phosphatase conjugate (Promega) was used as secondary antibody, thus expression was detected using BCIP/NBT substrate.

2.8.11 Dot blotting (YTHS-A)

A total of 5 µl of each bait sample or control was gradually dotted using a capillary tube, onto PVDF membranes, allowing sample to dry between each 1 µl application. Detection was as per Western immunoblot detection procedure using BM Chemiluminescence Blotting Substrate (POD). Yeast strain EGY48 was included as negative control while EGY48 containing pSH18-34/pSH17-4 served as a positive control expressing LexA.

CHAPTER 3

REGULATION OF CELL CYCLE-ASSOCIATED GENES BY LMP1

3.1 INTRODUCTION

This study set out to investigate aspects of cell cycle regulation mediated by EBV. To this end, the levels of mRNA of a range of cell cycle-related genes were examined by RPA in a selection of latency group I and group III cells as well as LCL cell lines. It is now generally accepted that the EBV latent gene products are responsible for the activation of the resting B cell and the induction of continuous proliferation. As LMP1 is a key effector of EBV-mediated transformation of B cells, the contribution of this oncoprotein to EBV-mediated deregulation of the cell cycle (in the absence of other latent viral proteins) was investigated, based on findings of the preliminary RPA experiments using a range of EBV-related cell lines.

3.1.1 The Cell Cycle

The cell cycle is a collection of highly ordered processes that result in the duplication of a cell. A critical feature of the cell cycle is that it precisely duplicates the cell, and this requires that cell cycle events be executed in proper sequence. Sophisticated cell control mechanisms must ensure that each round of DNA replication (S phase) is followed by cell division (mitosis/M phase) and that one phase should not follow until the other has been successfully completed. The cell cycle can conveniently be divided into four phases. Chromosome duplication occurs during S phase and chromosome segregation plus cytokinesis during M phase. A G_1 phase intervenes between M and S, while a G_2 phase separates S from M (Figure 4.1). The pathways that render one cell cycle event dependent upon the completion of another are called checkpoints. Checkpoints can be thought of as the set of intracellular conditions that must be satisfied for cell cycle progression to continue, and thus maintain the order and timing of cell cycle events. Checkpoints also monitor the integrity of DNA and mediate cell cycle arrest and repair processes in response to DNA damage (reviewed by Elledge, 1996; Weinert and Lydall, 1993; Murray and Hunt, 1993; Pines 1992; Coats and Roberts, 1996).

We now know that much of the regulation of the cell cycle in eukaryotes is conserved throughout evolution and in recent years a great deal of research interest has resulted in

significant advances in our understanding of these very sensitive and sophisticated control mechanisms. In particular, research interests are aimed at understanding the breakdown of these controls, which can result in a variety of pathological consequences, including tumorigenesis. Oncogenic processes exert their greatest effect by targeting particular regulators of G_1 phase progression (reviewed by Harper and Elledge, 1992; Weinert and Lydall, 1993; Hunter and Pines, 1994). During the G_1 phase, cells respond to extracellular signals by either advancing toward another cell division or withdrawing from the cycle into a resting state (G_0). Thus, interference with control mechanisms at cell cycle checkpoints can lead to uncontrolled proliferation of cells.

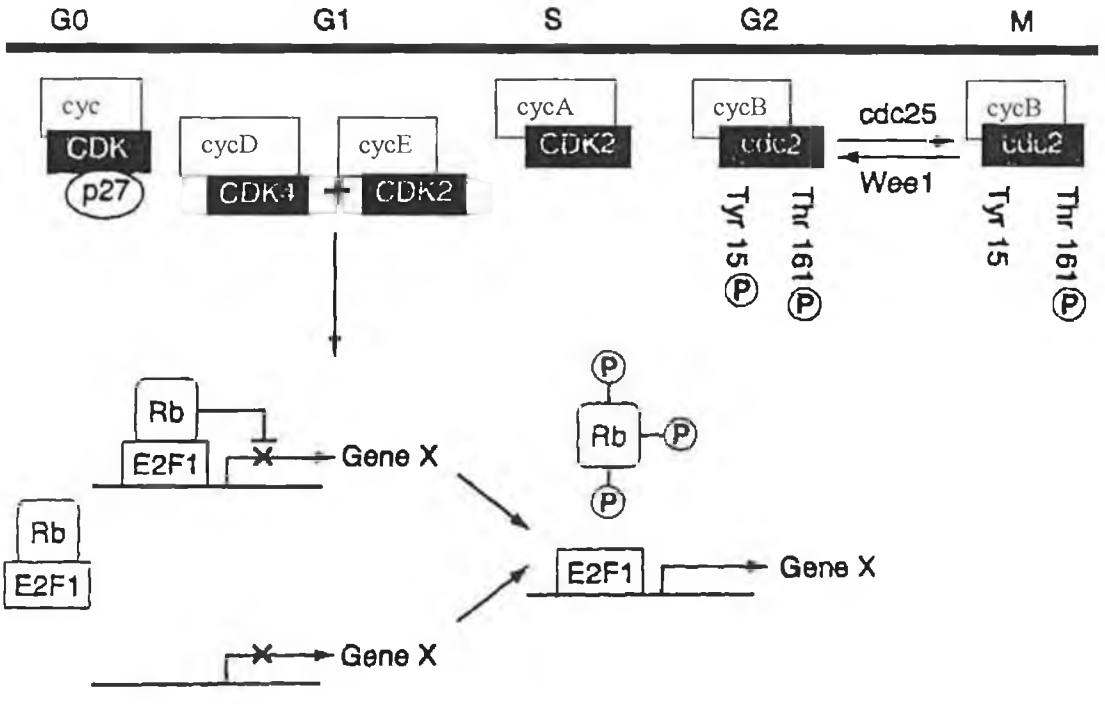


Figure 3.1 Schematic representation of the mammalian cell cycle. Cyc, cyclin; CDK, cyclin – dependent kinase. Adapted from Coats and Roberts, 1996.

3.1.2 Cyclin/cdk complexes

Central to cell cycle regulation are a set of serine/threonine protein kinases that are activated only when bound by a cyclin partner i.e. cyclin-dependent kinases (cdks / catalytic subunits) bind to cyclins (regulatory subunits) to form active cyclin-cdk complexes. Nine different cyclins have been identified to date and are designated A through I (For reviews, see Sherr, 1993; Pines, 1993). The asssociation of cdks 1 to 7 with particular cyclins are precisely-timed events in the cell cycle. Cyclin-cdk complexes form holoenzymes that phosphorylate the Retinoblastoma-family proteins (pRb, p107, p130). Phosphorylation results in inactivation of Rb proteins which have a central role in negative regulation of the cell cycle. The transcription factors E2F (1-4) and DP1 (1 and 2) which drive the DNA replication machinery are negatively regulated by direct interaction with pRb, p107 and p130. In this way, cdks drive cell cycle progression by removing the inhibitory effect of Rb proteins. The main cyclin/cdk complexes formed in vertebrate cells are cyclin D-cdk4 (G_0/G_1), cyclin E/cdk2 (G_1/S), cyclin A/cdk2 (S) and cyclin B1-cdk1 (G_2/M) (reviewed by Pines, 1994). Specific substrates for cyclin-cdk complexes include nuclear lamins, histones, oncogenes (e.g. c-*abl* and SV40 large T antigen), tumour suppressor genes (eg. retinoblastoma protein, Rb), nucleolin and others. The activated cdk can be inactivated through several pathways eg. levels of cyclins are regulated at the level of transcription as well as by targeted degradation via the ubiquitin pathway. Cdk activity is further regulated by activating or inhibiting phosphorylation, and by small proteins known as the cdk inhibitors or the CKIs (Xiong *et al*, 1993; Harper *et al*, 1993; El-Deiry, 1993).

3.1.3 Cyclin-dependent kinase inhibitors (CKIs)

Recent evidence suggests that regulated inhibition of the cdks is central to cellular responses such as differentiation, senescence, DNA damage and perhaps even apoptosis (Elledge *et al*, 1996; Grana and Reddy, 1995; Sherr, 1996). A group of small proteins

known as the cyclin-dependent kinase inhibitors (CKIs) are central to this regulation process. CKIs bind to cyclins, cdks or their complexes, thus inhibiting their activity at precisely-timed points in the cell cycle. When cells are exposed to external insults, such as DNA-damaging agents, negative regulation of the cell cycle occurs; CDK activities are inhibited by CKIs and the cell cycle is arrested in either G_1 or G_2 phase, thus preventing cells from prematurely entering into the next stage of the cell cycle before their DNA is repaired.

Mammalian cells possess two classes of CKIs which differ in structure, mechanism of inhibition and specificity (reviewed in Harper and Elledge, 1996; Sherr and Roberts, 1995; Xiong, 1996). The p21 or INK family of inhibitors consisting of p21 (CIP1, WAF1, SDI1, MDA-6, PIC1), p27 (KIP1) and p57 (KIP2) are general inhibitors of the G1S Cdks. Homology between family members is limited to a conserved amino-terminal 60 residue domain responsible for kinase binding and inhibition. p27 mediates growth arrest induced by transforming growth factor beta (TGFβ), contact inhibition or serum deprivation and is thought to play a critical role in negative regulation of cell division in vivo (Sherr, 1996). Recently, high level expression of p27 from adenovirus (Ad) vectors has been shown to induce apoptosis in tumour cell lines (Wang *et al*, 1997). The second class of CKIs, the INK4 (inhibitor of cdk4) family are specific inhibitors of cyclin D1/cdk4 or cdk6 complexes. This family of ankyrin-repeat proteins includes p16 (INK4a), p15 (INK4b), p18 (INK4c) and p19 (INK4d). CKIs vary in their ability to promote or protect against cell death i.e. while p16, p18 and p27 appear to be most effective at inducing cell death, p21 and p19 promote cell suvival (Schreiber *et al*, 1999).

It is not yet fully understood why there are so many CKIs and what specific roles the individual CKIs have in cell cycle regulation, differentiation and tumorigenicity but currently this is an area of active study. Mutations within the p16 gene and loss of p16 expression are frequent events observed in both tumour cell lines and in various carcinomas (Sherr, 1996). The p18 and p19 CKIs have not, as yet, been as well characterised. Both are frequently expressed in cells of the myeloid lineage (Schwaller *et*

al, 1997) and p18 is thought to play a pivotal role in terminal differentiation of late-stage B cells to plasma cells (Morse *et al*, 1997).

3.1.4 p21/WAF1 cdk inhibitor.

p21 was the first of the CKIs to be identified (Harper *et al*, 1993; Xiong, 1993: El-Deiry, 1993) and has since been found to share homology with p27 and p57 which together form a family of universal inhibitors of the G_1S cdks. p21 was originally documented as an inhibitor of cdk2, cdk4 and cdc2 (cdk1) kinase complexes (Gu *et al*, 1993; Harper *et al*, 1993; Xiong *et al*, 1993). More recently it was shown that p21 can also inhibit cdk3 and cdk6 kinases and that p21 is most effective towards G_1 cyclins (Harper *et al*, 1995). Indeed when overexpressed in transiently transfected cells, p21 can cause cell cycle arrest in a variety of cell lines (Guan *et al*, 1994; Harper *et al*, 1995; Medema *et al*, 1995), suggesting that it can interfere with cyclin-dependent activity in intact cells. p21 has also been implicated as an effector of the TGFβ growth inhibitory signalling pathway (Datto *et al*, 1995). This 21 kDa nuclear protein is a dual specificity inhibitor in that it not only binds to cdks but also associates with the DNA replication factor, PCNA via the former's unique carboxy-terminal domain (Warbrick *et al*, 1995). PCNA functions in both DNA replication and repair as a subunit of DNA polymerase delta. p21 therefore can directly inhibit DNA replication in the absence of cyclin-cdk complexes (Waga *et al*, 1994) and overexpression of this interaction domain in mammalian cells has been reported to reduce the fraction of cells found in the S phase cells (Luo *et al*, 1995). Strikingly, whereas in normal cells most of the cdk-cyclin complexes are found associated with p21 (and PCNA), this association is absent in most transformed cells (Xiong *et al*, 1993). An unusual feature of p21 function is that multiple inhibitor molecules are required for cdk inhibition, such that complexes containing a single inhibitor molecule are catalytically active, whereas those containing multiple p21 subunits are not. Changes in the stoichiometry of p21 appear to be sufficient to account for the conversion (Zhang *et al*, 1994).

The p21 gene contains binding sites for the p53 tumour suppressor protein in its promoter and studies have shown that the transcription of p21 can be directly regulated by p53 (Lee *et al*, 1995; Haendler *et al*, 1987). p53-mediated growth arrest as a result of genotoxic damage appears to be mediated at least in part by induction of p21 (El-Deiry *et al*, 1993; Dulic *et al*, 1994). Indeed, p21 has been defined as a cardinal mediator of p53-induced G_1 checkpoint control. Other reports have shown that p21 can be induced by p53-independent mechanisms (Michieli *et al*, 1994; Sheikh *et al*, 1994). For example, in cellular differentiation, p53-independent induction of p21 has been observed as an immediate early response to a variety of physiological and chemical stimuli. Various researchers have reported that p21 expression has a protective effect against apoptosis induced by p53 as well as other agents (Gorospe *et al*, 1997; Lu *et al*, 1998). However, despite extensive searches, very few tumours have been shown to have mutations in their p21 genes (Harper and Elledge, 1996) and p21-null mice appear to develop normally and show no increased incidence of neoplasia (Deng *et al*, 1995).

3.1.5 p53 and pRb tumour supressor genes

The fundamental importance of p53 as a tumour suppressor is underscored by the statistic that at least one in six of the population will develop cancer due to defective p53 function. p53 is a 53 kD nuclear multifunctional phosphoprotein which guards the stability of the genome by inhibiting cell proliferation when DNA damage occurs (Canman and Kastan, 1995). One way the p53 protein acts is by behaving as a transcription factor to upregulate specific target genes, which ultimately results in either cell growth arrest or apoptosis. These genes include mdm2 (which negatively controls p53 expression), GADD45 (involved in DNA replication), the pro-apoptotic bax gene and p21 which inhibits kinases responsible for G_1-S transition, thus arresting the cell cycle at the G_1 phase (reviewed by Milner, 1996). Wild type p53 protein has a very short half life and is usually not detectable with monoclonal antibodies in normal tissues. Mutant p53 proteins typically have an increased half-life, accumulate to high levels, and are detectable with mAbs. Mutations in the p53 gene have been detected in a wide variety of cancers (Levine *et al*, 1993), the majority of which inactivate the tumour

suppressing function of the protein (loss of function mutants), while others confer transforming activity (oncogenic mutants) (Dittmer *et al*, 1993). Elevated expression of p53 is a frequent finding in different tumour types (including Burkitt's Lymphoma), indicating that at least in some tumours. in addition to mutation of the p53 gene, high levels of mutant protein are required for malignant transformation in vivo (Farrell *et al*, 1991).

Deregulation of pRb function(s) is a fundamental characteristic of tumorigenesis (reviewed by Mulligan and Jacks, 1998). The three known members of the Rb family of proteins, in their activated state, associate with and modulate the activity of several cell transcription factors, including the E2F and DP gene families. In this way, by inhibiting transcription of genes required for DNA replication, Rb proteins act to block G1/S progression in normal cells. Thus, interference with this fundamental control mechanism can result in uncontrolled proliferation of cells. While pRb has a well-established role in tumour suppression (Rb-/- mouse embryos die within 13 –15 days of gestation), the activities of p107 and p130 in tumour suppression remain unclear. Interbreeding studies using mutant mouse strains have revealed significant functional overlap within the Rb gene family although distinct in vivo functions are also indicated, which are only recently beginning to emerge (reviewed by Mulligan and Jacks, 1998). In lymphocytes, p107 fully compensates for p130 deficiency, and the absence of both might lead to compensation by pRb and other as yet unidentified protein(s). Thus, despite their diversification and specialisation, currently available in vivo assays suggest that the pRb family of proteins act in a coordinated fashion to regulate at least some cellular functions (reviewed by Stiegler *et al*, 1998; Grana *et al*, 1998).

3.1.6 Epstein-Barr virus, tumorigenesis and the cell cycle

Increasing evidence suggests a critical role for mammalian cell cycle regulatory proteins in tumorigenesis. Progression through the mammalian cell cycle is controlled by the regulatory interplay between distinct positive and negative regulators (reviewed by Coats and Roberts, 1996, Murray and Hunt, 1993). This passage through the individual

phases of the cell cycle is affected by viral oncoproteins which can provide their host cells with additional growth stimuli, thereby extending their proliferative capacity. Virally transformed cells fail to cease proliferation in response to many growth-suppressing signals, implying that major cell cycle controls are lost in such cells.

In recent years, striking parallels have emerged in the strategies used by small DNA tumour viruses to transform cells. Two cellular pathways are disrupted by oncoproteins of Adenovirus, SV40 and the oncogenic strains of the human papilloma virus (HPV), involving interference with the p53 and pRb pathways. The discovery that the p53 dependent pathway is functional in EBV-immortalised cell lines (Allday *et al*, 1995) demonstrated that one arm of this common transformation strategy is not required by EBV to immortalise primary B lymphocytes, suggesting that EBV may differ significantly from the small DNA tumour viruses in this regard. Many aspects of EBV-mediated immortalisation resemble the normal programme of B lymphocyte activation, suggesting that EBV achieves the immortalisation of B lymphocytes by substituting for the stimuli these cells normally require for proliferation. EBV induces the same cell cycle regulating proteins as polyclonal stimuli in primary B cells, bypassing the requirement for antigen, T cells and growth factors (Kempkes *et al*, 1995). One model suggests that one component of EBV-driven cell proliferation involves the modulation of the activity of pRb and p107 (and potentially other substrates), following the activation of a normal cellular phosphorylation pathway. It is significant that pRb is not however functionally inactivated as a result of immortalisation by EBV as indicated by the absence of a rise in p16 levels which is normally subject to negative regulation by pRb (Cannell *et al*, 1996). Thus, it appears that EBV is able to immortalise primary B lymphocytes without functionally inactivating either p53 or pRb.

Mutations and elevated expression of the p53 tumour suppressor gene are two of the factors that can play a role in malignant transformation or tumour cell growth of Burkitt's Lymphoma. BL cells with p53 lesions have been shown to be relatively resistant to DNA-damaging drugs when compared to LCLs which induce transcriptionally active p53 as part of a pro-apoptotic response. Allday *et al* (1995)

found that BL cells remain relatively viable when challenged with drugs such as cisplatin. Mutations in p53 have been found in approximately 33% of BL biopsies and in at least 63% of BL cells lines. In 65% of BL cell lines studied the wild type allele is lost and the cells express the mutant p53 allele at elevated levels. In general, single nucleotide substitutions which result in amino acid changes are found. In these studies, no EBV protein was found to be associated with p53 and no correlation has been found between EBV status and p53 mutation status in BLs. Various different mutants of p53 have been observed to lose the suppressor function, to gain a dominant transforming activity or to be unaffected. In any case, in addition to mutation of p53, it appears that elevated levels of mutant p53 protein are required for transformation (Farrell *et al*, 1991; Balint and Reisman, 1996)

EBV efficiently converts resting human B cells into immortalised cell lines, referred to as lymphoblastoid cell lines (LCLs), in which the normal mechanisms that control cell cycle and apoptosis are permanently dysregulated. At least six viral genes are essential for growth transformation of resting B cells: the nuclear proteins EBNA-1, -2, -3A, -3C and EBNA-LP and the latent membrane protein, LMP1 (reviewed by Farrell, 1995) suggesting that a complex series of events are needed to override normal growth controls. Upon infection of resting B cells with EBV, cells enter the cell cycle, with DNA synthesis beginning at approximately 48 hours, accompanied by increased expression of cyclin E and PCNA, two established markers of G_1/S progression. These events are preceeded, however, by the expression of cyclin D2, which to date is the earliest cellular gene known to be activated by EBV infection. Cyclin D2 is normally undetectable in primary B cells. Expression of EBNA2 and EBNA-LP has been shown to be sufficient to induce cyclin D2 expression, thus cooperating to cause G_0 to G_1 transition during B cell immortalisation (Sinclair *et al*, 1994; reviewed by Farrell, P. 1995). Using quantitative RNAse protection assays, cyclin D2 mRNA was shown to be increased at least 100-fold as a result of infection of primary B cells with EBV. Similar fold increases were seen in cdk1, cyclin E and CD23 using RT-PCR, while *B-myb* and *c-myc* mRNA were upregulated by at least 10-fold in LCLs (Sinclair *et al*, 1994).

One study found that pRb, p130 and p107 were readily detectable by Western blot analysis of total protein lysates isolated from quiescent primary B lymphocytes. Following EBV-mediated immortalisation of these cells, the relative level of p130 protein was found to decrease, while in contrast, levels of p107 and pRb appeared to increase (Cannel *et al*, 1996). The apparent contradiction of an increase in negative regulators of the cell cycle may be explained by a decrease in the mobility of both p107 and pRb, which is characteristic of their hyperphosphorylation and inactivation. The changed mobility of pRb is an early event during immortalisation and is readily detectable by 52 hours post-infection.

EBNA2, one of the first genes expressed after EBV infection of B cells (Allday *et al*, 1989), is a transcriptional activator of viral and cellular genes and is central to the transformation potential of the virus. Using recombinant EBV in which the expression of functional EBNA2 is dependent upon the addition of oestrogen, it has been shown that EBNA2 does not perturb the physiological order of cell cycle progression but rather induces B cell activation and entry into the cell cycle by inducing and maintaining the expression of early G_1 regulating proteins, such as cyclin D2, cyclin E and cdk4, while cdc2 (cdk1) and cdk2 are constitutively expressed (Kempkes *et al*, 1995). Shortly after upregulation of cyclin D2, there is a significant shift of Rb to the hyperphosphorylated state. E2F1, a transcriptional activator of genes involved in DNA synthesis (which is maintained inactive by binding to the hypophosphorylated form of Rb) becomes detectable concomitantly with the modification of Rb. Expression of LMP1 and c-Myc preceed accumulation of E2F-1, suggesting that E2F-1 is not involved in transcriptional activation of c-myc after reactivation of EBNA2, but other members of the E2F family may be involved as the c-myc gene contains an E2F site in front of it's second promoter. In the absence of EBNA2 about half of the cells enter a quiescent, non-proliferative state whereas the others die by apoptosis, following transformation of primary B cells with this mutant virus. Growth arrest occurrs at G_1 and G_2 stages of the cell cycle and a role in terminating S phase can not be excluded, indicating that functional EBNA2 is required at different restriction points of the cell cycle. Since EBNA2 is a pleiotropic activator of the other EBNAs and the LMPs, many of the cellular events observed upon

EBNA2 activation here may be due to activation of viral genes downstream of EBNA2, in particular LMP1 which has the most profound effects on cell growth.

3.1.7 Effects of LMP1 on aspects of the cell cycle.

The EBV-encoded LMP1 protein plays an important role in the immortalisation of B cells infected with EBV. Expression of exogenous LMP1 in EBV-negative BL cells has been associated with an increased resistance to apoptosis induced by serum starvation (Henderson *et al*, 1991), an effect which has been attributed to upregulation of the level of Bcl-2 protein. The oncogenic activity of Bcl-2 appears to result from its ability to promote cell survival rather than cell proliferation. Another protein which may play a role in the LMP1-mediated pro-survival effect is A20, a zinc-finger protein that confers resistance to TNFα cytotoxicity (Henderson *et al*, 1991; Rowe *et al*, 1994; Laherty *et al*, 1992). It has been shown that LMP1 blocks WT p53-triggered apoptosis, but has no effect on G_1 cell cycle arrest induced by WT p53. p21 is probably an important downstream effector of p53-induced cell cycle arrest and/or apoptosis. Recent data clearly show that LMP1 does not interfere with p53-mediated induction of p21. WTp53 also induces expression of the pro-apoptotic gene, *bax*. As the ratio between bax and bcl-2 determines whether a cell will enter apoptosis or survive after receiving an apoptotic signal, it is thought that LMP1 may block p53-induced apoptosis by upregulating bcl-2 and thereby counteracting the apoptosis-promoting effect of bax (Okan *et al*, 1995). Arvanitakis *et al* (1995) have shown that the presence of WT EBV or LMP1 results in the loss of TGFβ-mediated growth inhibition in human B cells. In their study, LMP1 induced the expression of cyclin D2 (normal B cells or EBV-negative BL cells do not express D-type cyclins), thus maintaining pRb in the hyperphosphorylated (non-functional) form and allowing the cell to proceed through the cell cycle regardless of the presence of TGFβ. A study which employed tetracycline-regulated expression of LMP1 found that LMP1 had a cytostatic effect in the EBV-negative BL cell lines, DG75 and BJAB and the EBV-positive Akata cell line (Floettmann *et al*, 1996). The cytostatic effect was shown to be due to an accumulation of cells at the G_2/M phase of the cell cycle, suggesting a novel function for LMP1 in controlling the proliferation of EBV-

infected cells by regulating progress through G_2/M. These data did not necessarily contradict a previous report which found that LMP1 induces a G_1 to S transition (Peng and Lundgren, 1992) as DNA synthesis (S phase) may continually be stimulated in cycling LMP1-positive cells (Wang *et al*, 1988).

3.1.8 Tetracycline-regulated gene expression

In order to study the effects of LMP1 expression, an established tetracycline-regulated inducible expression system was employed. For comparison, expression of EBNA2 (also a main effector of EBV-mediated phenotypic change) was induced in a similar manner and included in initial experiments. The tetracycline regulated system used by Floettmann *et al.*, (1996) is based on that developed by Gossen and Bujard (1992), in which the gene of interest is cloned downstream of a promoter containing binding sites for the hybrid tetracycline-regulated transactivator (tTA). The plasmid tTA encodes a fusion protein of the sequence- specific DNA binding tetracycline repressor (TetR) and the C-terminal domain of the herpes simplex virus VP16 transactivator. A second plasmid contains the gene of interest cloned downstream of seven copies of the *Escherichia coli Tn10* tetracycline operator (tetO) contiguous with a CMV-IE minimal promoter. When tetracycline is present, it binds to the tTA preventing it binding to the promoter. Upon removal of tetracycline the hybrid TetR binds to the tetO site positioning the VP16 domain so that it can transactivate the CMV-IE promoter (Gossen and Bujard, 1992).

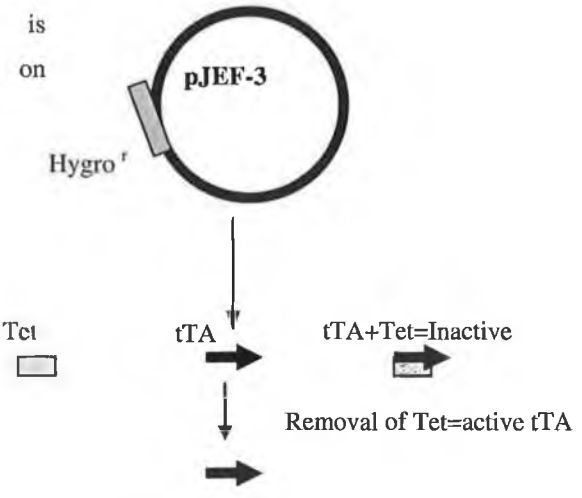
Stable cell lines containing plasmids in which EBNA2 and LMP1 expression was regulated by tetracycline were generated by Floettmann *et al.*, (1996) as follows: The tTA expressing plasmid pUHD15-1 was modified by the addition of a hygromycin resistance gene under the control of an SV40 promoter creating the drug-selectable tTA-expressing vector pJEF-3. A neomycin resistance gene under the control of an SV40 promoter was cloned upstream of the tTA responsive promoter of pUHD10-3 to create the responsive vector pJEF-4. The EBNA 2 coding with a 5' rabbit *β-globin intron* was

cloned into pJEF-4 to produce pJEF-31 (Figure 3.1). The LMP1 cDNA was inserted into pJEF-4 to produce pJEF-6. pJEF-3 was then transfected into DG75 to give the stable cell line DG75 tTA, which was then transfected with pJEF-31 producing the cell line DG75 tTA EBNA2 or pJEF-6 giving the cell line DG75 tTA LMP1 (Floettmann *et al.*, 1996). This system allowed examination of either EBNA 2 or LMP1 in the same cell background before and after induction of the EBV protein thus eliminating clonal variations which may occur between cell lines.

TETRACYCLINE REGULATED CELL LINES DG75 tTA EBNA2/DG75 tTA LMP1

The host cell line DG75 is stably transfected with two plasmids pJEF-3 and pJEF-31 or pJEF-6 which are selected during cell culture using the drugs Hygromycin and G418 respectively.

A tetracycline regulated transactivator **(tTA)** is constitutively expressed on pJEF3



Active tTA binds to a 5' regulatory region conatining a minimal promoter resulting in expression of EBNA2 or LMP1

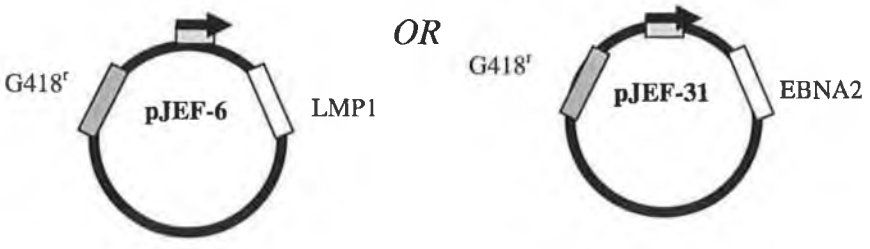


Figure 3.2 A schematic representation of the tetracycline-regulated gene expression system. The example given shows regulation of expression of LMP1 and EBNA2 in DG75tTA-EBNA2 and DG75tTA-LMP1 cells, respectively.

3.2 RESULTS

3.2.1 mRNA levels of a group of cell-cycle genes in various EBV-related cell lines.

This study set out to investigate EBV-associated changes to the expression of a panel of cell cycle-related genes in BL cells. In a preliminary experiment using two established isogenic EBV-positive BL cell lines, group I MUTU-BL and group III MUTU-BL, mRNA levels transcribed from the *Rb, p130, p107, p53, p57, p27, p21, p19, p18, p16* and *p14/p15* genes were compared using multiprobe ribonuclease protection assay (RPA; HCC-2 kit, Pharmingen). This approach permitted the simultaneous detection and quantitation of these mRNAs in a single sample and also enabled direct comparative analysis of RNA from these two cell lines by the inclusion of probes for transcripts from the two housekeeping genes *GAPDH* and *L32* (Figure 3.3). Group I BL cells (type 1 latency) express EBNA1 as sole viral protein; when serially passaged in vitro, they 'drift' to express all the known latency-associated viral proteins (including LMP1), when they become known as group III BL cells (type III latency). Group III BL cells have acquired many of the phenotypic characteristics of an immortalised LCL (Gregory *et al*, 1990). It can be seen from this experiment that significantly elevated steady state levels of *p21* mRNA were present in MUTU-III relative to MUTU-I cells. In each case *p21* bands were quantitated by densitometric scanning using two different exposure times. *GAPDH* and *L32* mRNA levels were similarly assessed to allow for differences in the amounts of total RNA analysed. The results showed a >10-fold higher level of *p21* mRNA in MUTU-III versus MUTU-I. The exact level of upregulation was difficult to establish accurately due to the very low levels of *p21* mRNA transcript in MUTU-I cells. In order to investigate if upregulated *p21* expression was a general feature of EBV-infected cells, *p21* mRNA levels from the EBV-negative cell line, BL41, were compared to those in it's EBV-superinfected derivative, BL41-B95-8, and to a spontaneous EBV-transformed normal LCL (IARC171), which is derived from the same patient as BL41. Barely detectable levels of *p21* mRNA were seen in BL41, which were significantly upregulated in BL41.B95.8 (Figure 3.3A). Although the level in BL41.B95.8 was significantly less than in MUTU-III, the fold upregulation was observed to be

approximately similar in the transition from MUTU-I to MUTU-III as that from BL41 to BL41.B95.8.

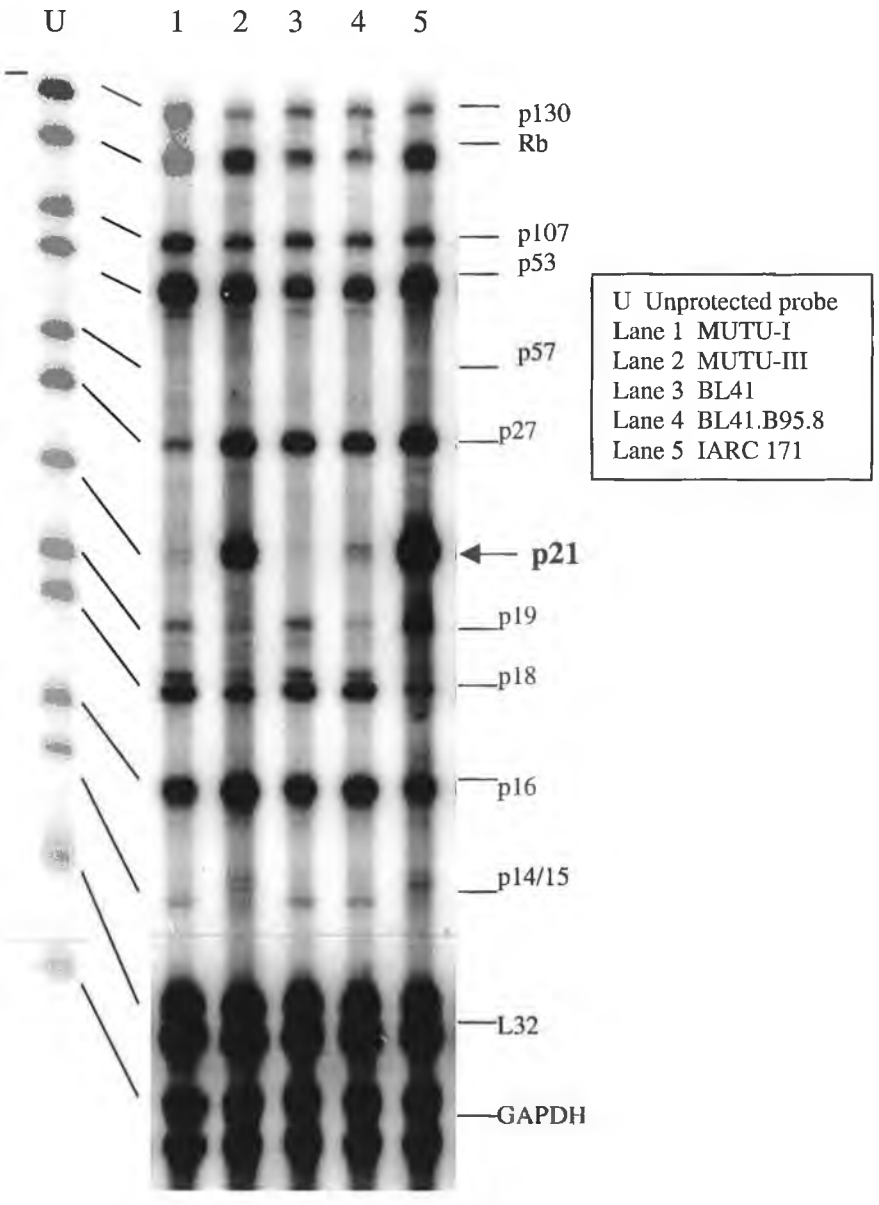


Figure 3.3 EBV-associated modulation of the steady state levels of mRNAs from cell cycle-related genes. **(A)** The steady state level of *p21* mRNA is elevated in EBV-infected LMP1-expressing B cells. Expression of the cell cycle-related genes *p130, pRb, p107, p53, p57, p27, p21, p19, p18, p16 and p14/15* in a range of EBV-related cell lines as detected by mutiprobe RPA. Unprotected riboprobes (lane U) are shown linked to their smaller RNAse-protected fragments which correspond to protected portions of mRNA in each sample. Details of cell lines in each lane are given next to each autoradiogram image.

Furthermore, the transition from group I BL74 BL cells to its spontaneously transformed LCL, IARC290B (derived from the same patient), showed a significant upregulation in levels of p21 mRNA. High levels of *p21* mRNA were also observed in another Group III BL cell line, Ag876-III (Figure 3.3B), and two other LCLs, X50-7 and OKU-LCL (not shown), while EBV-negative DG75 cells showed much lower levels of *p21* mRNA (Figure 3.3B).

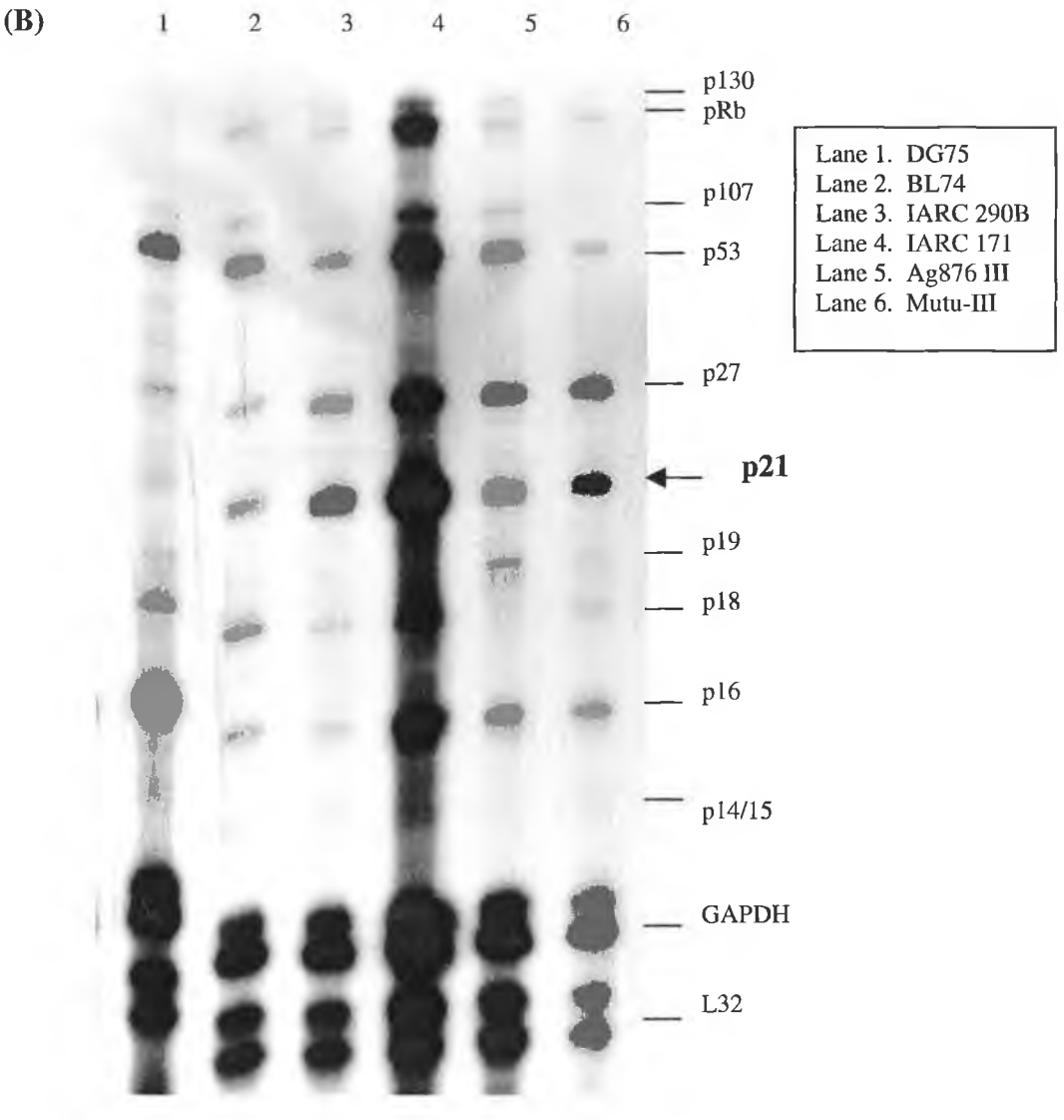


Figure 3.3 (B) as per (A) Levels of mRNA transcripts from the *p130, pRb, p107, p53, p57, p27, p21, p19, p18, p16* and *p14/p15* genes were compared in a range of EBV-related cell lines using total RNA in multiprobe RPA assays.

An interesting observation was also the decreased levels of *p19* mRNA in MUTU-III and BL41.B95.8 cells relative to their latency type I counterparts, while LCLs showed very low levels of *p19* mRNA. Transcript from the gene encoding the p18 protein is barely detectable in the three LCLs and is undetectable in Ag876 Group III cells, an interesting observation in that it is also down-regulated in MUTU-BL upon drift to the group-III phenotype (Figure 3.3A). This does not however appear to be a general feature of EBV-positive BL cells exhibiting a group-III phenotype in that the level of *p18* mRNA is relatively unchanged in BL41.B95.8 versus BL41 cells. Additionally, although elevated levels of *Rb* and *p27* mRNA and down-regulated levels of *p110* are seen in MUTU-III relative to MUTU-I, these effects are also inconsistent in that they are not observed in the BL41/BL41.B95.8 pair. mRNA from the cdk inhibitor, *p57,* was not detected in any of the cell lines analysed.

Protein lysates harvested from the same cells as those used for RPA analysis as well as a number of other B cell lines were examined for p21 levels by Western blot analysis using a mouse monoclonal anti-p21 antibody (Santa Cruz SC6246) (Figure 3.4). These results showed that, in general, higher levels of p21 protein were observed in LCLs and Group III BL cells relative to Group I BL cells, in agreement with RPA data. The corresponding levels of LMP1 protein are also shown (Figure 3.4A, lower panel) for comparison. Overall, these results demonstrate that stably elevated levels of *p21* mRNA and protein are a feature of EBV-infected B cells exhibiting type III latency. Due to the barely detectable levels of p21 mRNA/protein in MUTU-I and BL41 cells it was difficult to compare the fold increase in mRNA versus protein.

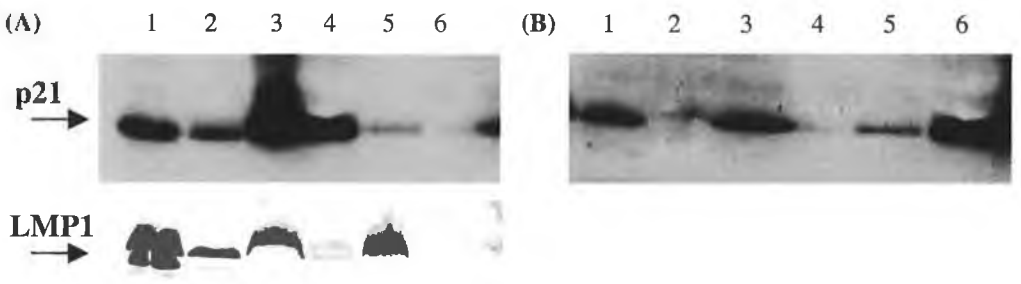


Figure 3.4 Western blot analysis of a range of EBV-related cell lines for p21 protein expression. (A) and **(B)** Protein extracts were prepared from the same cells as those in Figure 1 RPA assay and analysed along with some additional cell lines. For (A), upper panel and (B), proteins were separated on 15% SDS-PAGE gels and p21 detection was performed using an ECL detection protocol. For (A) lower panel, a 10% gel was used and LMP1 expression was assessed using an alkaline phosphatase conjugate.

(A) Upper and lower panels

Lane 1: BL72III
Lane 2: IARC307
Lane 3: X50-7
Lane 4: Ag876 III
Lane 5: BL41.B95.8
Lane 6: BL41

(B)

Lane 1: IARC 290B
Lane 2: MUTU-I
Lane 3: MUTU-III
Lane 4: BL41
Lane 5: BL41.B95.8
Lane 6: Ag876 III

3.2.2 Tetracycline-regulatable induction of LMP1/EBNA2 expression.

As both LMP1 and EBNA2 are known to be main effectors of phenotypic change in EBV-infected cells, it was therefore of interest to investigate if the expression of either of these viral proteins alone could contribute to an upregulation of p21 levels. To this end, an established tightly-regulatable expression system was employed to induce LMP1 in the EBV-negative BL cell lines, DG75tTA-LMP1 and BJABtTA-LMP1. EBNA2 expression was similarly induced in DG75tTA-EBNA2 cells. Briefly, the inducible promoter driving LMP1/EBNA2 expression contains binding sites for a hybrid tetracycline-regulated transactivator (tTA) that is constitutively expressed in the parental clone DG75-tTA and BJAB-tTA. Removal of tetracycline from the growth medium leads to tTA binding to the promoter and the expression of LMP1/EBNA2. Levels of induced LMP1 were monitored by Western blot analysis using the anti-LMP1 murine monoclonal antibody cocktail CS.1-4 (Rowe *et al*, 1992), while EBNA2 was detected

using the murine monoclonal antibody PE2 (Rowe *et al*, 1992). In these experiments LMP1 was normally detectable in induced DG75tTA-LMP1 cells by 18-24 hours, and remained detectable for up to at least 96 hours (Figure 3.5A). At 48 hours the level of LMP1 was comparable to that seen in the reference LCL, X50-7 (not shown). Levels of LMP1 were significantly higher in induced BJABtTA-LMP1 cells (Figure 3.5B) relative to DG75tTA-LMP1 cells, a finding which has been observed previously (Floettmann *et al*, 1996). EBNA2 was detectable in DG75tTA-EBNA2 cells by 24 hours and remained detectable up to at least 96 hours (Figure 3.5C). Total RNA and protein lysates were prepared simultaneously from cells harvested at the indicated time points and used as before in multiprobe RPA and Western blot analysis. In this way, RPA analysis permitted an investigation of the effects of LMP1 and EBNA2, when each was expressed as sole EBV protein, on levels of mRNA transcribed from the same group of cell cycle-related genes as outlined above.

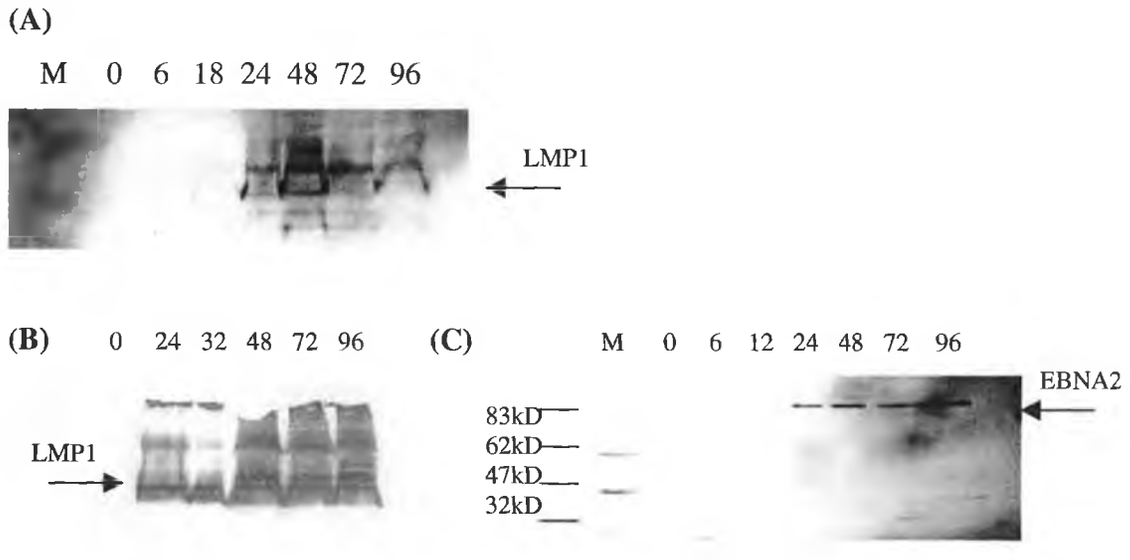


Figure 3.5 Tetracycline-regulatable induction of LMP1/EBNA2 expression. Cells were induced to independently express LMP1 or EBNA2 by reculturing cells in the absence of tetracycline. Cells were harvested and protein extracts were prepared for Western blot analysis at the indicated time points (hours) post-induction. In both cases detection was based on alkaline phosphatase enzyme activity. **(A)** Induction of LMP1 expression in DG75tTA-LMP1 cells. **(B)** Induction of LMP1 expression in BJABtTA-LMP1 cells. **(C)** Induction of EBNA2 expression in DG75tTA-EBNA2 cells. Lane M represents protein molecular weight markers. Predicted sizes of marker proteins are indicated on the left side of Figure (C).

3.2.3 LMP1 upregulates p21 expression in DG75 BL cells.

Levels of mRNA transcripts from cell cycle-related genes were compared over a 96-hour time course following induction of LMP1 expression in the EBV-negative BL cell line, DG75tTA-LMP1. An approximately 5 fold upregulation of p21 mRNA was observed, with a more subtle increase (about 2 fold) in p19 mRNA levels. In uninduced DG75 cells, the p21 mRNA levels were very low or undetectable, a finding which is consistent with p21 levels observed in other EBV-negative BL cell lines (Okan *et al*, 1995; Allday *et al*, 1995). Based on several individual induction experiments it was found that the level of p21 mRNA peaked at between 24 and 48 hours and remained high until at least 72 hours post induction (Figure 3.6A). The level of p21 mRNA was significantly higher in MUTU-III relative to that detected in DG75tTA-LMP1 cells 48 hours post-induction of LMP1 expression, implying that other EBV latent proteins may serve to further enhance p21 expression in MUTU-III cells.

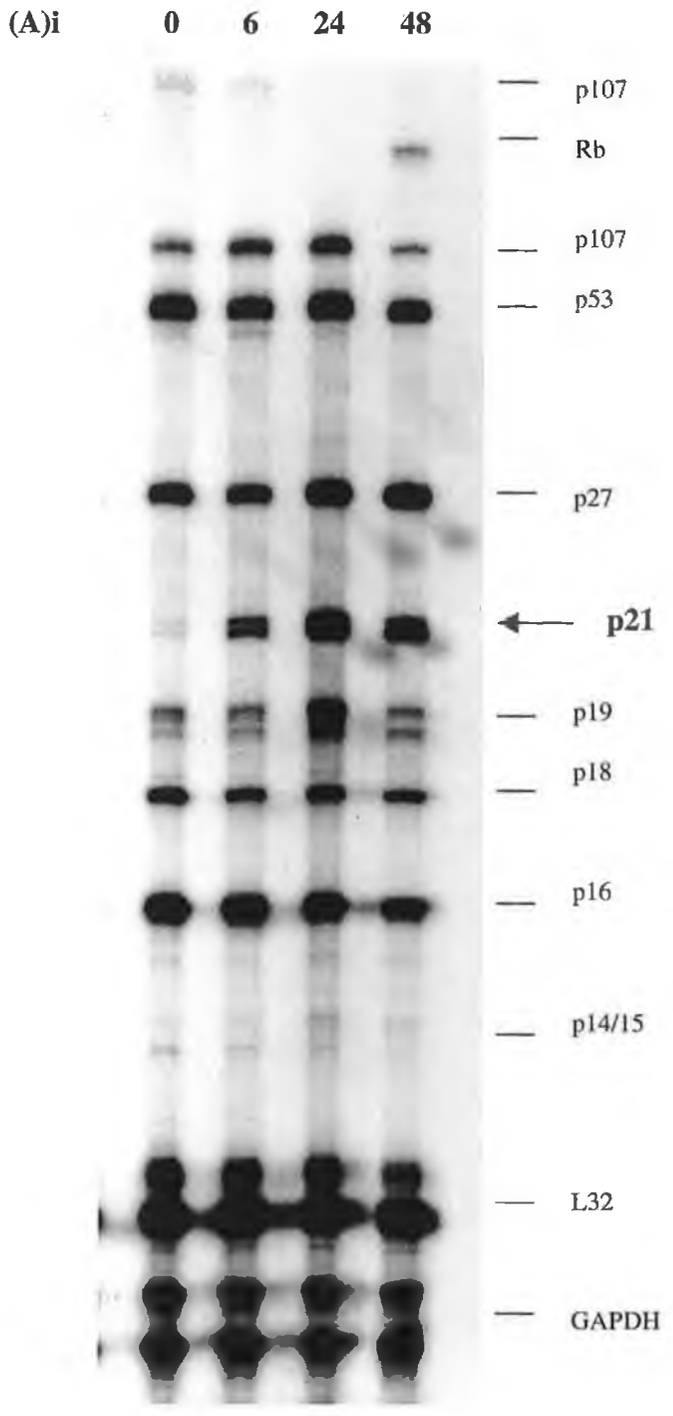


Figure 3.6 Upregulation of p21 expression by LMP1. LMP1 expression was induced in DG75tTA-LMP1 cells and both total RNA and protein extracts were prepared at the indicated time points (hours) post-induction. **(A)** (i) and (ii) RPA analysis performed as per figure 1 show two individual induction experiments each of which illustrate a several fold increase in p21 mRNA levels by 24-48 hours post-induction.

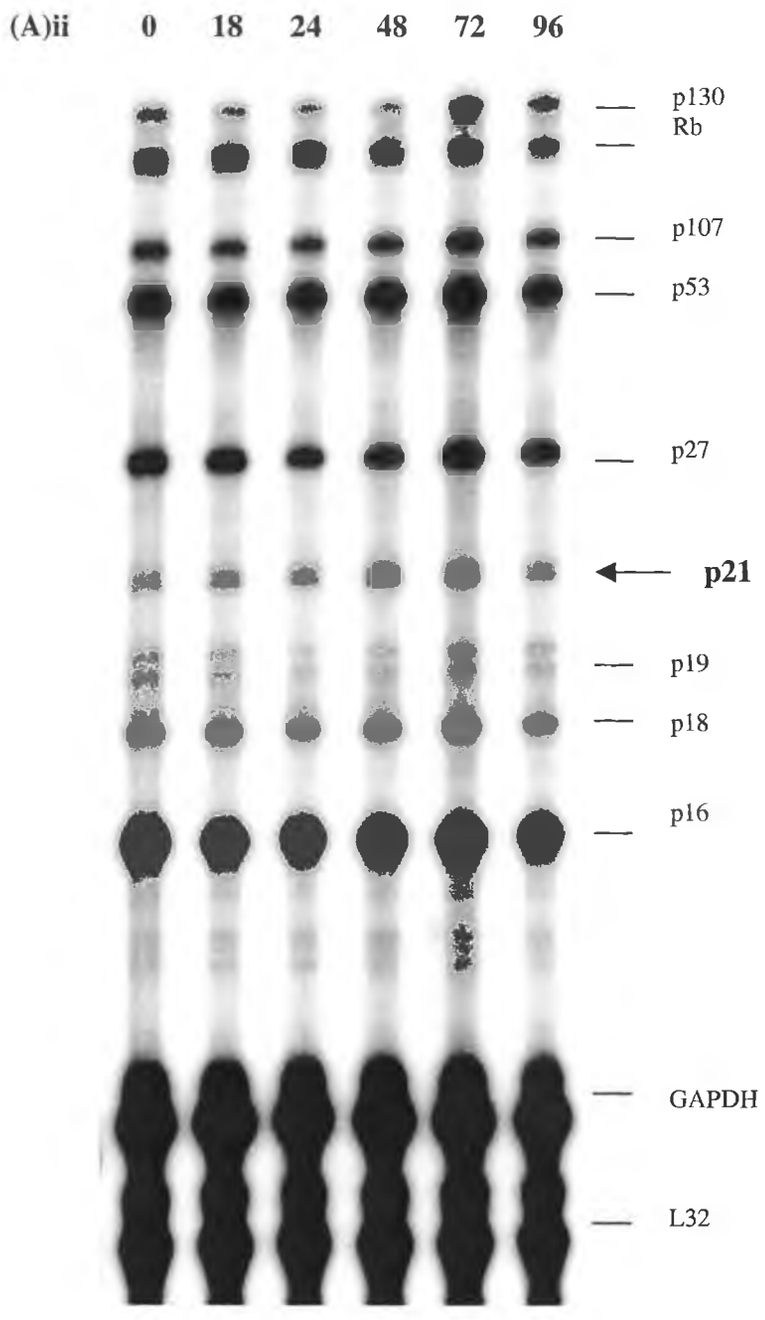


Figure 3.6 Upregulation of p21 expression by LMP1. LMP1 expression was induced in DG75tTA-LMP1 cells and both total RNA and protein extracts were prepared at the indicated time points (hours) post-induction. **(A)** (i) and (ii) RPA analysis performed as per figure 1 shows two individual induction experiments each of which illustrate a several fold increase in p21 mRNA levels by 24-48 hours post-induction.

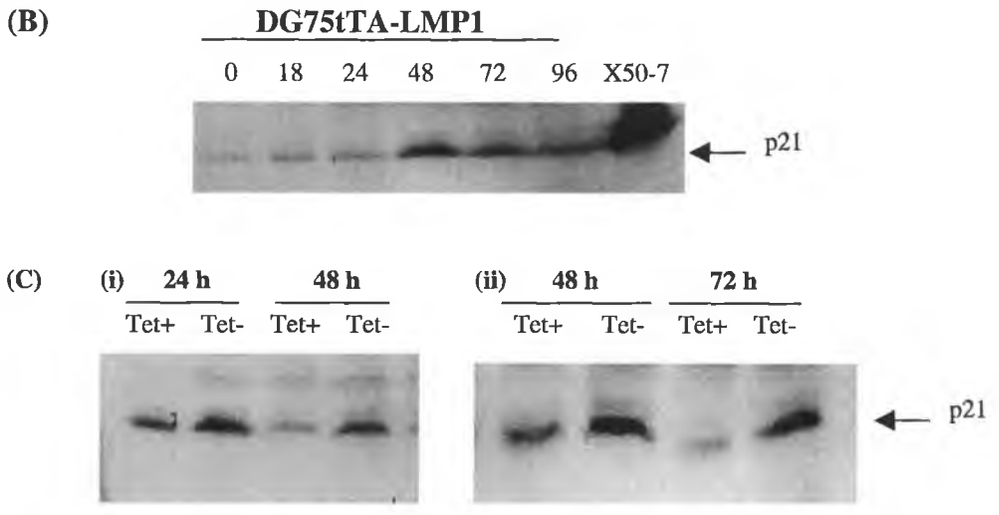


Figure 3.6 Upregulation of p21 expression by LMP1. LMP1 expression was induced in DG75tTA-LMP1 cells and both total RNA and protein extracts were prepared at the indicated time points (hours) post-induction. **(B)** Western blot analysis of p21 protein expression in response to induction of LMP1 expression. Corresponding LMP1 protein levels for this induction are shown in figure 3A. **(C)** (i) and (ii) represent cells which were maintained in the presence (Tet+) or absence (Tet-) of tetracycline for the indicated periods following induction of LMP1 expression in DG75tTA-LMP1 cells.

Detection of p21 protein expression by immunoblot revealed that LMP1-mediated upregulation of p21 mRNA was correspondingly elevated at the protein level (Figure 3.6B and C). By comparing levels of LMP1 protein and p21 protein in a range of LMP1-positive and –negative cell lines (illustrated in Figure 3.4A), it can be seen that, in general, high levels of p21 expression corresponded with expression of LMP1. Relative levels of p21 and LMP1 over 0 to 96 h post-induction are illustrated in Table 1. Viable cell counts demonstrate an LMP1-induced cytostasis. Clumping of cells is due to induction of expression of adhesion molecules by LMP1 (Wang *et al*, 1988b; Peng and Lundgren, 1992).

Time/h	Clumping	LMP1	p21 mRNA	p21 protein	Cell Counts x 10^6/ml
0	-	-	+	+	0.40
18	+	+	+	+	0.26
24	++	+	+	+	0.31
48	+++	++	++	++	0.41
72	++	+++	+++	++	0.80
96	++	+++	+	++	0.92

Table 3.1 Summary of the effect of LMP1 on levels of p21 mRNA and protein and on cell proliferation. Time/h implies time elapsed post induction of LMP1 expression.

3.2.4 EBNA2 expression in DG75 cells has no effect on p21 mRNA levels but levels of p19 mRNA are transiently upregulated.

In order to investigate whether EBNA2 may cooperate with LMP1 in the upregulation of p21 mRNA, EBNA2 expression was induced in a manner similar to that described above for LMP1 in the DG75tTA-EBNA2 cell line. Induction of EBNA2 expression was monitored by Western blot analysis of protein lysates prepared at the same time as RNA samples (see Figure 3.5C). Results of RPA analysis showed no change in the level of p21 mRNA as a result of EBNA2 expression. This result also served to rule out the possibility of an effect on p21 levels mediated by tetracycline withdrawal. The level of p19 mRNA was observed to be transiently upregulated by EBNA2, with a slight increase in levels of p18 mRNA. p19 increased slightly at 24 hours post-induction with a peak 4-fold increase detectable at 48 hours after which time the levels decreased, although elevated levels of p19 were still apparent at 96 hours (Figure 3.7). For comparison, low levels of p21 and p19 in DG75 cells can be seen in lane 8, same figure. Upregulated p19 mRNA levels were not observed to be a feature of EBV positive cells (see Figure 3.3). In fact, lower levels of p19 were observed in MUTU-III relative to MUTU-I cells and in BL41.B95.8 relative to BL41 cells.

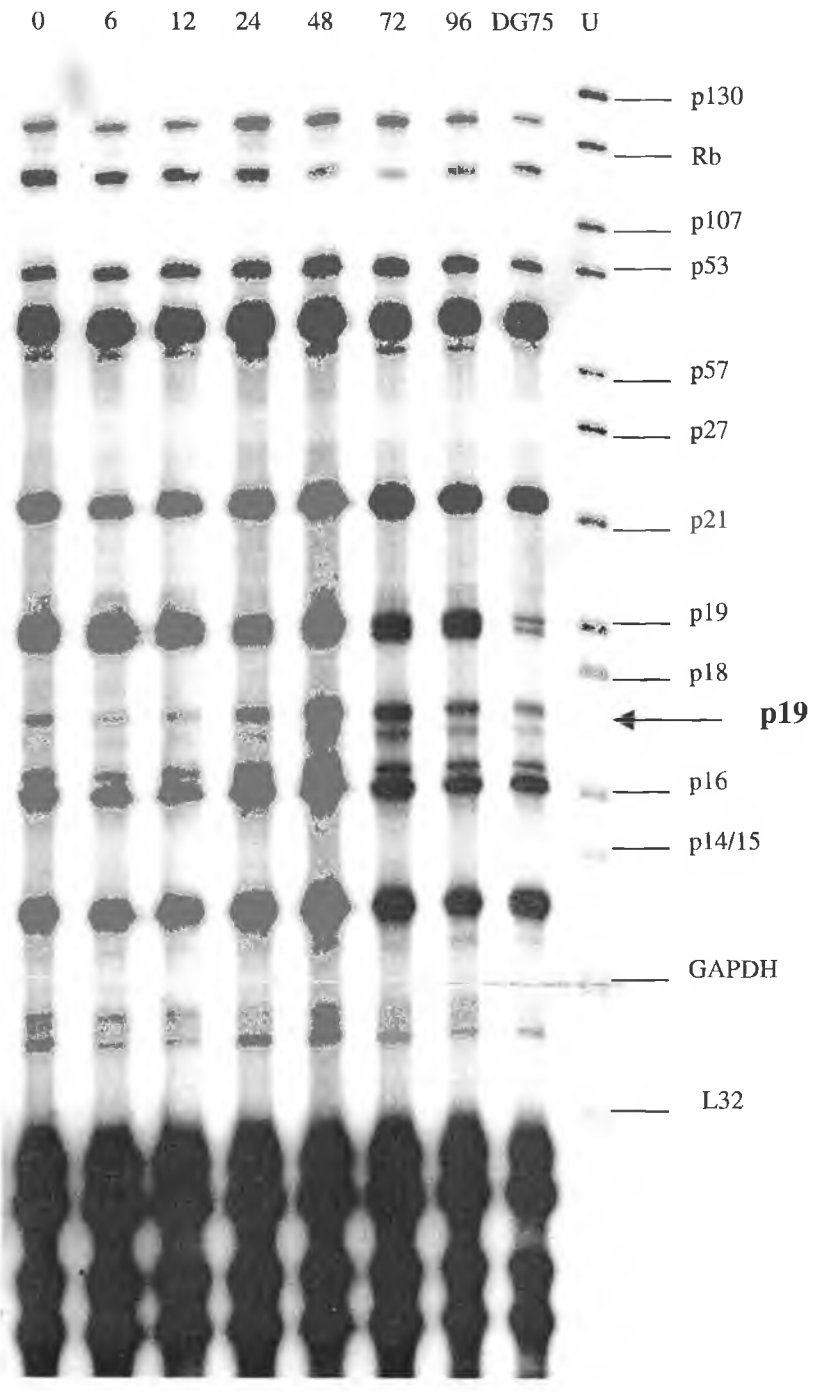


Figure 3.7 Transient upregulation of p19 mRNA levels by EBNA2. DG75tTA-EBNA2 cells were induced to express EBNA2 in a manner similar to DG75tTA-LMP1 cells. Total RNA samples were prepared at the indicated time points (hours) post-induction and analysed by multiprobe RPA as before. Lane 8 shows DG75 cells for comparison.

3.2.5 LMP1 does not upregulate p21 expression in BJABtTA-LMP1 cells.

The effect of LMP1 expression was also examined in the EBV-negative BL cell line BJABtTA-LMP1 in which expression of LMP1 is similarly repressed by the presence of tetracycline. RPA analysis showed that, when levels of GAPDH were taken into consideration, no increase in p21 mRNA was observed as a result of LMP1 expression, although BJABtTA-LMP1 cells did seem to express higher constitutive levels of p21 mRNA than DG75tTA-LMP1 cells (Figure 3.8). The level of p53 mRNA was similarly unchanged in response to LMP1 expression in BJABtTA-LMP1 cells. BJAB cells harbour a p53 mutation which is different to that found in DG75 cells (discussed later). Rb mRNA levels were observed to be markedly higher relative to other Rb family members (p107 and p130) in BJABTA-LMP1 cells than in the case of DG75tTA-LMP1 cells.

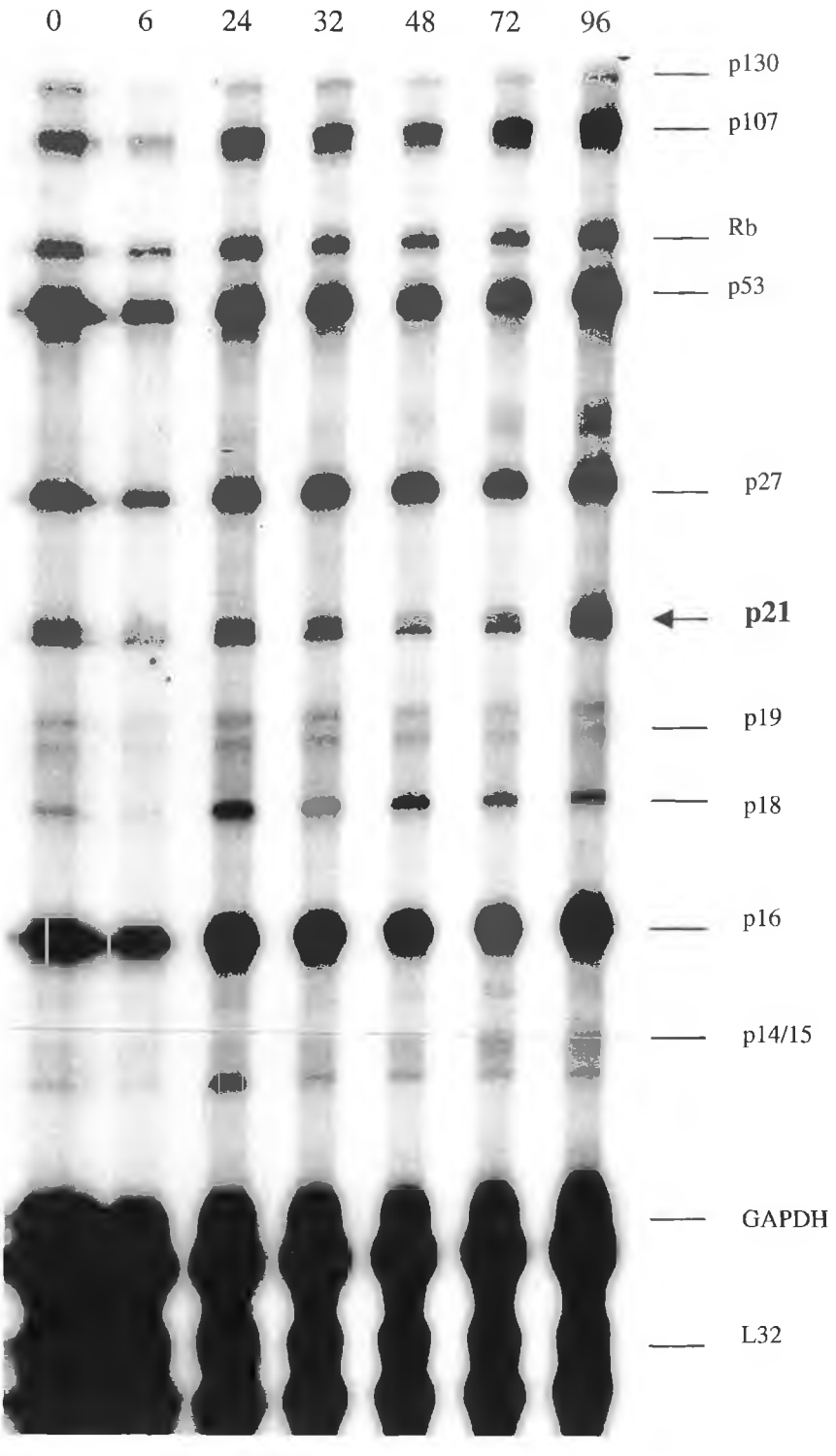


Figure 3.8 LMP1 does not upregulate p21 mRNA levels in BJABtTA-LMP1 cells. BJABtTA-LMP1 cells were induced to express LMP1 as for DG75tTA-LMP1 cells and total RNA samples were harvested at the indicated time points (hours) post-induction for RPA analysis.

3.2.6 LMP1 expression does not affect p53 levels in DG75tTA cells.

Changes in p21 expression are normally either p53-dependent or –independent. Most BL cell lines carry a mutant *p53* gene and several reports have shown that BL cells express many fold higher levels of *p53* mRNA and protein than LCLs (Finlay *et al*, 1988; Balint and Reisman, 1996). RPA and Western blot data presented here support these observations (Figures 3.3 and 3.9, respectively). Levels of *p53* mRNA were assessed in RPA assays in association with *p21*, which allowed comparative analysis of the different mRNA species within samples. Also, Western blot analysis was performed on the same protein lysates as were used for p21 and other analyses. The 53 kD protein was detected using a monoclonal anti-p53 antibody (Santa Cruz, SC-126) in conjunction with alkaline phosphatase detection. *p53* mRNA and protein levels were unchanged in response to LMP1 expression.

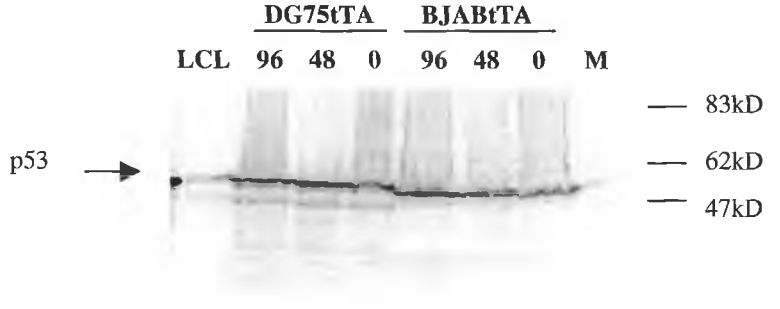


Figure 3.9 Western blot analysis of p53 expression. p53 protein levels were detected using the same extracts which were used for p21 protein detection, represented in Figure 3.4(B). Samples were separated on a 10% SDS-PAGE gel and probed with anti-p53 antibodies which were detected using an alkaline phosphatase method. LCL: IARC 290B; DG75tTA-LMP1 and BJABtTA-LMP1 cells were induced to express LMP1 for the indicated time periods; M: Molecular weight markers.

3.2.7 C-Myc protein levels in LMP1-positive and –negative BL cells.

It has recently been found that c-Myc can repress transcription from the p21 promoter (El-Deiry *et al*, 1999), levels of c-Myc protein were examined by Western blot analysis in both BJABtTA-LMP1 and DG75tTA-LMP1 cells following induction of LMP1 expression. C-Myc protein exists in two forms; the cytoplasmic form migrates as a band

of 48-50 kD on SDS-PAGE gels, while the 62-66 kD form is exclusively nuclear-associated. In these experiments, mainly nuclear protein was detected using a murine monoclonal IgG_1 antibody (Calbiochem). In these cell lines, the level of c-Myc protein has previously been shown to be decreased by approximately 50% as a result of LMP1 expression (Floettmann *et al*, 1996). Using the same protein lysates which were analysed for p21 levels, in both BJAB and DG75 cells, c-Myc protein levels show an approximately 2-fold decrease in c-Myc protein by 24 hours and begin to increase again soon afterwards returning to pre-induction levels by 96 hours (Figure 3.10A and B). MUTU-I cells showed a similar decrease in c-Myc protein levels relative to MUTU-III, as did BL41 versus BL41.B95.8 cells (Figure 3.10C)., while the LCL, IARC290B showed the lowest levels (not shown). BJAB cells differ from DG75 cells in that they lack the c-myc chromosomal translocation that is a marker for BL. This is interesting as it implies that LMP1-associated down-regulation of c-Myc levels occurs independently of the translocation.

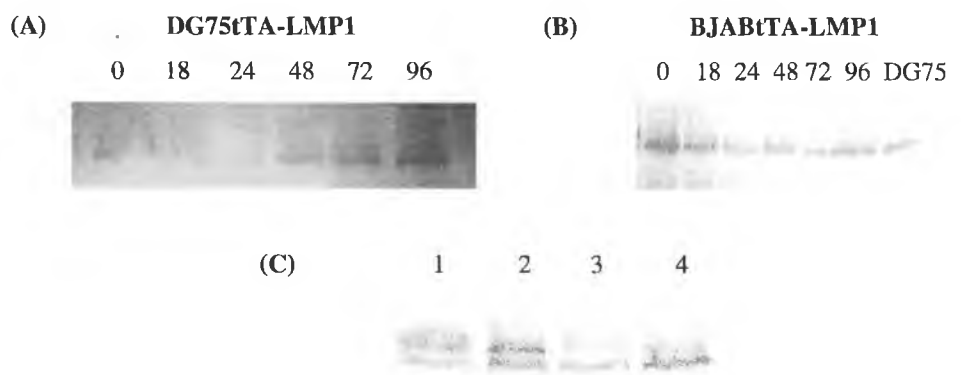


Figure 3.10 Western blot analysis of c-Myc protein expression. Levels of c-Myc protein in DG75tTA-LMP1 and BJABtTA-LMP1 BL cells induced to express LMP1 are illustrated in **(A)** and **(B)** respectively. Time in hours post-induction of LMP1 expression is given above each lane. **(C)** represents levels of c-Myc protein in LMP1-negative (BL41and MUTU-I) and LMP1-positive (BL41.B95.8 and MUTU-III) cells. Lane 1, MUTU-III; Lane 2, MUTU-I;
Lane 3, BL41.B95.8; Lane 4, BL41.

3.2.8 Cell type-specific effect of LMP1 on p21 expression.

LMP1 exhibits cell type-specific differences with regard to its effects on gene transcription. For this reason, it was of interest to determine whether or not p21 could be upregulated by LMP1 in two other cell types which are relevant to EBV, namely T lymphocytes and epithelial cells. To this end, p21 mRNA and protein levels were investigated using a tetracycline-regulated LMP1 expressing clone derived from the Jurkat T cell line and a stably transfected LMP1-expressing clone of the epithelial cell line C33A. The results showed that LMP1 did not exhibit any effect on p21 mRNA levels in either cell context (Figure 3.11), indicating that p21 upregulation by LMP1 may be a B cell-specific effect. p21 was barely detectable in Jurkat T cells and not detectable at all in C33A epithelial cells, regardless of LMP1 status. It is known, however, that many cell cycle regulators are expressed in distinct cell type-specific patterns. It is also interesting to note that one effect of LMP1 which is specific to epithelial cells is upregulation of the EGF receptor (Miller *et al*, 1995) and EGF-mediated growth suppression has been demonstrated to correlate with increased expression of p21 (Johannessen, 1999).

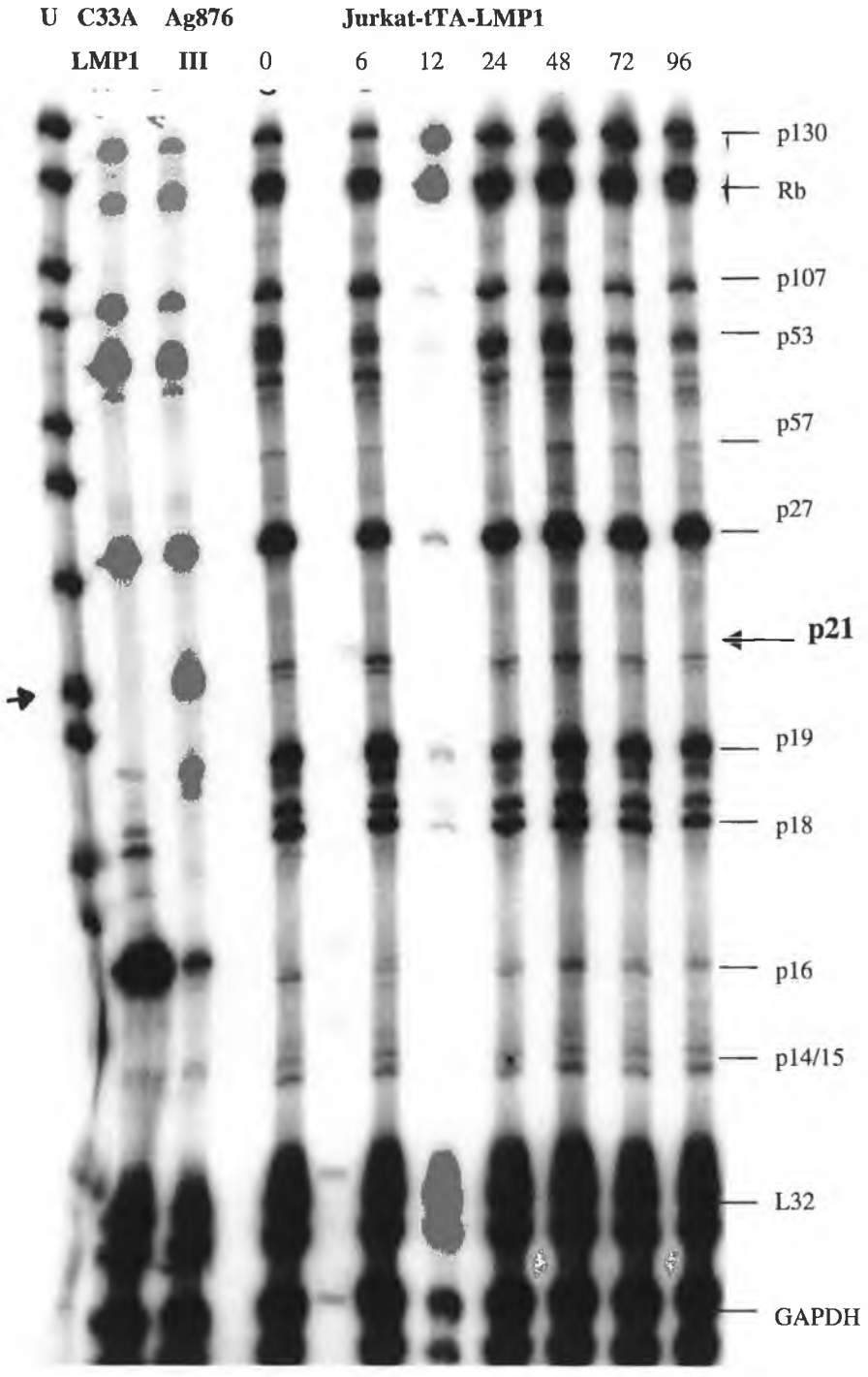


Figure 3.11 LMP1 does not upregulate p21 mRNA in either a T cell or an epithelial cell context. **(A)** Jurkat-tTa-LMP1 T cells were induced to express LMP1 in a manner similar to DG75tTA-LMP1 cells. Group III Ag876 BL B cells and C33A-LMP1 epithelial cells are included for comparison.

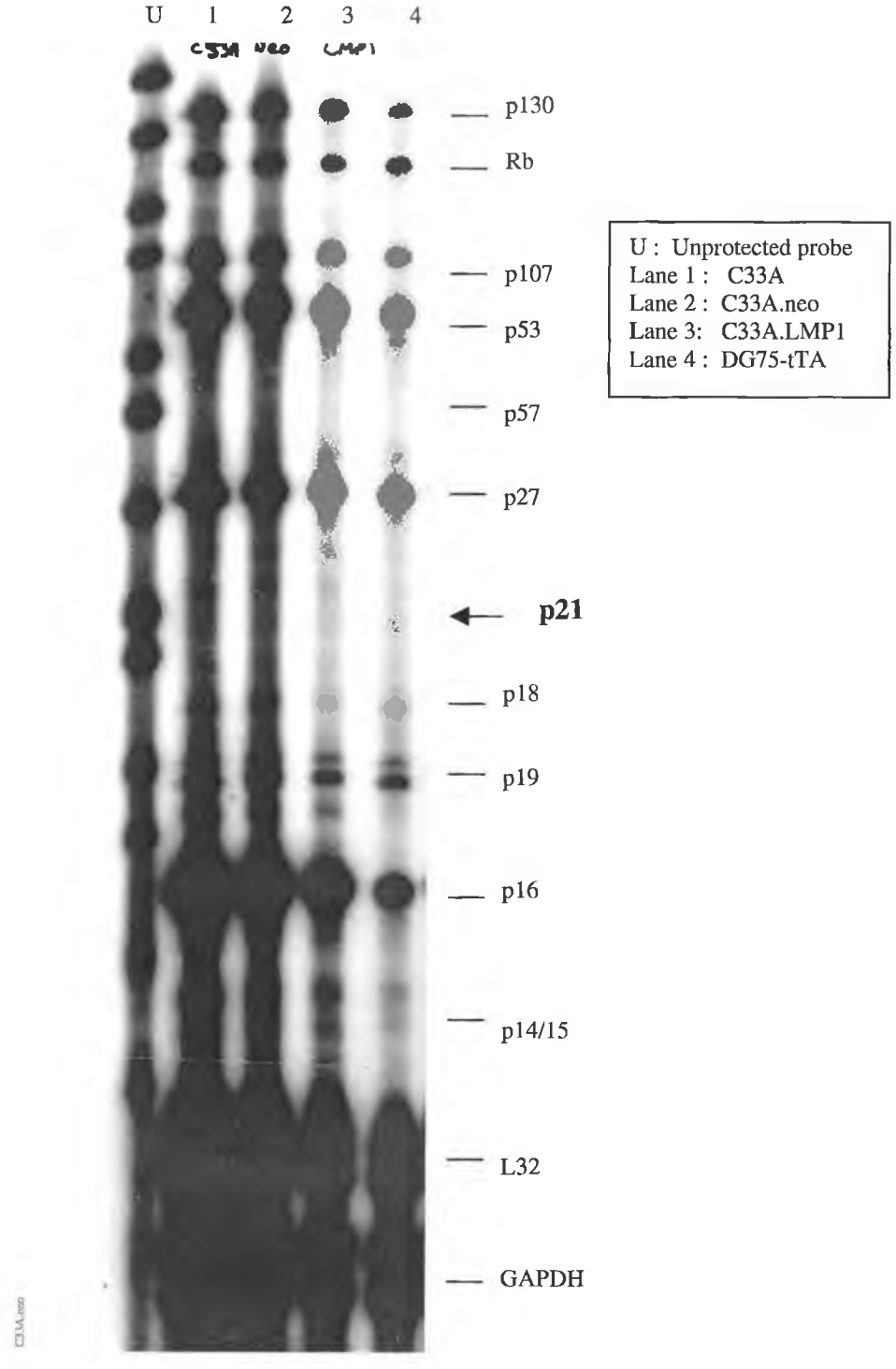


Figure 3.11 LMP1 does not upregulate p21 mRNA in either a T cell or an epithelial cell context. (B) C33A-Neo and C33A-LMP1 are stably transfected control and LMP1-expressing derivatives of the C33A parent line, respectively. Total RNA was harvested from each cell line or at the indicated times (hours) post-induction and analysed by multiprobe RPA as before.

3.2.9 Effect of LMP1 on p21 promoter activity.

The increase of steady state *p21* mRNA levels could be due either to an enhanced rate of transcription or stabilisation of previously transcribed *p21* mRNA or to a combination of both mechanisms. In order to investigate if increased p21 promoter activity was involved, a *p21* promoter-luciferase reporter construct, WWP-LUC (a gift from Dr. Bert Vogelstein; El-Deiry *et al*, 1993) was transiently co-transfected with a construct which constitutively expressed LMP1, under the control of the SV40 promoter. WWP-LUC comprises a 2.4 kb DNA sequence from the 5` regulatory region of the *p21* gene subcloned into pGL2-basic (Promega). The co-transfection experiments were carried out in DG75 cells, with the bfl-1 promoter used as a positive control for LMP1-mediated transactivation. No transactivation of the *p21* promoter was observed despite also using an alternative LMP-1 expression vector (pEF-LMP-1). The *bfl-1* promoter was transactivated 5-fold by LMP1 in these experiments (Figure 3.12). High levels of p21 promoter basal activity were observed in each experiment, which were several fold higher than the *bfl-1* control promoter, and comparable to or higher than luciferase activity from the SV40 promoter in the pGL-2 control promoter. Using the WWP-luc construct, others have reported a high basal level of promoter activity in different cell lines (Datto *et al*, 1995; Billon *et al*, 1999). A pair of *p21* promoter constructs, 0-luc (full-length 2.4 kb *p21* promoter) and 6-luc, which is identical to 0-luc except for 10 consecutive mutated bases (-43/-34) located near the TATA box. (Datto *et al*, 1995), were also employed in similar experiments. Alternative experiments involved transfection of p21 promoter constructs into uninduced or LMP1-induced DG75-tTA cells, where again no significant increase in luciferase activity was detected as a result of LMP1 induction. In one such experiment, using the WWP-LUC promoter construct, a 1.2 fold increase in promoter activity was detected, while a similar experiment using the 0-luc promoter construct produced a 1.5 fold increase in promoter activity (not shown). It was concluded that no significant transactivation of the p21 promoter was detectable in the context of DG75 cells under the conditions described above. Thus, if LMP1 affects the level of the p21 promoter activity, then the DNA sequence elements that may mediate this effect are most likely located elsewhere.

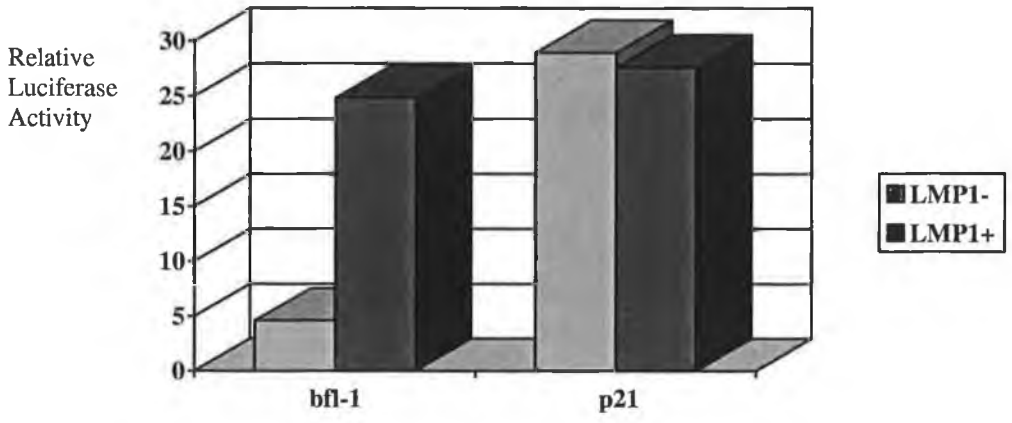


Figure 3.12 Effect of LMP1 expression on p21 promoter activity. DG75 cells were co-transfected with WWP-LUC (p21 promoter luciferase construct) plus pEF (LMP1-) or pEF-LMP1 (LMP1+). As positive control, cells were similarly co-transfected with the pGL2-bfl-1 promoter construct with pEF or pEF-LMP1, as before. After 48 hours, cells were harvested and used in luciferase assays..

3.2.10 Effect of LMP1 on stability of *p21* mRNA.

To determine whether the stability of *p21* transcripts might be enhanced as a result of LMP1 expression, half-life ($T_{1/2}$) studies of *p21* mRNA were performed by blocking overall transcription with an inhibitor of RNA polymerase II, actinomycin D (5 μg/ml) in uninduced and LMP1-induced DG75-tTA cells. Total RNA was extracted at each time point over a period of 20 hours as indicated in Figure 12A, and the decay of *p21* mRNA was followed by RPA analysis. The relative half-lives were calculated by plotting the best-fit semi-logarithmic lines generated from the relative amounts of *p21* mRNA as determined by RPA assay (Figure 3.13A). Initially, actinomycin D was added 24 hours after induction of LMP1, as at this stage the *p21* level is still increasing so the stabilisation effect may be easier to detect. Although the upregulation of *p21* mRNA had not yet peaked by this stage, an almost 2-fold stabilising effect was apparent in LMP1-induced cells over uninduced cells (Figure 3.13B). At 48 hours, the level of *p21* transcript was seen to have accumulated presumably as a result of increased stability, but

the difference in stability is now less apparent and at this stage *p21* levels soon begin to decrease again. The sudden drop in *p21* mRNA level immediately after inhibition of RNA synthesis in both induced and uninduced cells may be due to the very short half-life of *p21*, such that transcripts need some time to accumulate. Similar mRNA stability experiments were carried out in LCL X50-7 cells (Figure 3.12A), as well as in MUTU-I (not shown) and MUTU-III cells (Figure 3.12C). *p21* mRNA showed prolonged stability in X50-7 cells and in MUTU-III cells. It wasn't possible estimate the stability of *p21* mRNA in MUTU-I relative to MUTU-III cells due to the very low levels observed. This was despite extended exposure times of up to one week. In all cases, *p21* mRNA levels at each time point were quantitated by densitometric scanning. GAPDH bands were similarly assessed and used as a control for loading. Consistent with previous reports (Balint and Reisman, 1995), levels of *p53* mRNA was observed to be unchanged up to at least 10 hours in the presence of 5 μg/ml actinomycin D.

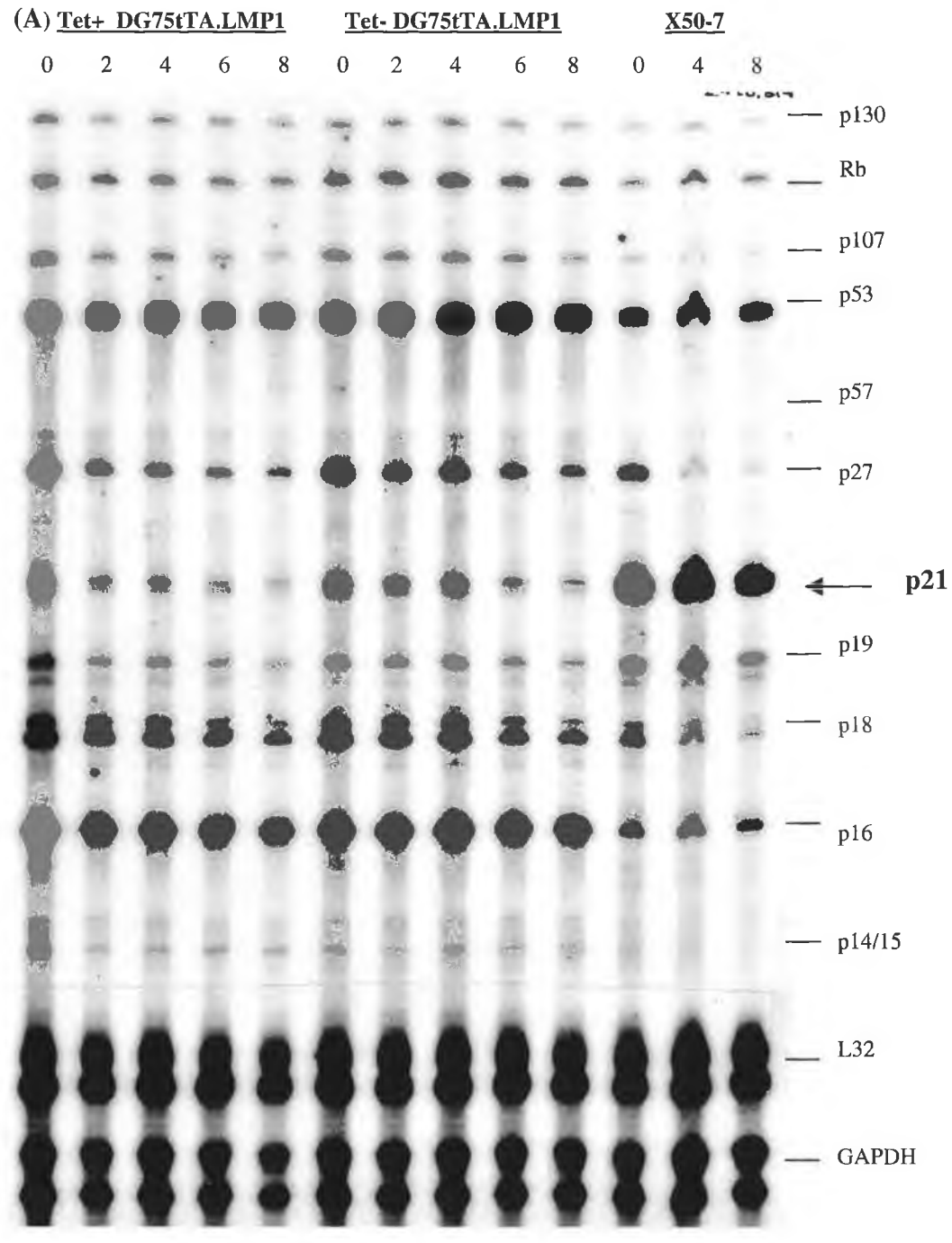


Figure 3.13 LMP1 expression in DG75tTA-LMP1 cells increases the stability of p21 mRNA. (A) DG75tTA-LMP1 cells were grown in the presence (LMP1-) or absence (LMP1+) of tetracycline for 24 hours before addition of 5 μg/ml actinomycin D. The cells were then further incubated for the times indicated before total cellular RNA was purified and assayed by RPA as before (lanes 1-10). For comparison, the higher stability of p21 mRNA in LCL X50-7 cells can be seen in lanes 11-13.

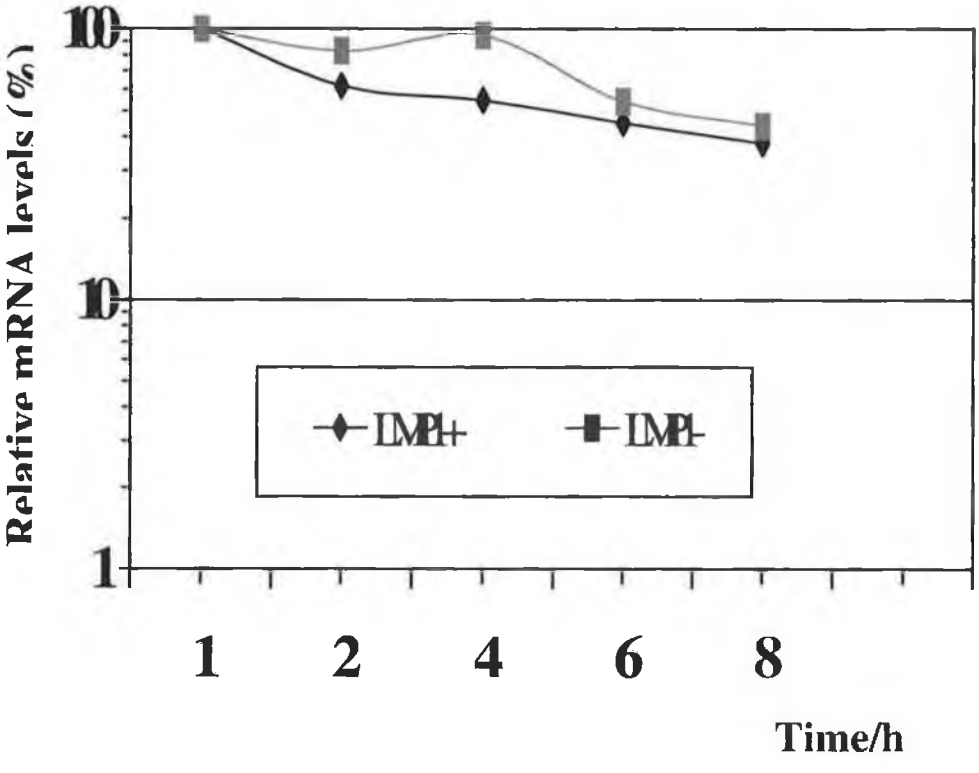


Figure 3.13 LMP1 expression in DG75tTA-LMP1 cells increases the stability of p21 mRNA. (B) In DG75tTA-LMP1 cells shown in (A), the intensity of each p21 band was normalised to the intensity of its corresponding GAPDH band in order to correct for loading. Values were then plotted as a percentage of the control (time 0 hours) and used to calculate half-lives.

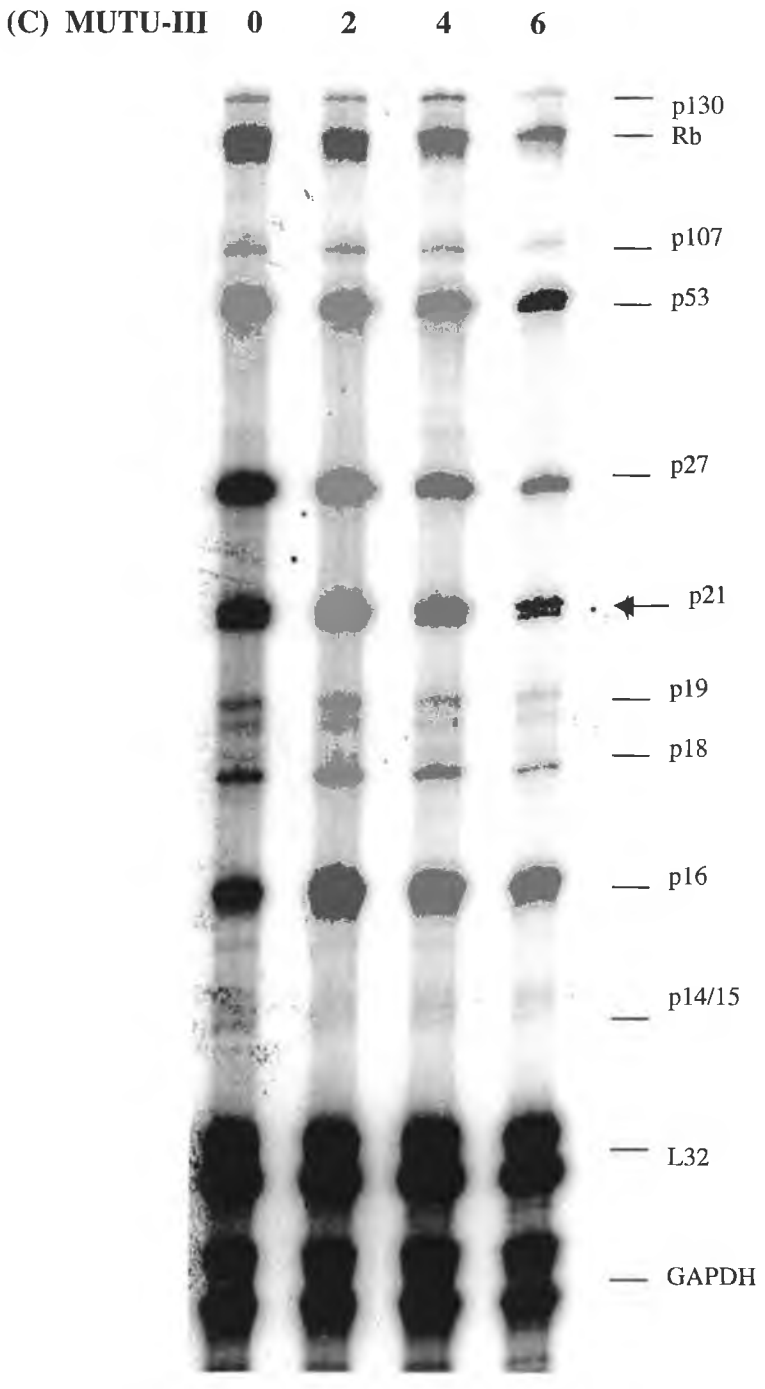


Figure 3.13 LMP1 expression in DG75tTA-LMP1 cells increases the stability of p21 mRNA.

(C) Stability of p21 mRNA in MUTU-III cells. MUTU-III cells were passaged in fresh media 24 hours before addition of 5 μg/ml actinomycin D. Cells were then harvested at the indicated time points as before and analysed by RPA.

As an independent measure of mRNA stability, the level of *p21* mRNA was monitored in the absence of any new protein synthesis. Addition of the protein synthesis inhibitor, cycloheximide (CHX) is sometimes used to determine whether low levels of mRNA transcripts are due to regulation of mRNA stability by a labile protein. At 48 hours post-LMP1 induction, 10 μg/ml CHX was added to or omitted from DG75tTA-LMP1 cells, which were then incubated for a further 30 hours with RNA samples taken at the indicated time points. Immunoblotting was used to confirm that LMP1 levels of expression were unaffected for at least 30 hours after CHX treatment (Figure 3.13A). It can be seen that *p21* mRNA levels accumulated a further >2 fold in cells treated with CHX versus untreated cells (Figure 3.13B). Historically, inhibition of protein synthesis using cycloheximide has been shown to cause stabilisation and accumulation of several messenger RNAs. Various interpretations are possible for this phenomenon: presumably a labile protein is involved in the degradation of these mRNAs, or the mRNA-degrading machinery is tightly associated with the ribosome. One possible conclusion from this particular experiment is that *p21* mRNA is normally degraded by a labile factor in DG75 cells, and that LMP1 may stabilise *p21* mRNA through interference with such a negative factor or group of factors.

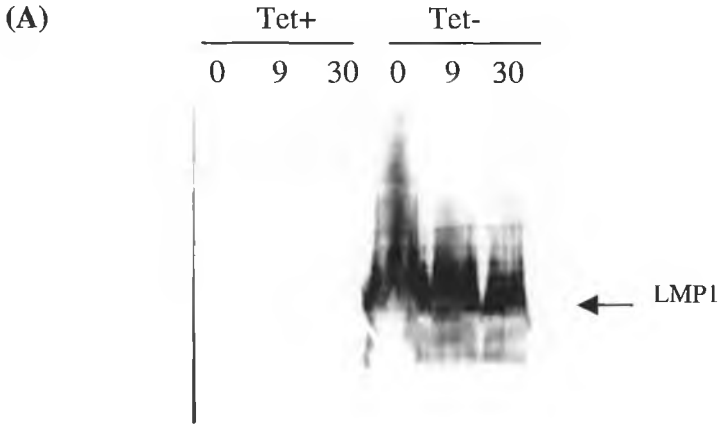


Figure 3.13 Effect of inhibition of new protein synthesis. **(A)** Expression of LMP1 is unaffected in the presence of 10 μg/ml cycloheximide (CHX) for up to at least 30 hours. DG75tTA-LMP1 cells were grown in the presence (LMP1-) or absence (LMP1+) of tetracycline for 48 hours before addition of cycloheximide. Protein extracts were then prepared at the indicated time points and analysed for LMP1 protein expression as described previously.

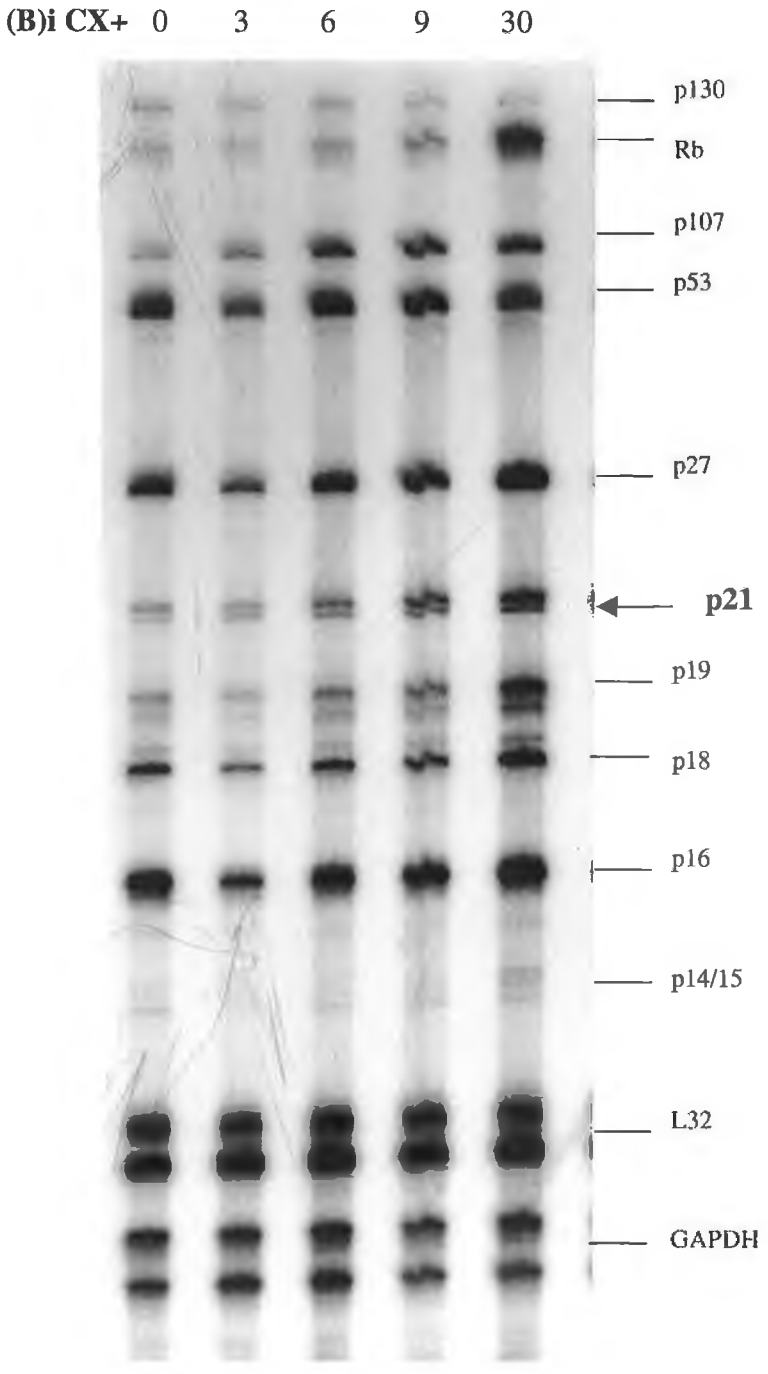


Figure 3.13 Effect of inhibition of new protein synthesis. **(B)** LMP1-induced *p21* mRNA transcripts accumulate in the presence of cycloheximide. DG75tTA-LMP1 cells induced to express LMP1 for 48 hours were incubated in the presence, B(i) or absence, B(ii) of 10 μg/ml cycloheximide. Total cellular RNA was harvested at the indicated times post-treatment and analysed by multiprobe RPA.

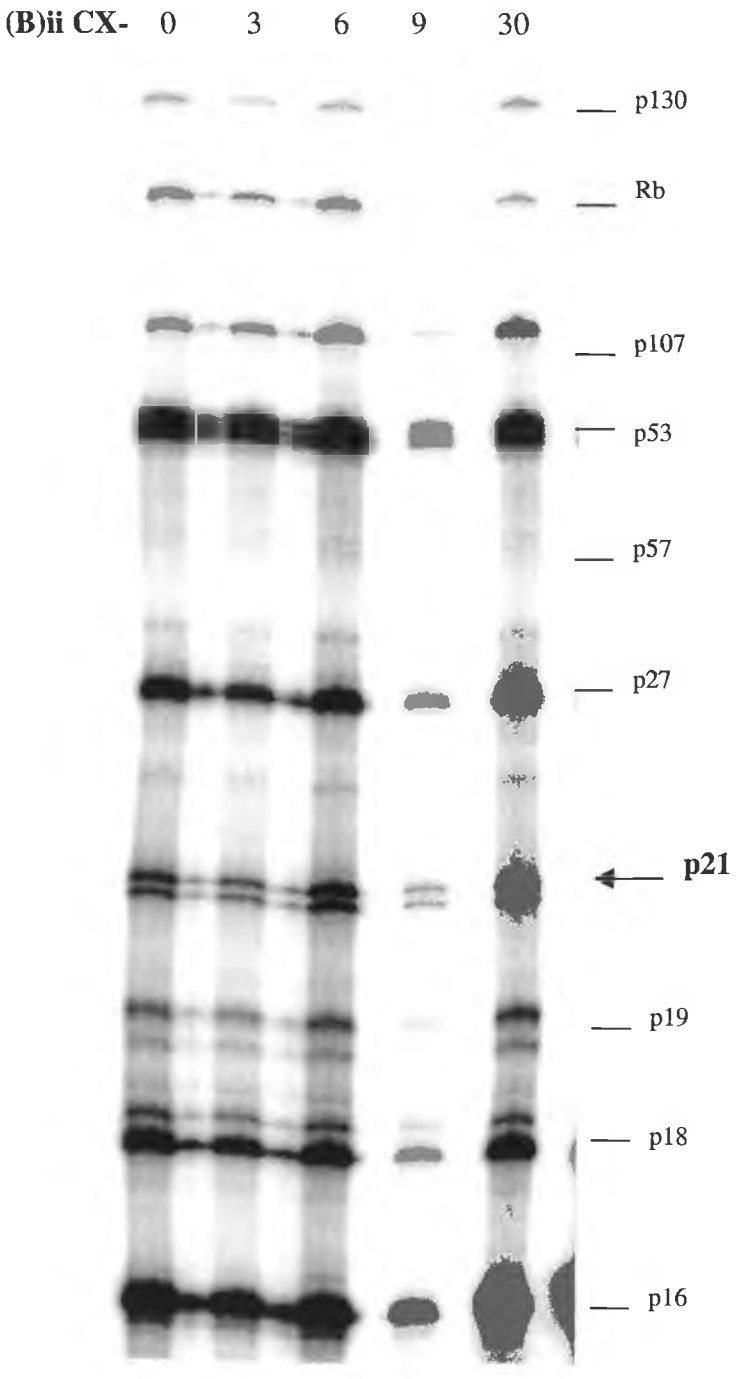


Figure 3.13 Effect of inhibition of new protein synthesis. (B) LMP1-induced *p21* mRNA transcripts accumulate in the presence of cycloheximide. DG75tTA-LMP1 cells induced to express LMP1 for 48 hours were incubated in the presence, B(i) or absence, B(ii) of 10 μg/ml cycloheximide. Total cellular RNA was harvested at the indicated times post-treatment and analysed by multiprobe RPA.

3.2.11 Effect of LMP1 expression on p21 protein stability

As mentioned above, Western blot analysis confirmed an upregulation of p21 protein by LMP1 in DG75-tTA cells, where levels were seen to peak at about 48 hours and remain elevated until at least 72 hours post-induction of LMP1 (Figure 3.5B). The stability of the p21 protein was examined in uninduced versus LMP1-induced cells at various times after the addition of cycloheximide (10 μg/ml) to inhibit de novo protein synthesis. Protein lysates were prepared at the times indicated in Figure 3.14 over 0-30 hours. No difference in p21 protein stability was observed in the presence of LMP1.

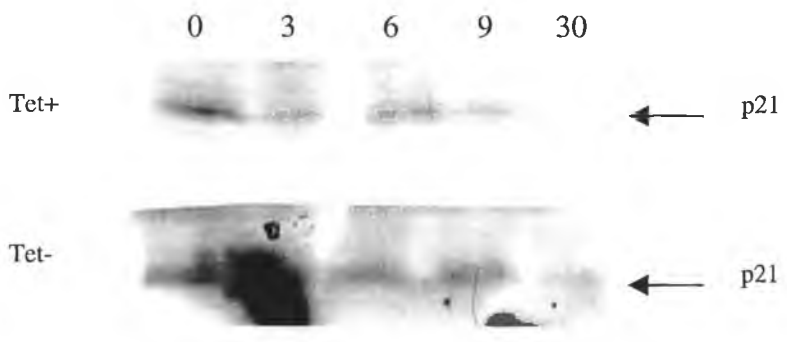


Figure 3.14 Stability of p21 protein is unaffected by LMP1 expression. Protein extracts were prepared at the same time as RNA samples used in RPA analysis in figure 12. Proteins were then fractionated on 15% SDS-PAGE gels and immunoblotted for p21 expression as described before. Bands were visualised by ECL and their relative intensities were quantitated by densitometric scanning. Times (hours) post addition of 10 μg/ml cycloheximide are given above each lane. Time 0 represents 48 hours post-induction of LMP1 expression.

3.2.12 Effect of PMA and staurosporine on p21 expression in LMP1-induced and uninduced cells.

A number of studies have linked regulation of p21 expression to the PKC pathway (Akashi *et al*, 1999; Zeng and El-Deiry, 1996; Lin *et al*, 1996). To explore the potential role of the protein kinase C (PKC) pathway in the observed increase in p21 levels mediated by LMP1, PMA and staurosporine were employed to stimulate and inhibit the PKC pathway, respectively. Firstly, in order to investigate the functional integrity of a PKC-mediated induction of p21 expression in the context of DG75 cells, cells were treated with 10 ng/ml PMA and protein lysates were prepared at 0, 1, 2, 3, 6 and 24 hours in the presence and absence of PMA. p21 protein levels were seen to elevate by 2 hours, continue to increase up to at least 6 hours and remain elevated up to 24 hours but perhaps longer as a result of stimulation by PMA (Figure 3.15A and B). Very high levels of p21 protein were observed in response to PMA treatment: levels exceeded those of the group III Ag876 cells by 6 hours post-addition of PMA. Somewhat similar patterns of rapid induction have been reported in other cell lines eg. in HL60 cells p21 was detectable by 3 hours, peaked at 24 hours and remained elevated for 72 hours when exposed to 60 ng/ml PMA (Schwaller *et al*, 1995). It was also confirmed that the induction process (which involves extensive washing of cells in PBS) had no effect on the ability of PMA to activate the PKC pathway in DG75 cells i.e. cells which were washed and cultured in the presence or absence of tetracycline were stimulated with PMA in a manner similar to unwashed DG75tTA-LMP1 cells (Figure 3.15B). In addition, the results suggest the absence of any cooperative effect of PMA and LMP1, as no difference was detected between levels of p21 protein in PMA-treated LMP1-positive cells and PMA-treated LMP1-negative cells. This may indicate that PMA and LMP1 are using the same signal transduction pathway which has become saturated as a result of PMA treatment.

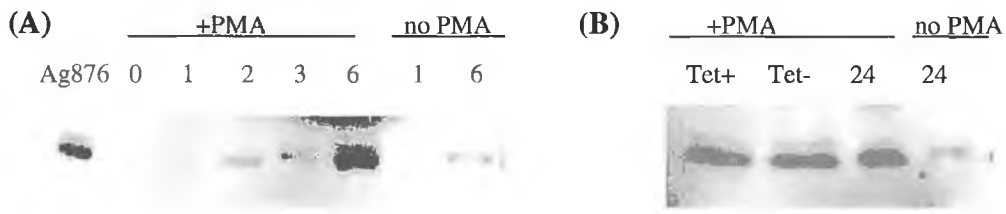


Figure 3.15 PMA treatment of DG75 cells stimulates p21 protein expression. **(A)** DG75 cells were incubated in the presence of 10 ng/ml PMA and protein extracts were subsequently prepared at the indicated times post-treatment. For comparison, group III Ag876 cells are loaded in lane 1. **(B)** Lanes 1 and 2 represent DG75tTA-LMP1 cells incubated in the presence (LMP1-) or absence (LMP1+) of tetracycline, where 10 ng/ml PMA was added at the point of induction. Lanes 3 and 4 correspond to the PMA+ and PMA- DG75 cells respectively at 24 hours as for (A).

Staurosporine (ST) is a potent, cell-permeable broad spectrum inhibitor of protein kinases and was used at a concentration of 0.1nM to inhibit the PKC pathway in uninduced and LMP1-induced cells. Treatment of LMP1-induced DG75tTA-LMP1 cells with ST was observed to inhibit the upregulation of p21 expression (Figure 3.16A). However, when protein lysates were analysed for expression levels of LMP1, it was found that LMP1 expression was similarly inhibited in the presence of ST (Figure 3.16B). The results using this inhibitor are, therefore, inconclusive with regard to the role of the PKC pathway in LMP1-mediated p21 induction. Nonetheless, the experiment did show that inhibition of LMP1 expression correlated with inhibition of p21 expression. In Figures 3.16A and B, it can be seen that relative levels of p21 protein correspond approximately to those of LMP1 for each particular cell sample. In the example shown, where LMP1 expression was not totally inhibited by ST after 24 h, the level of p21 is slightly higher in LMP1-induced cells than in uninduced cells.

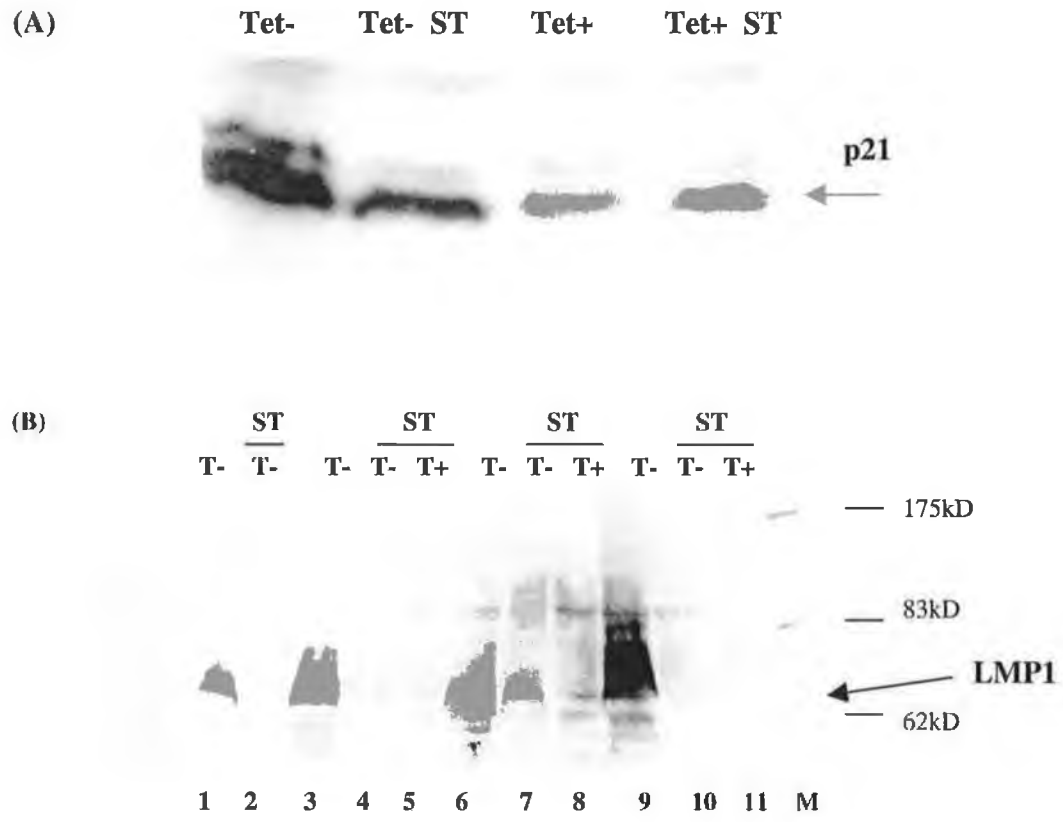


Figure 3.16 Staurosporine inhibits both p21 and LMP1 expression in DG75tTA-LMP1 cells.
(A) DG75tTA-LMP1 cells were incubated in the presence (LMP1-) or absence (LMP1+) of tetracycline for 24, 48 or 72 h, where 0.1 nM staurosporine (ST) was added to or omitted from cells at the point of induction. Lanes 1-4 represent protein extracts harvested at 24 h, separated on a 15 % SDS-PAGE gel, and immunoblotted for p21 protein expression as before. **(B)** Protein extracts prepared as for (A) but analysed for expression of LMP1 using a 10 % SDS-PAGE gel. Lanes 1 and 2: 72 h; lanes 3-5: 48 h; lanes 6-8: 24 h. Lanes 1-8 are from the same induction experiment. Lanes 6-8 correspond to extracts used in (A). Lanes 9-11 represent a different induction experiment at 24 h. Lane M: protein molecular weight markers.

3.2.13 Investigation of the role of the p38 MAPK pathway.

It was recently shown that activation of the p38 MAPK pathway was important in the coregulation of IL-6 and IL-8 production by LMP1 in HEK 293 cells (Eliopoulos *et al*, 1999). Activation of the MAPK pathway has also been linked with the regulation of p21 expression (Lin *et al*, 1996; Akashi *et al*, 1996). Therefore, experiments were carried out in order to investigate whether this pathway may be important in the LMP1-mediated upregulation of p21 expression. To this end, induced and uninduced DG75tTA-LMP1 cells were incubated in the presence of the specific p38 inhibitor, SB203580 (Calbiochem), at a concentration of 10uM or 20uM. SB203580 is a pyridinyl imidazole compound that has been shown previously to specifically inhibit p38 activity in response to a range of stimuli (Craxton *et al*, 1998; Hsu *et al*, 1999, Eliopoulos *et al*, 1999). As SB203580 was dissolved in DMSO, induced and uninduced cells were incubated with a corresponding volume of DMSO as negative control. In a similar manner to that observed with ST, 20 μM SB203580 inhibited the upregulation of p21 protein but also inhibited LMP1 protein production in this system (Figures 3.17A and B, respectively). In the presence of 10 μM SB203580, the level of LMP1 protein was unaffected and upregulated p21 protein was still detectable in these cells. However, it will be necessary to establish inhibition of the p38 MAPK pathway at this concentration of inhibitor using eg. ELISA assays for IL-6 or IL-8. Alternatively, p38 MAPK has also been reported to be required for CD40-induced gene expression in B lymphocytes. These results would suggest that the MAPK pathway is not important for LMP-1 mediated upregulation of p21 expression in DG75tTA-LMP1 cells, subject to confirmation of inhibition of the pathway under these conditions.

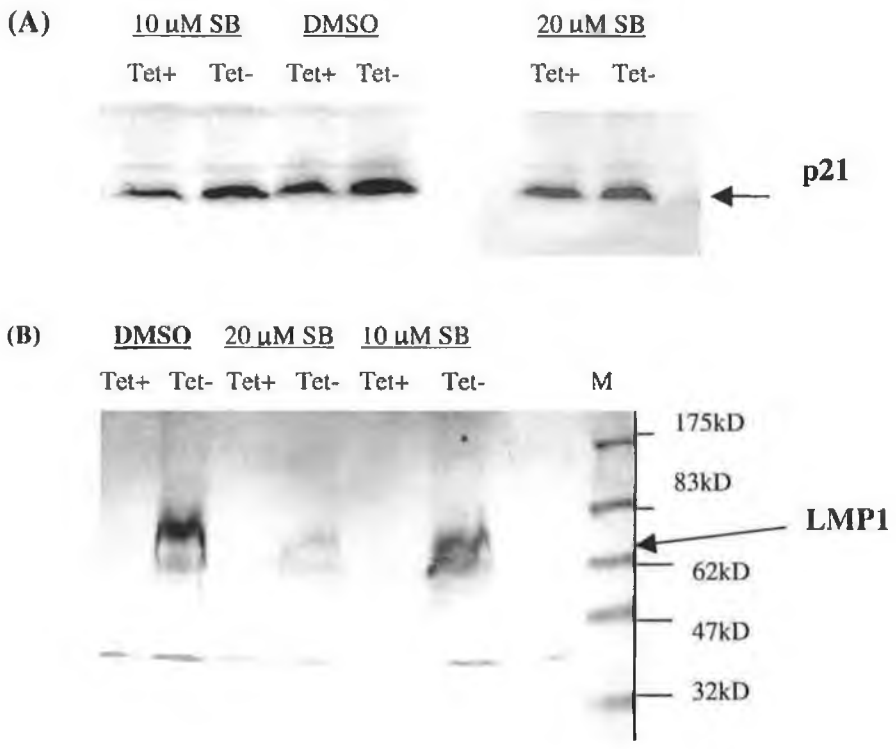


Figure 3.17 A p38 MAPK inhibitor inhibits both p21 and LMP1 expression in DG75tTA-LMP1 cells. **(A)** Cells were incubated in the presence (LMP1-) or absence (LMP1+) of tetracycline, protein extracts were prepared at 48 hours post-induction and immunoblotted for p21 expression. 10 nM, 20 nM SB203580 (specific p38 MAPK inhibitor) or 2.5 µl DMSO was added at the point of induction. Lanes 1 and 2: 10 µM SB203580; lanes 3 and 4: DMSO; lanes 5 and 6: 20 µM SB203580. **(B)** Protein extracts as for (A) were immunoblotted for LMP1 expression as described previously. Lanes 1 and 2: DMSO; lanes 3 and 4: 20 µM SB203580; lanes 5 and 6: 10 µM SB203580.

3.2.14 Rb levels and phosphorylation status in LMP1-positive and –negative BL cells.

pRb is regulated in part by inactivating phosphorylation events, which reverse it's growth suppressive effects. A decrease in the mobility of the 110kD pRb protein on SDS-PAGE gels has been documented as being characteristic of its hyperphosphorylation and inactivation. Increased levels of pRb protein with a shift to the phosphorylated form has been described as an early event in EBV-mediated

immortalisation of primary B cells (Cannell *et al*, 1996). Also, LMP1 has been shown to induce pRb hyperphosphorylation in EBV-positive cells (Arvanitakis *et al*, 1995). Using a mouse monoclonal anti-Rb antibody (Santa Cruz, SC-102) to detect pRb protein, the relative levels and phosphorylation status of pRb were compared in the EBV-negative BL DG75, group-III Ag876 and LCL IARC-290B (Figure 3.18A). As expected, Rb protein in Ag876-III and IARC-290B cells was mainly hyperphosphorylated, while DG75 pRb protein was mainly hypophosphorylated. The effect of LMP1 expression in DG75tTA-LMP1 cells on pRb was then investigated in the same protein lysates used for p21 and c-Myc analysis (Figure 3.18B). At 24 and 48 hours, when LMP1 levels were seen to peak, pRb was present mainly in the hyperphosphorylated form. At 72 hours, when the level of LMP1 protein has begun to decrease, a shift towards the hypophosphorylated form was observed, although most pRb was still hyperphosphorylated. Thus, in support of previous studies, the data indicate that LMP1 expression maintains Rb in the hyperphosphorylated state, and that a predominance of hyperphosphorylated Rb is a feature of group III BL and LCL cells.

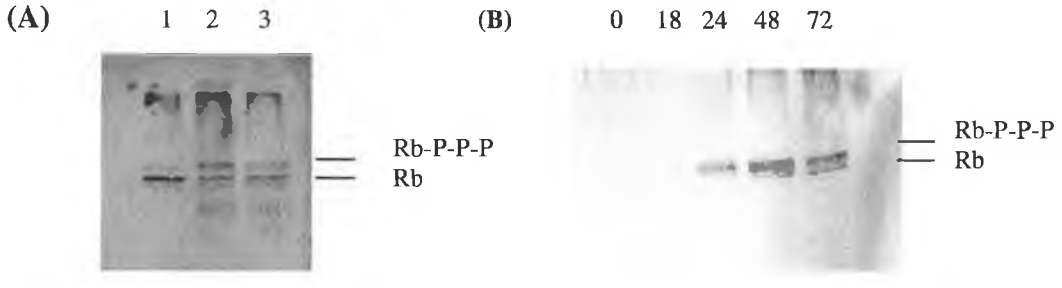


Figure 3.18 Western blot analysis of pRb. **(A)** Levels and phosphorylation status of pRb protein expressed by LMP1-positive (Ag876-III and IARC 290B) and –negative (DG75) cell lines are compared. Proteins were fractionated on 10% SDS-PAGE gels, immunoblotted using specific anti-pRb antibodies and bands were visualised by ECL. Hyperphosphorylated pRb, which migrates more slowly than hypophosphorylated pRb is indicated as pRb-P-P-P. Lane 1: DG75; Lane 2: Ag876-III; Lane 3: IARC290B. **(B)** pRb was investigated in a similar manner in DG75tTA-LMP1 cells induced to express LMP1. The times at which cells were harvested are indicated in hours post-induction.

3.3 DISCUSSION

In the absence of antigen-mediated mitogenic signaling, B lymphocytes in the peripheral circulation exist in a quiescent state. Infection with EBV drives quiescent B cells into a continuous state of proliferation by exploiting the normal cell pathway of activation (Cannell *et al*, 1996). The EBV LMP1 oncogene has a major role to play in this activation process. In mammalian cells, transit through the cell cycle is thought to be regulated by the action of specific protein kinase complexes, each comprising a cyclin and its associated cyclin-dependent kinase (cdk) subunit, which regulate the phosphorylation status of the retinoblastoma protein (pRb) (Sherr, 1993). Previous studies have indicated that EBV-mediated immortalisation drives the hyperphosphorylation of pRb, which involves the upregulation of a number of cyclins and cdks and the down-regulation of a subset of cdk inhibitors (cdkIs) (Cannell *et al*, 1996).

The purpose of this study was to examine the effect of expression of EBV latent proteins on aspects of the cell cycle. Initial experiments involved investigation of EBV-associated effects on a panel of negative regulators of the cell cycle, including two major tumour suppressor genes, *p53* and pRb, the less well-characterised pRb family members, *p130* and *p107* as well as members of both the *CIP* (*p21, p27* and *p57*) and *INK (p15, p16, p18* and *p19*) families of cdkIs. LMP1 and EBNA2 are two latent EBV proteins which are each critical to the transformation process. Thus, observations which were made in a range of latency group I and group III cells as well as several EBV-immortalised LCLs were further explored by independently inducing the expression of LMP1 and EBNA2 in the absence of expression of any other EBV proteins.

3.3.1 EBV-associated upregulation of p21 expression

Initial experiments using a range of EBV-related cell lines found that elevated p21 mRNA and protein was a feature of both BL and LCL cell lines expressing the full spectrum of EBV latent genes. In support of this, a previous report has described the

absence of p21 in primary B lymphocytes, which became detectable in LCLs following EBV infection and immortalisation (Cannell *et al*, 1996). However, the precise role of an EBV-mediated upregulation of p21 expression as part of the transformation process is not completely understood. On further investigation, it was found here that when expressed singly in an EBV-negative background, LMP1, but not EBNA2, had an important role to play in the upregulation of *p21* expression observed in latency group III and LCL cells (see Figures 4 and 5). The molecular basis of this effect was therefore further investigated.

Although EBNA2 did not contribute to elevated levels of *p21*, it was found that *p19* mRNA was transiently upregulated, with a slight increase in the level of *p18* mRNA. Both p18 and p19 mRNA/protein levels have been found to oscillate during the cell cycle, accumulating during S phase and remaining high throughout G_2/M (Hirai *et al*, 1995), suggesting that these proteins may play a role during later stages of the cell cycle. EBNA2 is known to be required at both the G_1 and G_2 stages of the cell cycle and EBNA2-deprived cells arrest at both G_1 and G_2 (Kempkes *et al*, 1995). EBNA2 has been found to induce B cell activation and entry into the cell cycle by inducing and maintaining expression of early G_1-regulating proteins, including cyclins D2, E and A, cdk4, the E2F-1 transcription factor as well as c-Myc. Overexpression of E2F-1 activates p19 synthesis in mouse embryonic fibroblasts, as does overexpression of c-myc. The mouse p19 promoter contains at least 2 potential E2F-1 binding sites, whereas c-Myc has not been demonstrated to bind the p19 promoter and its effects on p19 protein synthesis may well be indirect (reviewed by Inoue *et al*, 1999). The effect of EBNA2 expression on c-myc levels was not investigated in the results presented here.

3.3.2 Upregulation of p21 expression by LMP1.

The profound effect of LMP1 on cell growth and proliferation is well documented (Baichwal and Sugden, 1988; Henderson *et al*, 1991; Miller *et al*, 1995; Rowe *et al*, 1995). LMP1-mediated upregulation of p21 expression is consistent with evidence from previously documented reports. For example, LMP1 is known to have a protective effect

against apoptosis induced by serum starvation (Henderson *et al*, 1991). The range of apoptotic stimuli to which a cell may be subjected means that frequently, the levels of several anti-apoptotic proteins must be modulated to provide sufficient protection to the cell. The prosurvival effect of LMP1 expression on cells appears to exploit a number of different proteins which combine in a precisely-timed manner to provide protection. For example, LMP1-mediated Bcl-2 induction is a delayed response (2-3 days) (Henderson *et al*, 1991), while A20 appears to be an immediate and direct effect of LMP1 expression (Laherty *et al*, 1992), similar to that of Bfl-1 (B. d'Souza and D. Walls, submitted). p21 expression has been shown to have a protective effect against apoptosis induced by p53 as well as other agents (Gorospe *et al*, 1997; Lu *et al*, 1998). In a recent study which used adenovirus vectors overexpressing individual cdkIs in a range of cell lines, greatest cell death was found to occur as a result of p16, p18 and p27 overexpression, while p19 and p21 had a more cytostatic effect on cell proliferation (Schreiber *et al*, 1999). Thus, it may not be entirely surprising that *p19* mRNA is also slightly upregulated by LMP1 expression, while no change is seen in *p16, p18* and *p27* mRNAs (Figure 4A).

A previous study has found that LMP1 (but not EBNA2) had a cytostatic effect on B cells due to an accumulation of cells at the G_2/M phase of the cell cycle (Floettmann *et al*, 1996). This report was based on tetracycline-regulated expression of LMP1/EBNA2 in -DG75tTA cells, where cytostasis was observed as a transient effect, lasting about 4 days. Using the same DG75tTA-LMP1 cell line to induce LMP1 expression in the study presented here, viability counts were carried out at each time point and showed a similar transient cytostatic effect which corresponded approximately with p21 upregulation (see Table 1). It has been proposed that the functions of p21 (and p27) are limited to cell cycle control at the G_1/S phase transition and in the maintenance of cellular quiescence. However, several studies have begun to emerge which support a role for p21 at the G_2/M checkpoint also. For instance, p21 was found to transiently accumulate in the nucleus near the G_2/M boundary (pre-mitosis) while primary embryonic fibroblasts derived from p21-/- mice had significantly reduced numbers of pre-mitotic cells (Dulic *et al*, 1998). This data suggested that p21 promotes a transient pause in late G_2 that may contribute to the late cell cycle checkpoint controls. A further study indicated that both p53 and p21

were essential for maintaining the G_2 checkpoint in human cells (Bunz *et al*, 1998). Cells transfected with adenovirus vectors overexpressing p18, p19, and to a lesser extent p21, have been shown to accumulate in G_2/M. In contrast, cells overexpressing p16 and p27 induced an enrichment of cells in G_0/G_1 compared to mock and control cells (Schreiber *et al*, 1999).

3.3.3 p53-dependent and –independent regulation of p21 expression

p53 sites are known to exist in the p21 promoter and p21 is a major target of the p53 protein. Generally, p21 upregulation occurs by one of two distinct mechanisms: one pathway is p53-dependent and activated by DNA damage, while the other is p53-independent and can be activated by mitogens and cytokines (Michieli *et al*, 1994; Shiohara *et al*, 1996). EBV does not block the p53-dependent activation pathway initiated by DNA damage. Furthermore, during infection, viral gene transcription activates transcription of the *p53* gene and therefore primes, but does not trigger, the p53-dependent route to cell death. Indeed, LMP1 and EBNA2 have been shown to independently transactivate *p53* gene expression, through induction of NFkB activity in resting B cells (Chen and Cooper, 1996). In their study, an SG5-LMP1 expression vector produced a 5-fold increase while EBNA2 gave rise to a 2-fold increase in *p53* mRNA levels as assessed by RT-PCR. The failure of EBV-mediated upregulation of p53 to block cell division and/or to induce apoptosis was proposed to be due to EBV-induced increases in c-Myc or in other cell-cycle regulating proteins such as cyclin D2, cdc2 (cdk1) or cyclin E. In contrast, no change in p53 levels was detected as a result of induction of EBNA2 expression in oestrogen-dependent LCLs expressing a conditional EBNA2 fusion protein (Kempkes *et al*, 1995).

When BL41 cells, which carry a mutant *p53* gene, were transfected with a temperature-sensitive wild type *p53* gene, LMP1 was shown to protect from p53-mediated apoptosis without interfering with induction of p21 (Okan *et al*, 1995). Indeed it is known that none of the EBV genes expressed in type III latency possess the ability to block p53-induced p21 expression and cell cycle arrest at the G1/S boundary (Chen and Cooper,

1996). Based on this evidence, it still remains possible that LMP1 acts via p53 in upregulating p21 during primary B cell infection. The studies described above did not investigate the effect of LMP1 expression on levels of p21 in the absence of functional p53.

Previous studies have shown that among BL cell lines, some of the highest levels of *p53* mRNA and protein are found in DG75 cells. Consistent with these reports, in this study DG75 cells revealed high levels of *p53* mRNA and protein relative to other cell lines tested and in general BL cells showed higher levels than LCLs (Figures 1 and 7). Also, *p53* was highly stable in response to actinomycin D treatment (Figure 11). Elevated levels of *p53* mRNA are due to both an increased transcription rate (contributed to by a deregulated *c-myc* gene) as well as increased mRNA stability (Balint and Reisman, 1996). Normal p53 protein is usually difficult to detect due to its high rate of turnover. The high p53 protein levels observed in these cells are due to the stabilising effect of mutations. It is known that most BL cell lines carry a mutant *p53* gene and functional analysis of mutant *p53* alleles have been carried out in a range of BL cell lines (Farrell *et al*, 1991). In one study (which included BL41 and BJAB) it was found that none of the mutant *p53* genes tested gained a dominant transforming activity. Most cell lines lost the ability to suppress transformation except for the Ramos and BL37 mutants, while the p53 of Louckes and BL41 had reduced suppressor activity. The Ramos and BL37 mutations are located within the conserved box 4 region, which makes it likely that they are also significant. p53 protein was undetectable in Akata cells probably due to a very unstable truncated mutant protein. It was concluded from this data that at least most of the mutations studied were significant mutations of the p53 gene that contributed to the growth of the BL cell lines. The p53 gene in DG75 cells bears a 283 His-Arg mutation which is located in the sequence-specific DNA binding domain of the protein. Functional p53 has been shown to be necessary in mouse thymocytes for apoptosis induced by DNA damage. In this regard, a range of BL cell lines (including DG75) have been found to be relatively resistant to DNA damage when compared with LCLs, suggesting loss of function of the *p53* gene. Furthermore, El-Deiry *et al* (1993) have illustrated p21 transcription activation by wild-type but not mutant p53. In the data

presented here, expression of LMP1 in DG75 cells was found to have no significant effect on *p53* mRNA or protein levels and the increase in *p21* mRNA was not associated with any detectable promoter transactivation. In addition, a relatively moderate level of p21 upregulation was observed here: reports show that the levels of p53-independent p21 expression are much lower relative to p53-dependent expression (Zhang *et al*, 1994). For these reasons, data indicate that the observed LMP1-mediated upregulation of p21 is likely to be a p53-independent effect in BL cells, although this does not exclude a role for p53 during primary B cell immortalisation.

3.3.4 p21 expression is not upregulated in BJAB cells which lack the c-myc transclocation

Overexpression of c-myc has been found to repress the p21 promoter (Mitchell and El-Deiry, 1999), and since LMP1 is known to have a negative effect on c-Myc levels, then LMP1 could potentially use this route to effectively derepress the p21 promoter. Upregulation of p21 expression mediated by LMP1 was observed in DG75tTA-LMP1 but not in BJABtTA-LMP1 cells. BJAB differs from DG75 and most other BL cell lines in that it lacks the *c-myc* chromosomal translocation, which in general characterises both endemic and sporadic types of BLs. The (t8:14) or less frequently (t8:2) or (t8:22) translocations juxtapose the *c-myc* oncogene to Ig regulatory elements, resulting in constitutive expression of *c-myc*. Consistent with the findings of Floettmann *et al* (1996), an approximately 50% decrease in the level of c-Myc protein was found to be induced by LMP1 expression in both DG75 and BJAB cells. Thus, down-regulation of c-Myc by LMP1 appears to occur independently of the translocated state of the *c-myc* gene. Since no upregulation in p21 expression was detected in BJAB cells induced to express LMP1, this implies that c-Myc is probably not involved in p21 upregulation, although the possibility cannot be ruled out.

The translocated c-myc allele frequently (65%) carries mutations. The mutations occur in the N-terminal transactivational region of the protein which may alter the gene regulatory activities of the c-Myc protein. One of the potential regulatory targets of c-

Myc is the *p53* gene, which is also expressed at high levels in BLs. Studies in DG75 cells, indicate that c-Myc can transactivate the *p53* gene, which is reflected in the observed high levels of c-Myc protein and *p53* gene transcription rate in these cells, indicating that the transactivation activity of c-Myc, in this case, is unaffected. In contrast, in Ramos BL cells, the transcription rate of the *p53* gene has been found to be very low despite very elevated levels of c-Myc protein (Balint and Reisman, 1996). This is possibly due to a mutation in the transactivation domain of the c-Myc protein in Ramos cells. Comparably high levels of c-Myc protein and *p53* mRNA were observed in BJAB and in DG75 cells. However, it remains to be seen whether or not the transcription rate of the *p53* gene is also high in these cells.

Western blot analysis revealed reduced c-Myc protein levels in BL41.B95.8 cells relative to BL41 cells and in MUTU-III relative to MUTU-I cells (Figure 8C). Thus, in both cases, levels of c-Myc protein were reduced in the LMP1-positive cells. Moreover, very low levels of c-Myc protein were seen in LCL IARC-290B cells. As mentioned above, BL cells typically display very high levels of c-Myc protein. This is due to deregulated expression of the *c-myc* protooncogene which is believed to be the dominant factor responsible for maintainence of tumorigenicity in BL. In contrast, levels are usually relatively lower in latency group III and LCL cells (Balint and Reisman, 1996). In conclusion, these results support a negative effect of LMP1 expression on c-Myc protein levels. As a result of this effect, cells may become less dependent on aberrant *c-myc* expression and more responsive to autocrine proliferation signals. Thus, the transient cytostatic effect of LMP1 expression, reflected by a partial block in G_2/M, may function in controlling the rate of proliferation of cells and may include a role for p21.

3.3.5 LMP1 maintains pRb in the hyperphosphorylated state.

It has been reported that EBV drives phosphorylation of Rb (Cannell *et al*, 1995), and that EBV is able to prevent TGFβ-mediated down-regulation of pRb in a BL cell line (Arvanitakis *et al*, 1995). Furthermore, it was shown that LMP1 has an important role to play in maintaining Rb in the hyperphosphorylated state. Phosphorylation of Rb has the

effect of inactivating this critical negative regulator of the cell cycle. The data presented here describe findings consistent with previous studies, in that DG75tTA-LMP1 cells induced to express LMP1 produced mainly hyperphosphorylated Rb protein. In addition, mainly hyperphosphorylated pRb in group III and LCL cells contrasted with mainly hypophosphorylated pRb in EBV-negative DG75 BL cells. It is interesting to note here that p18, p19 and p21 vectors were shown to be relatively less effective at inhibiting pRb phosphorylation than their p27 and p16 counterparts (Schreiber *et al*, 1999). Thus, increased p21 and p19 expression may be consistent with maintaining Rb phosphorylation.

In summary therefore, in view of the known oncogenic properties of LMP1, an upregulation of a cell cycle inhibitor by LMP1 may initially appear to be a surprising finding. On closer examination, however, it seems that an increase in p21 levels may have a role to play in some of the previously observed effects of LMP1: for example, protection from apoptosis, transient cytostasis associated with an accumulation of cells in G_2/M and promotion of an inactive hyperphosphorylated Rb.

3.3.6 p21 promoter studies.

As detailed in the results section, no significant transactivation of the p21 promoter was detected as a result of LMP1 expression in DG75tTA-LMP1 cells. However, based on these experiments, it remains possible that LMP1 affects promoter activity through sequence elements which are located elsewhere.

3.3.6.1 p21 promoter

Known positive regulators of the p21 promoter include the SP transcription factors 1-6, AP2, E2F, Stat 1 and 3, interferon regulatory factors (IRF) 1 and 2, C/EBP alpha, while the GTPase RhoA has been found to repress the p21 promoter. Andrei *et al* (1998) have found that E2F1 and E2F3, transcription factors that activate genes required for cell cycle progression, are strong activators of the p21 promoter. In contrast, HBP1 (HMG-

box protein-1) a novel Rb-binding protein, can repress the p21 promoter and inhibit induction of p21 expression by E2F (Gartel *et al*, 1998). Both E2Fs and HBP1 regulate p21 through cis-acting elements located between nucleotides –119 to +16 of the p21 promoter, which also contains four Sp1 binding sites important for E2F activation. This suggests that the interplay between these positive and negative regulators may determine the level of p21 transcription in vivo.

Several Sp-1 sites as well as an Sp-3 site have been mapped to specific sequences within the p21 promoter. While Sp1 and Sp4 have been described as activators of transcription, the role of Sp3 in the stimulation or repression of transcription is not clear eg. Sp3 has been reported to repress Sp1-mediated transcriptional activation (Hagen *et al*, 1995). A detailed functional analysis of the p21 promoter defined a 10 base pair sequence which is sufficient to drive TGF-beta-mediated transactivation. This TGF-beta responsive element (RE) was found to bind specifically to several proteins in vitro, including Sp1 and Sp3 (Datto *et al*, 1995). Sp1 binding sites have been described in many promoters and consequently Sp1 has become known as a house-keeping transcription factor whose activity is necessary solely for the basal transcription of many genes. However, several Sp-1-like proteins have since been identified, all of which are capable of interacting with an Sp-1 consensus site. Thus, such consensus sequences may serve as sites for the interplay of several differentially expressed transcription factors. Sp-1 is also a critical factor in regulating transcription mediated by Rb, found for example in the Rb control elements (RCE) of the c-Fos and TGF-beta1 promoters. It has therefore been suggested that the TGFbetaRE may represent an RCE and that by maintaining Rb in a hypophosphorylated state, TGF-beta may be exerting its effects on the p21 promoter through Rb. Induction of p21 would in turn lead to a further increase in the hypophosphorylated form of Rb, thus establishing a positive feedback loop between p21 and Rb, ensuring an effective G_1 cell cycle arrest.

3.3.6.2 Effect of LMP1 expression on p21 promoter activity levels.

No significant transactivation of the p21 promoter used in this study was detected in the presence of LMP1. An observation which was made, nonetheless, was a high basal p21 promoter activity, comparable to that of SV40 enhancer promoter sequence, which was observed using 3 alternative p21 promoter luciferase reporter constructs. These levels were several fold higher than levels of activity of the bfl-1 promoter (see Figure 3.11). The half-life of *p21* mRNA in untreated human cells is short (normally <1 hour). Nevertheless, while the basal level of *p21* mRNA is low, promoter activity is usually quite high, (reflecting the high rate of decay) (Schwaller *et al*, 1995; Esposito *et al*, 1997). This allows even a modest increase in stability to result in the rapid accumulation of *p21* mRNA, a mechanism which is clearly suited to high turnover molecules such as p21.

Using a common stimulator, previous findings have varied using the 2.4 kb p21 promoter constructs depending on the cell line under study. For example, p21 promoter luciferase reporter activity was induced in K562 cells treated with TPA (Zheng *et al*, 1996). Also, TPA induction of *p21* mRNA in ML1 cells signaled new *p21* RNA synthesis. In contrast, similar experiments conducted in the promyelocytic HL60 cell line, concluded that TPA regulation of p21 expression was mainly post-transcriptional (Schwaller *et al*, 1995). In addition, the period of incubation of cells can be important with regard to detection of changes in promoter activity. One study, for example, found no significant induction of transcription activity after 2 hours in the presence of TPA even though *p21* mRNA and protein levels had begun to accumulate by this stage, and that no increase in the rate of transcription was observed until 12 hours (Esposito *et al*, 1997). A different study reported that TPA activated promoter activity after 24 hours treatment of cells (Biggs *et al*, 1996). Specific sites within the p21 promoter have been localised as targets of certain activators of the PKC pathway. Phorbol esters and okadeic acid have been shown to activate the p21 promoter through Sp1 sites (Biggs *et al*, 1996). Similarly, other reports have identified binding sites within the p21 promoter which are targeted via p53-independent signal transduction pathways. For example it has been shown that either IFN-gamma or EGF induces p21 transcription through signal

transducers and activators of transcription (STAT) proteins; this activation was blocked by inhibitors of PKC (Karras *et al*, 1997; Suzuki *et al*, 1997). Thus, it appears that at least in some instances activation of the PKC pathway triggers an increased transcription rate of the *p21* gene. In other instances, post-transcriptional mechanisms are important.

3.3.7 Mechanism of transcriptional regulation of *p21* gene by LMP1

Investigations into the mechanism of upregulation of p21 expression by LMP1 revealed an important role for mRNA stabilisation. This conclusion was based on the following observations: p21 mRNA was shown to have a short half-life in unstimulated tumour cells in agreement with the findings of others (Schwaller *et al*, 1995; Esposito *et al*, 1997; Akashi *et al*, 1999; Johannessen *et al*, 1999); the stability of 21 mRNA was enhanced in the presence of LMP1; no significant transcriptional activation of the p21 promoter was observed in LMP1-induced cells, and finally p21 transcripts accumulated in the presence of cycloheximide. Also, since no accumulation of the p21 protein was seen as a result of inhibition of protein synthesis by cycloheximide, it appears likely that regulation at the post-translational level is not important.

The steady state level of mRNAs in the cell is dependent on both the rates of transcription and decay. Thus, a coordinated balance between transcriptional and post-transcriptional events is required. Metabolism of eukaryotic mRNA occurs in both the nucleus and cytoplasm of the cell. The importance of mRNA stability in the accumulation of mRNA transcripts has been highlighted, especially with regard to those with short half-lives (Ross, J. 1995). Changes in the stability of specific mRNA afford an extremely rapid mechanism to change the levels of their encoded proteins. Clearly, it is important that proteins involved in regulation of the cell cycle must be able to undergo rapid changes in response to various positive and negative stimuli of cell proliferation. Thus, it is not surprising that many cell cycle-related proteins demonstrate a significant role for mRNA stabilisation in their regulation. For example, topoisomerase II alpha and c-myc are regulated during cell cycle-related events at least in part by stabilisation of their mRNAs (Hanson *et al*, 1994; Goswami *et al*, 1996). Various extracellular stimuli

such as protein synthesis inhibitors and stimulators of PKC are able to stabilise c-myc RNA (Dani *et al*, 1984).

Experiments described here revealed an almost 2-fold increase in the half-life of p21 mRNA which was mediated by LMP1 in DG75tTA-LMP1 cells. A high promoter activity in the case of p21 implies that small increases in stability become more significant, allowing a more rapid accumulation of mRNA transcripts. Thus, the rapid changes which are required in the case of many regulators of the cell cycle are possible.

Several examples of stabilisation of p21 mRNA have been documented, with regard to a range of stimulators of p21 expression. For instance, in the human ovarian cancer cell line, SKOV-3, PMA markedly stabilised p21 mRNA, increasing it's $T_{1/2}$ from <1 hour to >4 hours, and this was found to be the most important mechanism of upregulation of p21 in this cellular context (Akashi *et al*, 1999). A different study revealed a longer $T_{1/2}$ of p21 mRNA (approx. $2_{1/2}$ hours in A431 cells) which was doubled to 5 hours in the presence of EGF (Johannessen *et al*, 1999). Upon longer exposure times in this study, the stability of the p21 protein was also increased by about 3 fold, while transcription rate was only slightly increased at 1.2 fold. These results reflect a p53-independent effect, as the p53 protein is non-functional in A431 cells due to a point mutation. In the same study, the findings in A431 cells were in contrast to MCF-7 cells with normal p53, in which the half-life of p21 mRNA was not increased upon addition of EGF. Furthermore, p21 was shown to be increased in a p53-independent manner by TNF alpha in the human myeloid leukaemic cell line, KG-1; this induction occurred in the absence of new protein synthesis, being due mainly to a 5-fold stabilisation of p21 mRNA (Shiohara *et al*, 1996). In this case transcription rate was increased by a factor of 1.4. In A431 cells, TNF alpha had no effect on the stability of p21 mRNA, unlike KG-1 cells, but did increase protein stability on prolonged incubation. This probably explains a more rapid induction of p21 protein by EGF than by TNF alpha (Johannessen *et al*, 1999). Similarly, RNA stability was highlighted in the enhancement of p21 expression in normal human fibroblasts (WI38) after exposure to IL-1 (Osawa *et al*, 1995).

Esposito *et al* (1999) have recently confirmed that a p53-independent pathway for p21 induction in response to oxidative stress exists. Moreover, they showed that TPA-induced and serum-induced increases of p21 mRNA expression are, at least in part, due to the activation of the same pathway, and involve changes in the intracellular oxidative environment. Stability of mRNA was enhanced 3-fold while no transactivation of the p21 promoter was detected following EtMal (dielthymaleate)-induced oxidative stress. Evidence suggests that many other p53-independent inducers of p21, such as PDGF, EGF, TNFalpha and gamma-rays may be modulated by the cells's redox state. Thus mRNA stability would seem to be a very common mechanism of p53-independent upregulation of p21. p21 mRNA has also been shown to be stabilised in a p53-dependent manner: mRNA stabilisation was the main mechanism of upregulation in colorectal carcinoma cells exposed to UV light (Gorospe *et al*, 1998).

Regulation of mRNA stability is so far poorly understood. However, it is known that cis-acting elements within the mRNA molecule can be recognised by regulatory proteins, and *cis*-elements have been demonstrated to modulate mRNA stability both positively and negatively (reviewed by Ross, 1995; Beelman and Parker, 1995; Sachs, 1993). Numerous mechanisms exist to degrade mRNA effectively. For example, deadenylation triggers decapping, thus exposing the mRNA to 5' to 3' degradation. Alternatively, decay may be initiated independently of deadenylation by sequence-specific cleavage of the mRNA. The latter pathway is more likely to be relevant to p21 mRNA degradation. Many labile RNAs such as those for granulocyte/macrophage-colony-stimulating factor (GM-CSF), TNF alpha, IFN gamma, IL-2 and IL-3 usually contain an adenosine- or uridine-rich element, characterised by 3 or more copies of the pentanucleotide, AUUUA, in their 3'UTRs and the stability of these mRNA molecules have been shown to be regulated by external stimuli (Shaw and Kamen, 1986; Sachs, 1993; Akashi *et al*, 1994; Ross, 1995; Beelman and Parker, 1995; Chen and Shyu, 1995). It was recently shown that PMA could stabilise more effectively a reporter RNA containing 3 or more repeats of the AUUUA motif in the 3'UTR than those with 2 or less. The p21 mRNA has 3 repeats of the AUUUA motif (El-Deiry *et al*, 1993), and it is likely that PMA may stabilise p21 mRNA through the AU-rich region in it's 3'UTR (Akashi *et al*, 1999).

Other labile mRNAs coding for oncogenes such as c-Myc and cytokines also have AUUUA repeats in their 3'UTR (Akashi *et al*, 1992; Shaw and Kamen, 1986). Indeed, there are several observations which suggest increased levels of AU-binding protein interacting with AU-rich elements in PMA-treated cells (Shaw and Kamen, 1886; Lindsten *et al*, 1989).

LMP1 is known to increase intracellular calcium levels and several studies suggest that the transient accumulation or redistribution of calcium may also be one of the mechanisms for the stabilisation of mRNAs by phorbol esters and PKC activation. A calcium ionophore, A23187, stabilised mRNAs coding for IL-3 and G/M-CSF through a pathway which requires AU-rich elements in the 3'UTR (Iwai *et al*, 1993). Redox is also known to modulate the stability of mRNAs coding for cytokines and oncogenes by the AU-binding protein (Miller *et al*, 1993) and treatment of cells with PMA causes oxidative stress (Esposito *et al*, 1997). Although it is likely that p21 is stabilised through the AU-rich region in the 3'UTR, at least via a PMA-induced pathway, further studies are required to determine the mechanism resulting in the stabilisation of this transcript.

No mammalian RNAse which might be programmed to degrade specific mRNAs has yet been identified. Thus, the molecular or enzymological mechanism underlying the stabilisation of p21 mRNA is not understood, although the degradation of mRNA appears to involve endoribonucleases. It is likely that a wide variety of endonucleases with different cleavage specificities exist, allowing specific enzymes to be limited to individual mRNAs or classes of mRNAs. In this way, their presence would allow for specific control of the decay rate of these transcripts. For example, endonucleolytic cleavages have been defined in vitro for the albumin mRNA (Dompenciel *et al*, 1995) and in the coding region of the c-myc mRNA (Bernstein *et al*, 1992). In some cases, the rate of endonucleolytic cleavage is modulated by the activity of protective factors that bind at or near the cleavage site and compete with the endonuclease. Therefore, in certain situations, it appears that the endonuclease is constitutively active and the accessibility of the cleavage site is regulated (Binder *et al*, 1994). Alternatively, examples have been documented where endonuclease activity appears to be directly

regulated (Silverman, 1994), due to the enzyme normally existing in an inactive state. Since treatment of LMP1-induced cells with CHX to inhibit new protein synthesis resulted in an enhanced accumulation of p21 mRNA, which was absent from control cells with no CHX (Figure 12B), this may suggest regulation of mRNA stability by a labile protein. It is possible, therefore, that LMP1 may either enhance protective factors of cleavage sites or alternatively, induce a direct effect on mRNA degrading enzymes. Future studies on the pathway of p21 mRNA degradation, identification of the specific endoribonucleases involved and the effects of LMP1 on these activities will contribute to a greater understanding of the mechanism of stabilisation.

3.3.8 Signal Transduction Pathways

As mentioned previously, it is becoming increasingly evident in recent years that several p53-independent p21 activation routes exist. Some recent studies demonstrated that p21 could be induced in cells lacking a functional p53 (Akashi *et al*,1995; Shiohara, 1996). Some of these signals include serum stimulation, treatment with the growth factors PDGF, FGF, treatment with okadeic acid, butyric acid, retinoic acid, Vitamin D3, TPA, treatment with the cytokines G-CSF, IL-6, IFN-gamma, or treatment with transforming growth factor beta (Michieli *et al*, 1994, Steinman *et al*, 1994; Sheikh *et al*, 1994; Jiang *et al*,1994; Elbendary *et al*, 1994; Datto *et al*, 1995; Zhang *et al*, 1995). Experiments have also provided evidence for p53-independent regulation of p21 in vivo, and have implicated a role for its expression in growth arrest associated with terminal differentiation (Halevy *et al*, 1995; Parker *et al*, 1995; El-Deiry *et al*, 1995; Macleod *et al*, 1995). However, despite the continuing emergence of data supporting significant roles for p53-independent routes, the molecular events and the signal transduction pathways involved in these instances of p21 induction remain unclear.

The LMP1 protein has many properties which are suited to signal transduction, as outlined in section 1.5.4. For example, activation of the NFkB transcription factor by LMP1 is important in several instances (eg. induction of A20). LMP1's ability to induce NFkB, however, is not required for it's oncogenicity (Mitchell and Sugden, 1995) and

thus, NFκB is likely to mediate many but not all of the changes in gene expression that are affected by LMP1. LMP1 has been shown to induce expression of the EGF receptor and A20 molecule in epithelial cells (Miller *et al*, 1995). Upon stimulation with EGF, these cells demonstrate enhanced tyrosine phosphorylation of downstream targets of the EGFR and exhibit enhanced growth in serum-free media. LMP1 is also known to signal by its direct association with TNF receptor-associated factors (TRAFS), but these TRAF molecules do not mediate most of LMP1's induction of NFkB activity (Sandberg *et al*, 1997). CD40 is one of a range of cell surface activation antigens whose expression is upregulated by LMP1. CD40 also signals by binding TRAF molecules and indeed parallel roles have been suggested for the CD40 protein and LMP1 in signal transduction pathways. The expression of CD40 in carcinomas but not normal epithelial cells and also in EBV-infected NPC tissue suggests that signals from CD40 may be involved in progression to malignancy. A recent study of CD40-mediated signaling revealed that CD40-mediated signals induce resting B cells to accumulate p21, while cycling B cells required B cell receptor and CD40-mediated signals to maintain increased expression of p21 (Mullins *et al*, 1998).

LMP1 also activates the JNK pathway, and both NFkB and JNK signaling are, in some instances, downstream events of activation of the protein kinase C (PKC) pathway. PKC is a widespread family of kinases responsible for many diverse and critical cellular functions, including aspects of cellular growth and metabolism (reviewed in Wilkinson and Hallam, 1994). For example, PKC phosphorylates various transcription factors and, depending on the cell type, these induce or repress synthesis of certain mRNAs. The NFkB inhibitor protein IkB is also a substrate of PKC. Phosphorylation of IkB by PKC releases NFkB which can then migrate to the nucleus where it induces transcription of target genes. Many other examples exist which illustrate the fundamental role of PKC in controlling cell growth. PKC consists of a family of at least 10 isoenzymes which differ in their structure, co-factor requirement and substrate specificity. The conventional PKCs (cPKC), PKC α, β_I, β_{II} and γ are activated by Ca^{2+}, phospholipids and DAG or phorbol ester, and are thus Ca^{2+}-dependent enzymes (Wilkinson and Hallam, 1994).

LMP1 has been shown to induce increased intracellular calcium levels which may therefore link LMP1 to activation of PKC isoenzymes.

Several studies have linked the PKC pathway to regulation of p21 expression (Michieli *et al*, 1994; Akashi *et al*, 1995). For example, PMA induced a relatively low level of p21 which delayed cell cycle progression but failed to induce cell cycle arrest in SKOV-3 cells (Akashi *et al*, 1999). Thus the p53 independence of p21 expression by PKC may occur at subsaturating levels which may be unable to arrest the cell cycle. Similarly, the relatively moderate level of upregulation of p21 observed in DG75 cells as a result of LMP1 expression, was found to simultaneously induce a transient slowing of cell proliferation in the absence of any loss of viability. As mentioned previously, this cytostatic effect has been shown to correlate with a transient block in G_2/M. Furthermore, the involvement of p21 in integrating the PKC signaling pathway to the cell cycle machinery at the G_2/M checkpoint has recently been described by Akashi *et al*, 1999. Thus, a number of lines of evidence suggest a potential role for the PKC pathway in LMP1's observed effects on p21 levels.

LMP1 is known to activate the MAPK pathway. Moreover, Lin *et al* (1996) have shown that activation of MAP kinase (MAPK) can induce p21 expression. Activation of the MAPK signaling pathway via PKC is an important mechanism for several biological events, such as apoptosis and PKC regulates the MAPK pathway alone or with other mechanisms (Hall-Jackson *et al*, 1998). Activation of PKC leads to the accumulation of p21 transcripts through a p53-independent pathway and activation of the MAPK signaling cascade is required for the induction of p21 by PMA (an activator of PKC). Evidence presented in the same study suggested that the induction of p21 occurred mainly through stabilisation of p21 mRNA and protein i.e. at the post-transcriptional level. The physiological role of the MAPK-dependent, p53-independent pathway of p21 mRNA induction is unknown. It is possible that it's transient induction in response to mitogenic stimuli results in inhibition of cdks thereby providing a protective role against inappropriate and premature transition from G_1 to S phase. Evidence strongly suggests

that MAPK promotes cell survival upon oxidative stress, through the induction of p21 (Akashi *et al*, 1999).

In addition to the Ras/MAPK/ERK pathway, LMP1 was more recently demonstrated to also activate the p38 MAPK pathway to coregulate IL-6 and IL-8 production (Eliopoulos *et al*, 1999). p38 is a member of the MAPK superfamily activated by stress signals and implicated in cellular processes involving inflammation and apoptosis. LMP1-mediated p38 activation occurs through both CTAR1 and CTAR2 domains of the protein and appears to be mediated by the adaptor protein, TRAF2. p38 activation has been observed in response to a variety of stimuli and requires phosphorylation of a closely spaced tyrosine and threonine residue in the activation domain of the protein (for reviews, see Ip and Davis, 1998; Kyriakis and Avruch, 1996). Among the downstream targets of p38 are the heat shock protein 27 (hsp27) and the transcription factors ATF2, Elk-1, CHOP/GADD153 and Max.

Specific inhibitors were used to show that compounds that efficiently block LMP1-mediated NFkB activation in Rat-1 cells do not impair its ability to signal on the p38 axis, and conversely, inhibition of inhibition of p38 activity (SB203580 inhibitor) does not influence NFkB binding, indicating divergence of signals (Eliopoulos, 1999). However, the possibility of LMP1-mediated NFkB transactivation being a target for p38 could not be excluded as preliminary data indicated that SB203580 induced a small inhibition in LMP1-mediated NFkB transcriptional activity. Transactivation of the IL-8 promoter occurred as a result of LMP1-mediated p38 activation and binding of ATF2 (a downstream target of p38 activation) to the promoter was demonstrated, in association with c-Jun proteins. The ability of LMP1 to induce c-Jun phosphorylation through activation of JNK suggests that this kinase pathway may also contribute to modulation of IL-8 expression. JNK, p38 and NFkB have been shown to be involved in a similar complex regulation of the E-selectin promoter. Thus, it is possible that activation of p38 MAPK plays a significant cooperative role in regulating additional LMP1 activities. Recently, the p38 inhibitor SB20350 was used in B lymphocytes to demonstrate that the p38 MAPK pathway is required for CD40-induced proliferation. As LMP1 signaling

closely resembles that of the CD40 TNFR, this observation lends further support to a role for p38 MAPK in LMP1-mediated gene regulation.

One study which examined a range of human tumour cell lines following treatment with serum, TPA or okadeic acid (OA) suggests the involvement of multiple signaling pathways in the regulation of p53-independent activation of p21. For example, cell type specific differences, variable patterns of p21 activation and different consequences with respect to cell cycle arrest were observed. Also, regulation was found to occur at both transcriptional and post-transcriptional levels depending on which form of stimulation was used. PKC was a requirement in the TPA- but not OA-mediated induction of p21. TPA is a known activator of the PKC pathway, but there are also examples of TPA induction of gene expression by a PKC-independent pathway. Zheng *et al* (1996) concluded a critical role for PKC function in TPA- (but not in OA-) induction of p21 expression, by using staurosporine to specifically inhibit the PKC pathway.

Some preliminary experiments were carried out to investigate the role of the PKC pathway in LMP1-mediated upregulation of p21 expression. Treatment of cells with PMA confirmed the functional integrity of the PKC pathway in upregulation of p21 protein in DG75 tTA cells. It was also shown that the upregulation of p21 was prevented using the PKC inhibitor, staurosporine. However, on further investigation, it was found that LMP1 expression was similarly inhibited by ST, such that no conclusions could be made regarding the potential role of the PKC pathway in this instance. Nonetheless, as can be seen from Western blot results in Figure 15, the experiment did confirm that inhibition of LMP1 expression in induced cells correlated with inhibition of p21 expression. Although inhibition of a cellular response by ST and other general PKC inhibitors has frequently been cited as confirmation of a PKC-mediated event, the use of such inhibitors has been criticised in the past for their lack of selectivity (Wilkinson and Hallam, 1994). ST is a broad spectrum inhibitor of protein kinases, which has various effects depending on the concentration used (Tamaoki *et al*, 1986; Katira *et al*, 1993). ST has been reported to block the activity of both src-related and receptor tyrosine

kinases, thus preventing any firm conclusion to be made as to the likely role of PKC. For this reason, alternative relevant inhibitors were explored.

Treatment of DG75tTA-LMP1 cells with SB203580 to inhibit p38 MAPK activity produced a result similar to that obtained after staurosporine treatment i.e. it was found that both p21 and LMP1 expression were inhibited. Thus, as before, the results were inconclusive with regard to the role of the MAPK pathway in LMP1-mediated upregulation of p21 expression.

3.3.9 Future studies

The findings of the investigations detailed in this report suggest a number of potentially useful further experiments. Clearly, possibilities exist for a somewhat similar progression of experiments charcterising changes in levels of other cell cycle-related proteins observed in the original RPA data eg. the EBV latent nuclear antigen, EBNA2 upregulated mRNA levels of the p19 cdkI about 4 fold.

As already mentioned, it will be important to elucidate the mechanism of p21 mRNA stabilisation as effected by LMP1. It may also be interesting to determine whether or not LMP1-mediated upregulation of p21 expression requires new protein synthesis. Independence of intermediate protein synthesis is one characteristic feature of primary response genes, also known as immediate early genes (Herschmann, 1991). In phorbol ester-induced HL-60 cells, p21 induction was demonstrated to occur independently of intermediary protein synthesis (Schwaller *et al*, 1995). However, the p53-independent induction of p21 mRNA by Raf signalling in mouse fibroblasts was reported to depend on protein synthesis and therefore was not an immediate-early response (Sewing, 1997). In these experiments, cells were treated with external stimuli (such as PMA) and CHX was added to prevent de novo protein synthesis from the point of stimulation, after which mRNA accumulation was followed using Northern Blot or RPA analysis. The tetracycline-regulated expression system, however, is dependent on de novo LMP1 protein synthesis and therefore is not suitable for such analyses. An alternative

expression system, in the form of a stably-transfected LMP1 fusion protein, may be useful for control studies. In these cells, the cytoplasmic tail of the LMP1 protein is fused to the membrane domain of the NGF receptor, such that activation of LMP1 signal transduction can be achieved by addition of NGF to the growth media, without requiring de novo synthesis of LMP1 protein (Schwenger *et al*, 1998). Obviously, this system may also prove more suited to a range of other applications. Similarly, chimeric molecules in which the extracellular and transmembrane regions of CD2 and CD4 have been linked to the cytoplasmic tail of LMP1 have been used to investigate LMP1 signaling following antibody-induced aggregation of the chimera (Kyriakis *et al*, 1996, Eliopoulos *et al*, 1999).

A number of alternative PKC inhibitors may be explored in order to confirm or exclude a potential role for PKC in this signal transduction pathway. For example, Calphostin C is a general PKC inhibitor, while more specific inhibitors such as Rottlerin, which specifically inhibits PKC-delta, may help to identify which PKC isoenzyme(s) are most important. It is thought that PKCξ may be the key isoenzyme in signaling to NFkB (Diaz-Meco *et al*, 1993). Also, a number of derivatives of PMA which are known to be poor stimulators of PKC (eg. 4-o-methyl-PMA, 4alpha-PDD) may be useful controls. Some researchers subject cells to prolonged exposure to PMA to reduce PKC activity, thus making them resistant to repeated exposure. In view of the fact that LMP1 protein expression was inhibited by both ST and SB203580, an alternative expression system may need to be employed to overcome this problem. For example, the stably-transfected NGF receptor-LMP1 fusion referred to previously would not require new LMP1 protein synthesis.

RPA gives no indication as to the size or number of mRNA transcripts. For this reason, Northern blots provide useful additional information. Preliminary Northern blotting experiments have detected an RNA band which is upregulated in MUTU-III cells relative to MUTU-I cells and in LMP1 induced cells relative to uninduced cells (results not shown).

The level of upregulation of p21 expression may be critical to its observed effect. Cdk-cyclin complexes require the presence of a single bound p21 molecule for kinase activity, while several bound p21 molecules exert an inhibitory effect. This may imply that subtle changes in p21 levels can have significantly different effects on cell cycle progression. It can be important therefore to determine if an observed increase in the protein expression level exceeds the threshold required to inhibit cdk activity. For example, as a result of exposure of EBV-immortalised LCLs to gamma irradation, p21 protein levels are increased by a relatively moderate amount. Without any change in cdk2 levels, the amount of p21 associated with cdk2 is dramatically increased, such that a significant reduction in cdk2 associated kinase activity is observed (Cannel *et al*, 1998). It has been shown that in EBV-infected cells, small increases in p53 and p21 led to cell cycle arrest at the G_2/M boundary, but not to apoptosis; moderate increases resulted in growth arrest at the G_1/S boundary, also without apoptosis; and large increases also induced apoptosis (Chen and Cooper, 1998). These results revealed further unanticipated complexities in cell cycle regulation, and show that critical levels of these proteins exist which determine cell fate.

Immunoprecipitation experiments may be performed in the future in an attempt to detect functional p21 protein in the context of its binding to other cell cycle proteins. p21 is a universal inhibitor which preferentially associates with cyclin-cdk complexes as opposed to cdks in the unbound state, eg. cyclin-D-cdk2/cdk4 and cyclinE-cdk2 (important during G1 phase); cyclinA-cdk2 (required for ongoing DNA replication) (reviewed by Sherr, 1994; Hunter and Pines, 1994). cdk2 and cdk4 monoclonal antibodies (a gift from Bioresearch Ireland) have already been tested to determine detectable levels in the relevant cell lines (not shown). It would be possible to isolate complexes containing p21 and cdk2 or cdk4 from the relevant protein lysates by immunoprecipitating with the relevent cdk antibody and subsequently immunoblotting with anti-p21 antibody. The immunoprecipitated complexes could then be assayed for kinase activity, using eg. Histone H1 and GST-Rb substrates. This would help to ascertain whether p21 levels had exceeded the threshold required to inhibit kinase activity. It may be interesting to note if any differences are observed in the type of complexes with which p21 is preferentially

associated and in their relative kinase activites in the different cell lysates under study. For example, is the level of LMP1-mediated upregulation of p21 sufficient to inhibit cdk2-associated kinase activity? In EBV-immortalised LCLs, at the basal (EBV-induced) level of expression, p21 does not prevent the formation of active cdk2-containing kinase complexes, whereas, following activation of the p53 pathway following exposure to gamma irradiation, the induced p21 associates with cdk2 in an inhibitory manner (Cannell *et al*, 1998). It is important to recognise, however, that p21 is a dual inhibitor in that it has been demonstrated to inhibit DNA synthesis and cell cycle progression without interfering with the function of cdk/cyckin complexes (Waga *et al*, 1994) i.e. p21 also associates with the DNA replication factor, PCNA, in an inhibitory manner.

The observations made in these experiments occurred in the context of BL cells. As EBV infects primary B cells, it would be useful to transfect an LMP1 expression vector into primary B cells and to isolate RNA/protein for analysis as before. It would be possible to co-transfect LMP1 with a CD2/GFP marker gene into tonsillar B cells for immunomagnetic sorting after 24 hours. Under these conditions, assuming a transfection efficiency of 5%, the transfected cells can be sorted to 80% purity. This should be sufficient to detect the p21 upregulation. In addition, LMP1 mutants are available, which could be used in the same set of experiments to assess the functional domains of LMP1 involved in the upregulation.

3.3.10 In Summary

Taken together, the data presented here suggest that elevated levels of p21 mRNA and protein are a general feature of group III EBV-positive cells and that LMP1 has an important role to play in this effect. LMP1 was observed to upregulate the p21 gene in a B cell-specific manner. Stabilisation of mRNA was found to be important which permits rapid changes in levels of p21 mRNA/protein due to the high basal activity of the p21 promoter. Preliminary data suggest that LMP1 may upregulate other target genes by stabilisation of their mRNAs (B. d'Souza and D. Walls, unpublished), so this may be

a common pathway used by LMP1. Further studies will be required to elucidate the mechanism of mRNA stabilisation involved. Data presented here indicate that LMP1 acts through a p53-independent pathway in upregulating p21 expression and that c-myc is probably not involved. Although activation of the PKC signal transduction pathway seems likely, the pathway, as yet, remains undetermined.

p21 is part of a complex network of regulatory signals in the highly sophisticated control of cell cycle progression. Originally described as a gene induced in response to p53 and upon cell senescence, p21 has subsequently been shown to be subject to modulation by a variety of effectors involving different mechanisms of regulation. Novel experimental data have been presented here which reflect recent surprising findings that several proteins that are considered to negatively regulate cell cycle progression (p21, p53, pRb, p107) are up-regulated during EBV-mediated B cell activation (Cannell *et al.*, 1996; Allday *et al.*, 1995; Szekely *et al.*, 1995). As yet, the contributions of each to interruption of normal cell cycle events in EBV-infected B cells or their mechanisms of upregulation are poorly understood. It is clear that further studies are required both to characterise the observations described in this study and to determine their role in the EBV strategy of deregulation of cell cycle events. EBNA2 was not found to be involved in the upregulation of p21 levels, but perhaps other EBV latent genes have a role to play. Elevated levels of p21 may contribute to cell survival and the control of cell proliferation rate, which appear to be important functions of the LMP1 protein.

CHAPTER 4

A STUDY OF THE FUNCTIONS OF THE EBNA3 PROTEINS

4.1 INTRODUCTION

This chapter describes two alternative approaches taken in the study of the functions of the EBNA3 proteins (see section 1.5.6). Firstly, the yeast two hybrid system was employed to screen for cellular proteins which potentially interact with EBNA3B. In the second approach, it was proposed to generate a stable cell line which could be induced to express EBNA3A in response to tetracycline withdrawal using DG75tTA cells (see section 3.1 for an outline of the principal of this system).

4.2 THE YEAST TWO HYBRID SYSTEM.

4.2.1 History and Principle

Specific interactions between proteins form the basis of many essential biological processes. Additionally, transforming proteins of tumour viruses in many cases exert their effect through their interactions with cellular proteins; for example, the SV40 large large tumour (T) antigen binds to the cellular proteins p53 and pRb (DeCaprio *et al*, 1988). Consequently considerable effort has been devoted to the development of methods for the assay of such interactions. Typically, many of these interactions have been detected by using co-immunoprecipitation experiments in which antibody to a known protein is used to simultaneously precipitate target as well as associated proteins. Such biochemical methods however result only in the identification of the apparent molecular mass of the associated proteins and obtaining cloned genes for these proteins is often a difficult process. One approach which has circumvented this problem is the use of purified labelled proteins as probes against bacterial expression libraries where a positive signal for an interacting protein is accompanied by the availability of the corresponding gene. An alternative approach which has grown in popularity over the past decade is use of the yeast two hybrid system, a simple and sensitive means to identify proteins that bind to a protein of interest or to delineate domains or residues critical for an interaction.

The concept of a novel yeast-based two hybrid genetic assay for detecting protein-protein interactions was first introduced by Fields and Song in 1989, and was derived from three experimental observations. Firstly, Brent and Ptashne (1985) demonstrated that the activation domain of Gal4 (a yeast transcription factor) can be fused to the DNA binding domain (DBD) of *E. coli* LexA to create a functional transcription activator in yeast. Second, Ma and Ptashne (1987) built on this work to show that the DBD does not have to be physically on the same polypeptide as the activation domain i.e. the activation domain could be brought to DNA by interaction with a DBD. Fields and Song (1989), working independently of Ma and Ptashne, made the seminal suggestion that protein interactions could be detected if two potentially interacting proteins were expressed as chimeras. Two yeast proteins, SNF1 and SNF4, were used to make a SNF1 fusion to the DBD of Gal4 and a SNF4 fusion to the Gal4 activation domain. They demonstrated that the strength of the SNF1-SNF4 interaction was sufficient to allow activation through a Gal4 DBD. From this, they suggested the feasibility of selecting interacting proteins by performing screens of cDNA libraries made so that library-encoded proteins carried activating domains (Fields and Song, 1989).

Based on these findings, the yeast two hybrid system (YTHS) exploits the finding that most eukaryotic transcription activators are modular. Thus, the ability of a pair of interacting proteins to bring a transcription activation domain into close proximity with a DNA binding domain (DBD) that regulates the expression of an adjacent reporter gene is used to indicate an interaction (Fields and Song, 1989; Chien *et al*, 1991). The principle of the system is summarised in Figure 4.1. Briefly, the DNA binding domain targets the hybrid protein to its binding site, where noncovalent interaction with another protein tethers the activation domain to the upstream activation sequences (UAS), activating transcription of a specific reporter gene. The latter protein is normally encoded by a pool of plasmids in which total cDNA or genomic DNA is ligated to the activation domain.

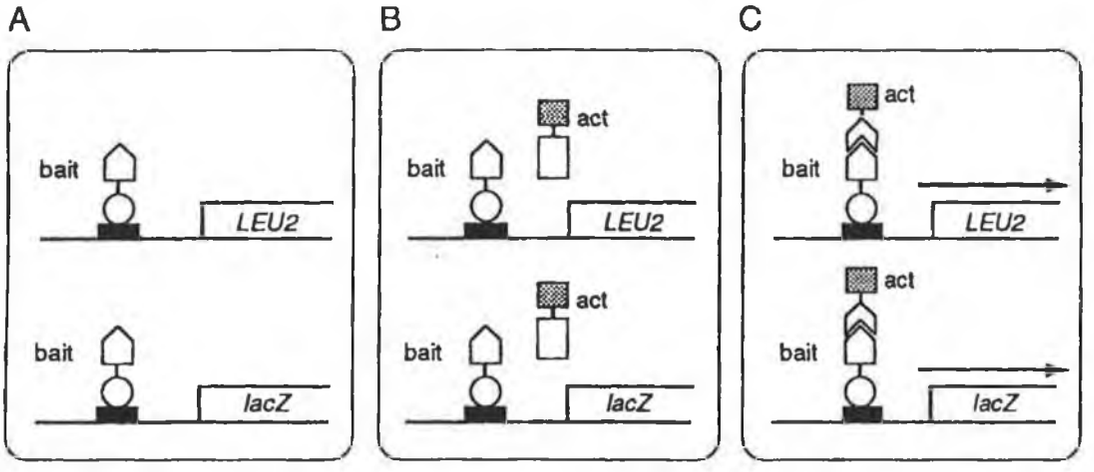


Figure 4.1 The interaction trap. **(A)** An EGY48 yeast cell containing two lexA operator-responsive promoters, one a chromosomally-integrated copy of the LEU2 gene (required for growth on –Leu media), the second a plasmid bearing a GAL1 promoter *lacZ* fusion gene (causing yeast to turn blue on media containing X-gal). The cell also contains a constitutively expressed chimeric protein, consisting of the DNA-binding domain of LexA fused to the probe or "bait" protein, shown as being unable to acivate either of the two reporters. **(B)** and **(C),** EGY48/pbait-containing yeast have been additionally transformed with an activation domain (ACT)-fused cDNA library in pJG4-5, and the library has been induced. In (B) the encoded protein does not interact specifically with the bait protein and the two reporters are not activated. In (C), a positive interaction is shown in which the library-encoded protein interacts with bait protein, resulting in activation of the two reporters (arrow), thus causing growth on media lacking leucine, and blue colour on media containing X-gal. Symbols: black rectangle, lexA operator sequence; open circle, LexA protein; open pentagon, bait protein; open rectangle, library protein; shaded box, activator protein. Adapted from Gyuris *et al*, 1993).

In most cases, the well-characterised yeast transcription factor Gal4 and the DBD of *E. coli* LexA are used in constructing the fusions, and the *E. coli lacZ* gene is often used as the reporter (Chien *et al*, 1991; Durfee *et al*, 1993 and Gyuris *et al*, 1993). Both Gal4 and LexA bind as dimers and the reporter genes contain several copies of the binding site. These DNA binding and activation domains (AD) can function at either end, but for ease of cloning are usually placed at the amino end. If they lack an endogenous nuclear localisation sequence, vectors usually include a heterologous signal sequence (for a review of the two hybrid system, see Fields and Sternglanz, 1994). A flow chart for performing an interaction trap is represented in Figure 4.2.

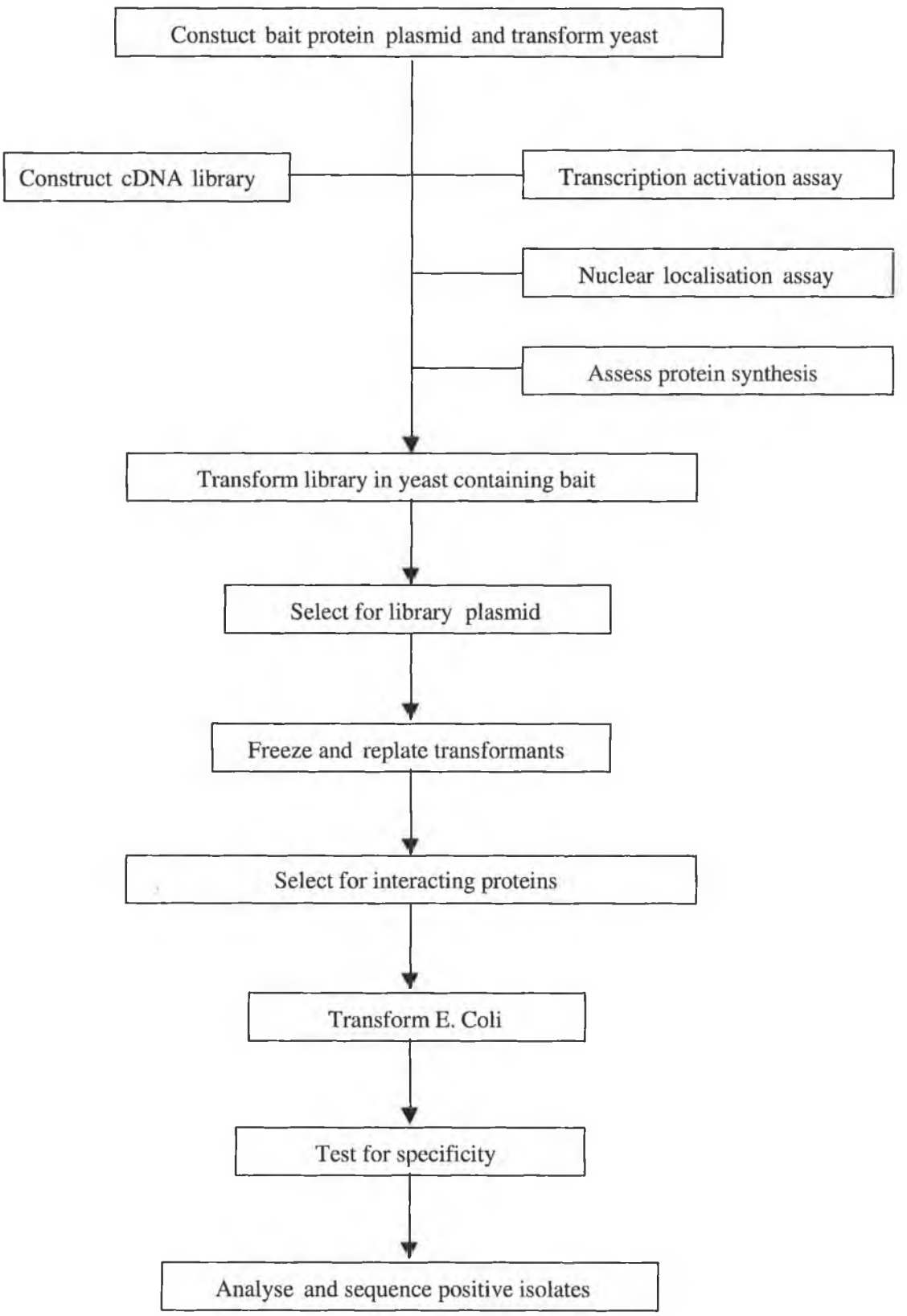


Figure 4.2. Flow chart for performing an interaction trap.

Thus, though systems differ in their specifics, all have three basic components:

- yeast vectors for expression of a known protein fused to a DBD,
- yeast vectors that direct expression of cDNA-encoded proteins fused to a transcription AD,
- yeast reporter genes that contain binding sites for the DBD.

Different systems use different reporter genes, but usually two alternative genes are utilised to help reduce false positives. As mentioned above, one commonly-used reporter gene is *E. coli lacZ* which produces blue colonies on plates or filters containing X-Gal. A second reporter gene which encodes an enzyme required for the biosynthesis of an amino acid may also be used. For example, use of *LEU2* or *HIS3* allow for selection of cells that grow on media lacking the relevant amino acid and are particularly helpful when screening libraries. The activation-tagged cDNA-encoded proteins are expressed either from a constitutive promoter or from a conditional promoter such as that of the *GAL1* gene. Use of a conditional promoter makes it possible to quickly demonstrate that activation of the reporter gene is dependent on expression of the activation-tagged cDNA proteins.

While the two hybrid system is most often used in yeast, it should work in any eukaryote and has been used in mammalian hosts (Vasavada *et al*, 1991; Fearon *et al*, 1992). However, the yeast-based system has numerous advantages, including the ease of transformation, the convenience of retrieving plasmids, and the availability of nutritional markers and well-characterised reporter genes for direct selection. Finally, endogenous yeast proteins are less likely to bind a mammalian target protein to prevent its interaction with a protein encoded by a library. No endogenous yeast proteins bind to the LexA operators.

4.2.2 Applications

The YTHS has three major applications. The system has been widely used to test known proteins for interaction and also to define domains or amino acids critical for an interaction. Finally, perhaps the most powerful current application involves the screening of libraries for proteins that bind a protein of interest. Typically, libraries are constructed in which total cDNA derived from an organism or tissue is fused to a sequence encoding a TA domain. These libraries generally contain $>10^6$ inserts, although only one sixth of these are likely to be in the correct orientation and reading frame. For example, the p21/CIP1 cyclin-dependent kinase (cdk) inhibitor was identified by screening a library with cdk2 (Harper *et al*, 1993). Similarily, screening with cdk4 detected the p16 cdk inhibitor (Serrano *et al*, 1993). The sensitivity of the method has been illustrated by the detection of certain enzyme-substrate interactions which had not been detected using alternative methods, eg. binding of Ras with the protein kinase Raf was detected using the yeast two hybrid system (Votjek *et al*, 1993) but had not been observed by co-immunoprecipitation. Also Raf binding of the Ikb protein which can be phosphorylated by Raf yields a signal in this system (Aelst *et al*, 1993). It seems likely that transient interactions can trigger transcription to produce a stable mRNA that can be repeatedly translated to yield a reporter protein. This type of amplification leads to a detectable signal even when the initiating interaction cannot be observed in vitro. Other interactions with the basal transcription machinery may help stabilise a weak protein-protein interaction.

Several industrially significant uses of YTHSs have emerged. Firstly, some important targets for pharmaceutical intervention have been identified using these techniques and it is thought that their extension in the future will allow the development of new drugs. For example, new nuclear hormone receptors were identified by Seol *et al* (1994). Ligands for these receptors are likely to be biologically active and may well have pharmaceutical significance. Another industrial application involves searching for compounds that modulate protein interactions. This is based on the premise that compounds that weaken a given interaction would diminish expression of reporters. Furthermore, in two hybrid systems, the strength of activation generally correlates with the strength of interaction.

Mutations in either interacting protein that diminish binding, and thus reporter activity, can indicate residues in protein-protein contact. This permits the determination of specific residues involved in a given protein-protein interaction.

4.2.3 Disadvantages and Limitations.

While a wide range of protein-protein interactions may be detected using the YTHS, some limitations exist. Despite the inclusion of a nuclear localisation signal in the bait plasmid, some proteins cannot be imported into the nucleus and thus are clearly not suitable. Also, improper folding of bait proteins in the yeast cell precludes their use in this assay. Interactions which are mediated by post-translational modifications may not be detected; for example, certain phosphorylation reactions may not occur in yeast. Similarly, proteins which are glycosylated and/or contain disulphide bonds are generally not compatible with a nuclear-based system. For this reason, it may not be possible to reproduce interactions involving extracellular proteins, thus, the assay may be of limited use in analysing receptor-ligand interactions which usually occur outside the cell. Nonetheless, the intracellular domains of membrane receptors will probably function in the YTHS. Despite these limitations, proteins are more likely to be in their native state in a yeast-based system than those produced in bacterial systems.

The broad applicability of the YTHS partially depends on the fact that most proteins do not contain activation domains. Nevertheless, in some cases a bait induces transactivation due to the presence of true ADs while in many cases random domains can cause activation. In these cases, a truncated form of the protein may be used or the residues responsible for transactivation may be deleted. However, it is often impossible to tell what effect this might have on it's ability to bind other proteins. Problems have also been encountered where the transcription factor domain blocks accessibility of either interacting protein. In these cases, the orientation of the hybrid can usually be reversed.

Despite the extensive control measures in place, false positives remain a significant problem associated with use of the YTHS. Having passed a dual selection screening process, certain library plasmids activate reporter gene expression, independent of

interaction with the bait protein. In many cases, this is caused by a library plasmid encoding a protein involved in transcription, but in other cases the explanation remains unclear. Such positives can usually be eliminated by assaying the positive library plasmids against hybrids of the DBD fused to other unrelated bait proteins. In this case, the more non-specific fusion proteins which are used the better. In view of these problems, careful design of bait, proper use of controls and confirmation of results with an independent method are essential for the successful use of the YTHS.

4.2.4 In Conclusion

In spite of the problems and pitfalls which have become more evident in recent years, the YTHS has nonetheless provided a useful tool over the past decade both in the study of interactions between known proteins and in the identification of new proteins which interact with known target proteins. The yeast two hybrid system has been particularly useful for studying proteins that control the cell cycle, that regulate transcription and function in oncogenesis and tumour suppression. It seems likely that, given its high sensitivity and broad applicability, this system and other hybrid protein systems will find application in many areas of research. Interaction technology may be of utility for assigning function in genome applications; eg. to assign function to unknown proteins, to assign proteins to ordered genetic pathways and even to find genes altered in disease states (see Mendelsohn and Brent, 1994 for applications to biotechnology research.). Thus, it seems likely that interaction technology will continue to have a large impact on many areas of basic and applied biological research.

4.2.5 Aims

The aim of this work was to screen relevant cDNA libraries for protein-protein interactions involving the EBNA-3 family of proteins, in particular, EBNA-3B. It was

proposed to prepare constructs which would allow screening for interactions involving EBNA-3A, EBNA-3B or EBNA-3C, but to conduct initial screening using the EBNA-3B construct. In the event of detection of any positive interactions, EBNA-3A and –3C could then be screened for specific association with the isolated interactor. Although members of the same family, amino acid homology is limited and so interactions specific to each family member as well as common interactions are quite likely. Furthermore, the YTHS could subsequently be used to more precisely delineate domains/amino acids critical for binding. In each case, amino-terminal fragments of EBNA-3 were chosen to use as bait in the screening assay due to the existence of transcription regulatory domains in the carboxy-terminal end (see Figure 4.3). In addition, previously identified protein-protein associations involving the EBNA-3 family have been largely confined to the amino-terminal end.

For the purpose of these experiments, the YTHS was employed to screen two alternative cDNA libraries. This necessitated the use of two systems which differ quite significantly in methodology. For convenience, they will be referred to as YTHS-A (developed by Golemis *et al*, 1993) and YTHS-B (developed by Harper *et al*, 1993). In each case, the relevant library was screened with an amino-terminal fragment of the EBNA-3B gene, in an attempt to identify proteins with which the latter interacts. Materials and methods used in the YTHS are described in sections 2.1 and 2.8, respectively. Solutions are detailed in Appendix.

4.3 YTHS-A

4.3.1 Strategy

The first yeast two hybrid system (YTHS-A) employed was based on a system which owes it's development to work primarily by Jeno Gyuris and Erica Golemis (Golemis *et al*, 1993). This system was employed to screen a library prepared from the cDNA of a human foetal lung fibroblastic cell line, WI-38, for potential interactors with a 1575bp amino terminal fragment of EBNA-3B. The YTHS-A method consisted of three critical

components (reviewed by Mendelsohn and Brent, 1994). First, a vector (pEG202, Figure 4.4) for expression of the protein of interest, EBNA-3B, fused to the LexA DBD, referred to as the bait plasmid, or pBait. Second, the yeast strain EGY48, which has two LexA-responsive reporter genes. A yeast *Leu2* derivative that has its upstream regulatory sequences replaced with LexA operators allowed transcription of the Leu gene to be measured by the ability of the strain to grow in the absence of leucine. The other reporter gene, *lacZ*, provided a secondary assay of activation, as well as some quantitative information about the interaction. Third, a library plasmid (pJG4-5, Figure 4.5) directs the conditional expression of cDNA proteins fused at their amino-termini to a moiety containing three domains: a nuclear localisation signal, a transcription activation domain and a HA epitope tag. The cDNA-encoded protein is expressed from the yeast GAL1 promoter, which is induced by galactose and repressed by glucose. The use of a galactose-inducible promoter makes it possible to determine that the leucine prototrophy is dependent upon cDNA expression. Thus, an advantage of this particular system is that cells containing true interactors will grow on media lacking leucine only if it contains galactose but not when it contains glucose.

4.3.2 Preparation of bait fusion construct, pAS-3B-525.

A cloning strategy was designed to construct a LexA-EBNA-3B fusion which would express an amino-terminal portion of EBNA-3B fused to the C-terminus of LexA. This involved insertion of the appropriate EBNA-3B coding region (Figure 4.3) into the pEG202 plasmid (see Figure 4.2 and section 2.1 for plasmid description) which constitutively expressed EBNA-3B fused to amino acids 1 to 202 of LexA (includes the DNA binding and dimerisation domains). Although it does not contain a yeast nuclear localisation sequence, LexA and most LexA fusions will enter the nucleus. Nevertheless, a derivative of pEG202 which contains an additional NLS (pNLEX) was included in similar cloning experiments.

One important requirement of the YTHS is that the bait should be transcriptionally inert. Therefore, selection of a portion of EBNA-3B for use as bait in the interactor hunt was

based on minimising the likelihood of inappropriate transcriptional activation, while retaining the domains most likely to be involved in protein-protein interactions. To this end, a 1575 bp amino terminal fragment of EBNA-3B was subcloned into pEG202 and pNLEX downstream of the LexA DNA binding domain. This region of EBNA-3B excludes a C-terminal proline-glutamine-rich domain (see Figure 4.3) which has been shown in EBNA-3C to contain a transactivation domain (aa 724-826) similar to the transactivation domain in the mammalian transcription factor, Sp-1 (Marshall *et al*, 1995). Other transcriptional regulatory domains have been delineated in the C-terminal portion of the EBNA-3 proteins, including a strong repressor domain identified in EBNA-3C (aa280-525). Furthermore, this bait construct retained the putative RBP-Jk binding site which lies between aa181-257 (Robertson *et al*, 1996).

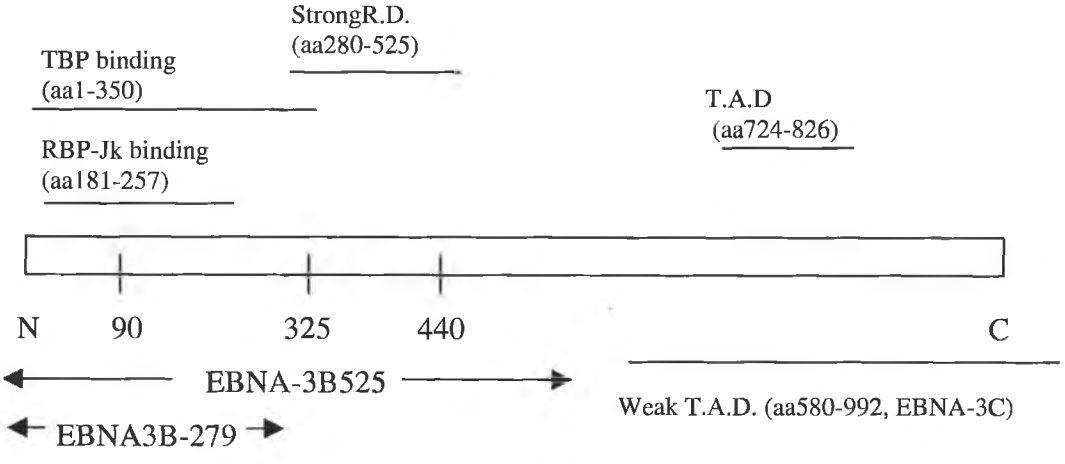


Figure 4.3 Functional domains of the EBNA-3B Protein. Approximate location of RBP-Jk binding domain and putative transcription regulatory domains of the 933aa EBNA-3B protein are shown (see also figure 1.7). TBP : TATA-binding component of TFIID; R.D : Repressor domain; T.A.D : Transactivation domain. (Cohen and Kieff, 1991; Le Roux *et al.*, 1994; Marshall and Sample, 1995). Arrows indicate regions of EBNA-3B included in bait protein. Dashes indicate known functional EBNA3 domains. Line sizes are approximate to domain sizes.

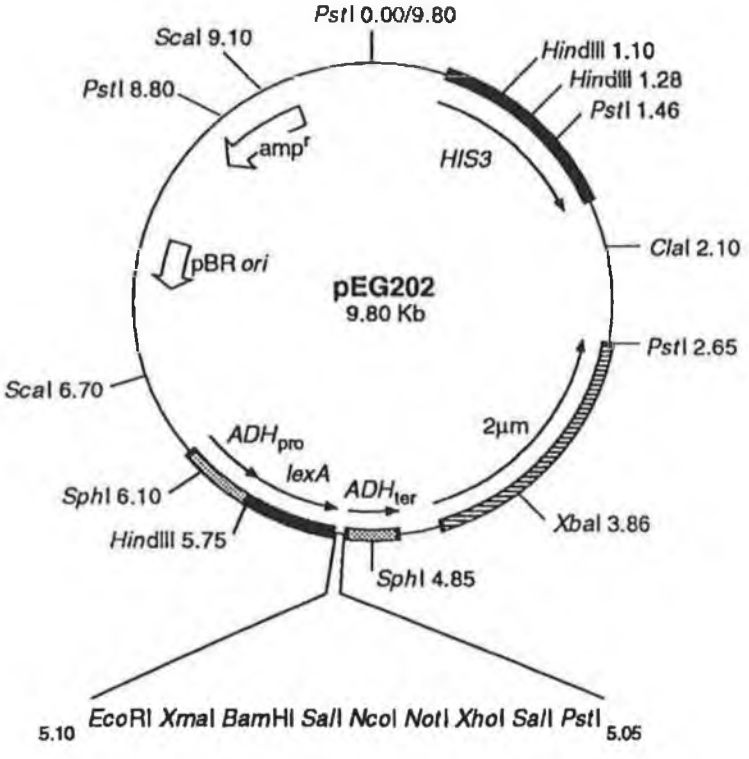


Figure 4.4 pEG202 expression vector. pEG202 uses the strong constitutive alcohol dehydrogenase promoter (ADH_{pro}) to express bait proteins as fusions to the DNA binding protein LexA. Restriction sites available for insertion of coding sequences are shown immediately upstream of the AHD_{ter}. This plasmid contains the HIS3 selectable marker and 2 µm origin of replication to allow propagation in yeast, and the ampicillin resistance gene (amp^r) and the pBR origin (ori) or replication to allow propagation in E.coli. Numbers indicate relative map positions.

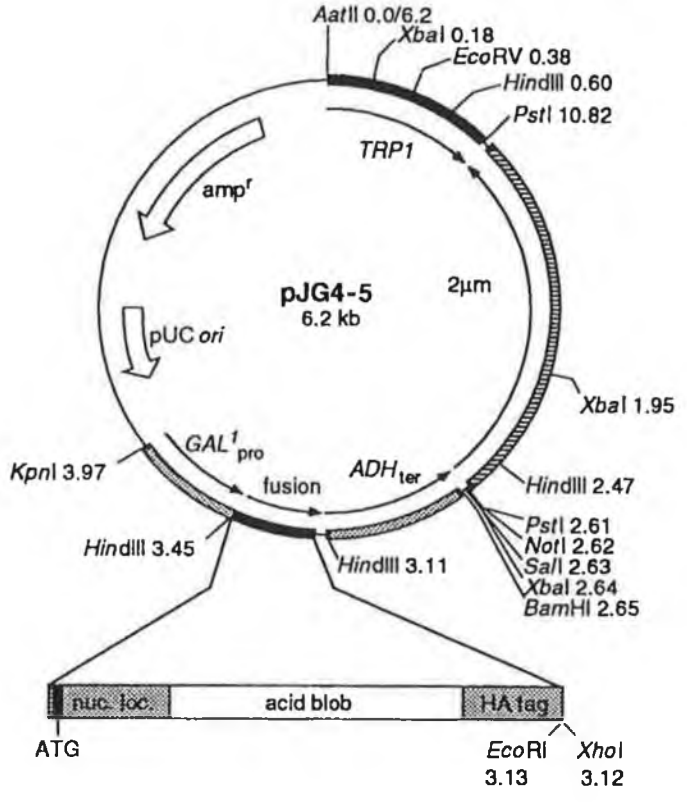


Figure 4.5 pJG4-5 library plasmid. pJG4-5 (Gyuris *et al.*, 1993) expresses cDNAs or other coding sequences inserted into the unique EcoR1 and Xho1 sites as translational fusions to a casette consisting of the SV40 nuclear localisation sequence (nuc. loc.), the acid blob B42 and the haemagglutinin (HA) epitope tag. Expression of sequences is under the control of the GAL1 inducible promoter. Numbers indicate relative map postitions.

The 1575 bp N-terminal fragment of EBNA-3B was amplified by PCR from p7CMVE4 using the primers and protocol detailed in sections 2.1 and 2.3 (see Figure 4.6). In order to allow insertion of EBNA-3B into the *Bam*H1 site of vectors, PCR primers were designed to incorporate a *Bam*H1 restriction site at each end of the PCR product.

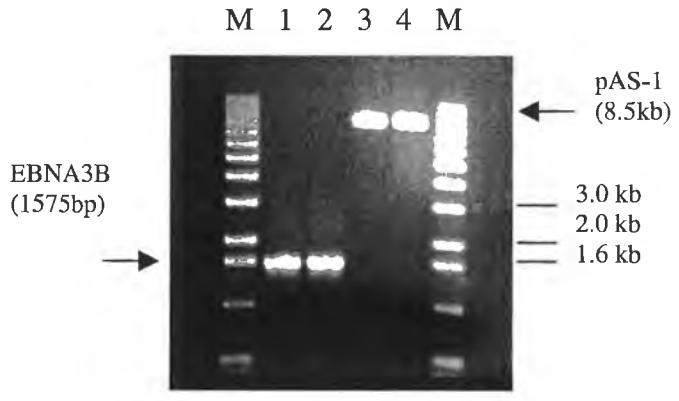


Figure 4.6 Electrophoresis of EBNA-3B PCR products (0.8% agarose gel).
Lane M: 1 kb markers; Lanes 1 and 2: EBNA-3B 1575 bp PCR product; Lanes 3 and 4: *Bam*H1-linearised pAS-1 (8.5kb). Arrows indicate DNA used in cloning experiments; dashes indicate DNA size markers.

EBNA-3B PCR product was cut with *Bam*H1 enzyme to give sticky ends which were then ligated with the *Bam*H1-linearised vectors. Following transformation of *E.coli,* recombinant clones were characterised by *Bam*H1 restriction analysis (Figure 4.7).

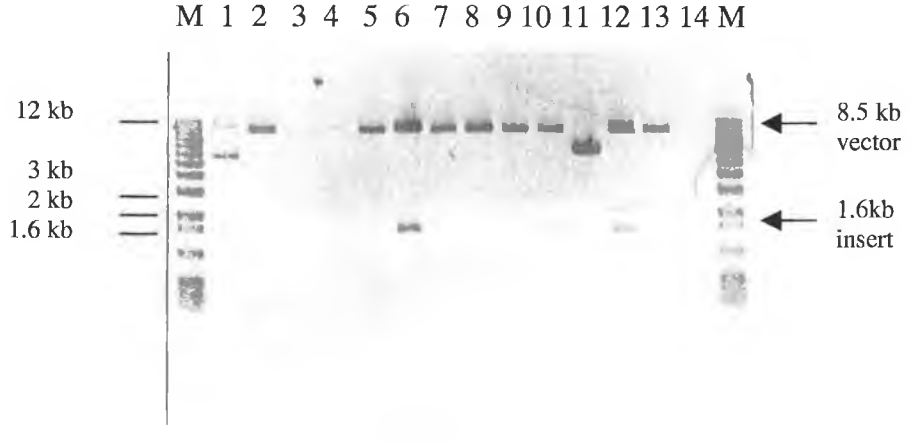


Figure 4.7 Identification of constructs containing EBNA-3B insert. *DH5α E.coli* were transformed with ligations of EBNA-3B and pEG202/pNLEX. Recombinant colonies were assessed by *Bam*H1 digestion of DNA minipreps, which were then electrophoresed on a 0.6% agarose gel. Plasmids which contain a 1.6 kb insert can be seen in lanes 6, 10 and 12. Arrows indicate digested DNA; dashes indicate DNA size markers.

To determine which recombinants contained EBNA-3B inserted in the correct orientation, two independent restriction analyses were performed (as for all subcloning experiments). For example, with reference to Figure 4.8 below a single *Nco*1 site is present in the multiple cloning site of pEG202/pNLEX, located downstream of the C-terminal end of the EBNA-3B insert, while EBNA-3B525 contains 2 *Nco*1 sites., incorrect orientation of insert gives a band of ~950 bps which is absent in correctly orientated inserts .

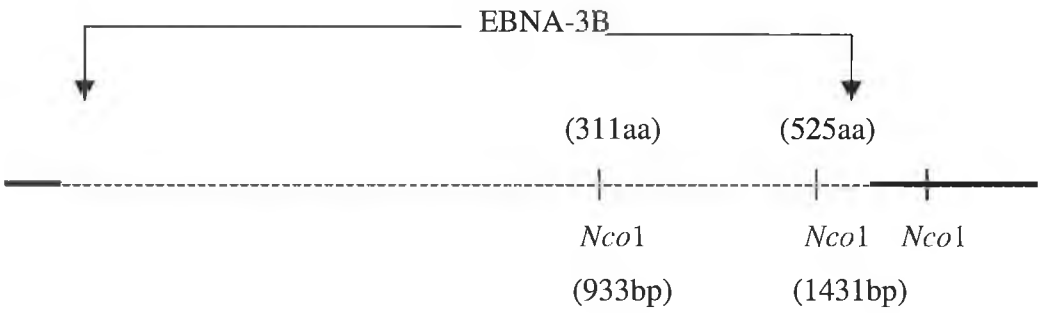


Figure 4.8 Schematic representation of Nco1 sites in EBNA-3B inserted into vector. Blue lines represent EBNA-3B sequences, while black lines represent pEG202 vector sequences.

4.3.2 Truncation of EBNA-3B525 bait construct to give EBNA-3B311 bait in pEG202/pNLEX.

Shorter LexA fusion proteins were derived from pEG-3B-525 and pLEX-3B-525 constructs by Nco1 restriction. Referring to Figure 4.8(A) again, it can be seen that this produces a 933 bp N-terminal EBNA-3B fragment inserted at the *Bam*H1 site of pEG202/pNLEX to give pEG-3B-311 or pLEX-3B-311. Each encodes the first 311 amino acids of the EBNA-3B gene product. The result of this manipulation is illustrated in figure 4.9(A) by *Bam*H1 excision of inserts, and confirmed in an independent restriction using Pst1 in Figure 4.9(B). Digestion with *Pst*1 gives a 5.35 kb band in

pEG202/pLEX-3B-525 which is reduced to a 4.75kb band as a result of loss of approximately 600 bp in pEG202/pLEX-3B-311.

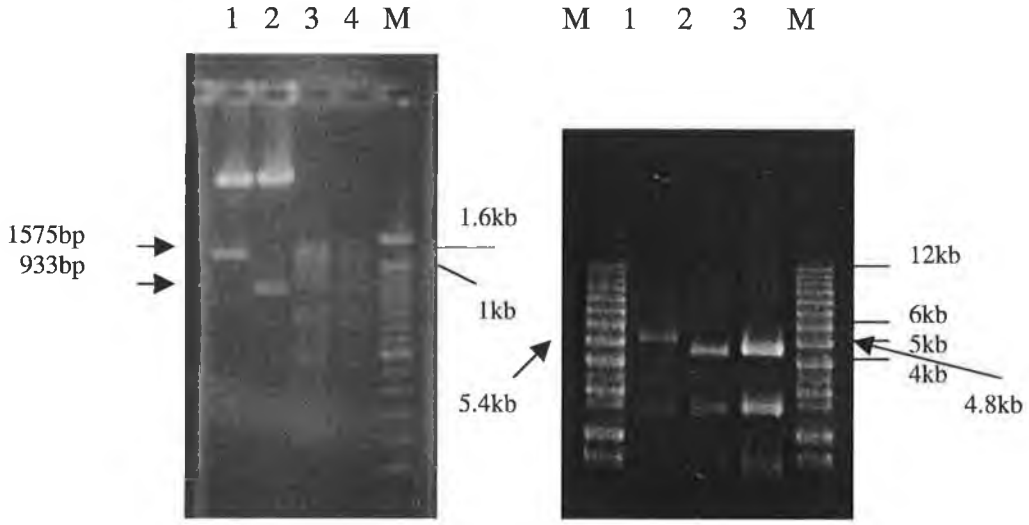


Figure 4.9 Truncation of pEG-3B525 bait construct to give pEG-3B311.

(A): BamH1 restriction of pEG-3B525 / pEG-3B311 (1.8% agarose gel).

Lane 1 : pEG-3B525 (1575 bp); Lane 2 : pEG-3B311 (933 bp); Lane M : 100bp Markers

(B): *Pst*1 restriction of pEG-3B525 / pEG-3B311 (0.6% agarose gel).

Lane M: 1 kb Markers; Lane 2: pEG-3B525, *Pst*1 digest; Lanes 3 and 4: pEG-3B311, *Pst*1 digest

Arrows indicate digested recombinant DNA fragments; dashes indicate DNA size markers.

In conclusion, 4 individual EBNA-3B baits were constructed as follows:

pEG-3B-525 : 1575 bp insert (encoding 525aa) in pEG202.

pEG-3B-311 : 933 bp insert (encoding 311aa) in pEG202.

pLEX-3B-525 : 1575 bp insert (encoding 525aa) in pNLEX.

pLEX-3B-311 : 933 bp insert (encoding 311aa) in pNLEX.

4.3.4 Characterisation of Baits.

4.3.4.1 Transcription activation assay

This assay was used to verify that baits did not activate transcription of the reporter genes. Using X-gal filter lift assays for activation of the *lacZ* reporter gene, positive controls were observed to be blue within an hour, while negative control and all baits tested showed no colour change after overnight incubation at 30°C. Transformant strains containing baits were also tested for their ability to grow in the absence of Leu.

EGY48	Carbon source	Growth on Leu-	LacZ
pSH18-34/pSH17-4	Glucose	-	-
pSH18-34/pSH17-4	Galactose	++	++
pSH18-34/pRFHM-1	Glucose	-	-
pSH18-34/pRFHM-1	Galactose	-	-
pSH18-34/pBait*	Glucose	-	-
pSH18-34/pBait*	Galactose	-	-

Table 4.1 Transcription activation assay. *pBait denotes that each of the following baits were tested: pEG-3B-525; pLEX-3B-525; pEG-3B-311; pLEX-3B-311.

The data in Table 4.1 confirm that none of the bait plasmids express a fusion protein that is transcriptionally active. As both reporter genes are controlled by gal-dependent promoters, therefore any transactivation should be apparent only when galactose is provided as the carbon source (see positive control). For this reason, glucose plates were included in the experiment as negative controls.

4.3.4.2 Repression assay (Nuclear localisation assay).

The repression assay was used to confirm that bait fusion proteins are capable of entering the nucleus and binding LexA sequences. This assay is based on the observation that LexA and and non-activating LexA fusions can repress transcription of

a reporter gene which has two lexA operators positioned between the TATA box and upstream activating sequence in pJK101. Thus, repression of β-galactosidase activity is used as an indication of nuclear localisation and interaction with LexA operators (Brent *et al*, 1994)). A quantitative β-galactosidase liquid assay was used to determine levels of activity.

S. cerevisiae EGY48	β-gal activity*
PJK101	730 U
pJK101/pRFHM-1	377 U
JK101/pLEX-3B-311	229 U

Table 4.2 Repression Assay (Nuclear Localisation Assay). *Units of activity are based on a duplicate average for each transformant.

The results indicated that the pLEX-3B-311 bait expressed a protein that is localised to the nucleus and binds LexA operators, as suggested by the 3.1-fold decrease in β-galactosidase activity (Table 4.2). This level of repression was greater than that produced by the control construct. It has been reported that 2-fold repression indicates >50% operator occupancy by the bait. Other baits tested gave no detectable activity but extension of incubation times in β-gal assays may have allowed detection of lower levels of activity.

4.3.4.3 Western immunoblotting.

It is generally recommended to assay for the production of full-length LexA fusions as occasionally some fusion proteins will be proteolytically cleaved by endogenous yeast proteases. Extracts from yeast cells harbouring the bait plasmid are usually immunoblotted with either an antibody to LexA or one specific to the protein fused to LexA to detect a protein of the expected molecular weight (Golemis *et al*, 1994). As no anti-EBNA3B antisera were available, a rabbit polyclonal anti-LexA antiserum (a gift from Dr. Luke O'Neill, University of Dublin) was used. Full-length LexA-525 and

LexA-311 baits were expected to appear as 57 and 81 kd proteins respectively. Although the correct size proteins could not be confirmed by Western blot, dot blot analysis was used to detect the expression of LexA protein in each bait (not shown). EGY48 containing pSH18-34/pSH17-4 was included as positive control, for expression of LexA protein while empty EGY48 was used as negative control. These results did not confirm that full-length fusion proteins were being expressed but did provide evidence of the expression of LexA proteins in the sample preparations, and confirmed the capacity of the detection system to detect LexA.

4.3.5 Interactor hunt

As baits were deemed likely to be suitable for use in a library screen, an interactor hunt was initiated using the pLEX-3B525 construct as bait. Selection of positive interactors using this system was based on detection of clones which exhibited galactose-dependent growth on medium lacking leucine, and galactose-dependent β-galactosidase activity. To this end, a two-step approach for selection was employed whereby library transformants were first harvested and frozen, before plating for selection of interactors. Thus, the first step involved introducing the library (Trp+) into the EGY48 selection strain which contained the reporter plasmid, pSH18-34 (Ura+) and the bait plasmid, pLEX-3B-525 (His+). As a much higher transformation efficiency was required here than for the transactivation and repression assays, an alternative high efficiency protocol was used. Library transformants were then selected on plates containing Leu+ medium with glucose as the carbon source (YNB/Glu-U-H-T+L) (Step 1). After 4 days growth at 30°C, transformants were then harvested, washed, and stored as glycerol stocks at -80°C in a total of 2 ml media.

Plating efficiency was determined in order to estimate an appropriate amount of stock to plate when screening for positive interactors. Using the two-step approach, plating efficiency was determined after the first plating by preparing a series of dilutions of transformants in galactose-containing Leu+ medium. In this way, plating efficiency was estimated to be in the region of 5 x 10^6 CFUs/100 µl frozen cells. Transformation

efficiency was calculated to be approximately 2.5 x 10^6 CFUs/μg DNA. Having determined plating efficiency, transformants could then be plated, as required, on media lacking leucine (YNB/gal-U-H-T-L) (Step 2). Growth in galactose-containing medium was carried out to induce expression of the cDNA-encoded proteins. Thus, primary isolation of putative interactors was based on detection of any interactions which transactivated the *leu* reporter gene. A small-scale plating allowed a rough estimation of the total number of putative interactors i.e. ~4% of stock gave a total of 51 colonies on Leu- plates containing galactose as carbon source. This result corresponds to a figure in the region of 1200 putative interactors in the total stock of library transformants.

In the two-step method, transformants isolated on Leu- medium in the initial plating must be screened for false positives by examining the Leu2 and lacZ phenotypes of the interactors. Growth on media lacking leucine must be shown to be galactose-dependent and the transformants must also exhibit galactose-dependent β-galactosidase activity. Firstly, the Leu+ yeast were streaked for single colonies to isolate them from contaminating Leu- yeast that were present when the Leu+ colony was forming. These were then patched onto a glucose master plate, from which four new replica plates were made in order to test for lacZ expression and galactose dependence. This phenotyping required two leu- plates and two X-gal plates; one Leu plate and one X-gal plate contain galactose to induce cDNA expression (plus raffinose to enhance growth), while the other leu- plate and X-gal plate contained glucose to repress cDNA expression. It was necessary to grow the yeast on glucose master plates to shut off cDNA expression before replica plating because galactose-dependence of Leu+ and lacZ+ phenotypes may sometimes be masked if there is sufficient message and protein product from the activation tagged cDNA protein to allow the yeast to grow on leu- glucose for several generations and turn blue on glucose X-gal without further cDNA expression. Despite this step, however, when Leu positive clones in this study were isolated and tested for galactose dependence, no clones appeared to exhibit galactose-dependence, that is, all clones grew on in the presence of either glucose or galactose. Thus, a summary of the interactor hunt results is represented in Table 4.3 below.

	Transformants	Leu +	Gal-dependent Leu+
No. of cDNA library clones	1×10^8	1200	-

Table 4.3 Summary of YTHS-A interactor hunt results.

Normally, yeast that grow on Leu-/galactose but not on Leu-/glucose medium, and that turn blue on galactose X-gal plates but remain white on glucose X-gal plates (i.e. those that are galactose-dependent Leu+ and lacZ+) are picked for further characterisation.

This type of false positive result has been previously documented and appears to occur with certain baits for reasons that aren't completely understood (Brent, 1994). One possible explanation is the presence of a Leu+ contaminant, but this is probably unlikely to account for all of the false positives. A number of investigations into this result were subsequently carried out. Yeast miniprep DNA was used in PCR reactions to amplify cDNAs from a random sample of 10 clones using primers complementary to the regions flanking the site of insertion of cDNAs (BCO1 and BCO2, see section 2.1). No PCR product was observed on agarose gel electrophoresis which indicates either a technical problem with the PCR reaction (a positive control for these primers was not available) or perhaps a leu-positive contaminant. Library DNA was investigated in two ways. Firstly, a sample of library DNA was used in PCR reactions as above. Secondly, library DNA was digested with *Xho*1/*Eco*R1 in order to excise the cDNA inserts. Unexpectedly, results of these analyses failed to show an intact library of the anticipated complexity. This implied that reduced quality of library DNA may have contributed to the observed results.

4.4.1 YTHS-B

4.4.1 Strategy

An alternative version of the YTHS was also employed in an attempt to identify proteins which interact with EBNA-3B. As human B lymphocytes are a main cellular target of EBV infection, a cDNA library prepared from the mRNA of EBV-transformed human B lymphoctes (Durfee *et al*, 1993) provided an ideal pool of potential interactors with which to screen EBNA3B. The system presented here represents a modification of a system (Durfee *et al*, 1993) originally developed by Harper *et al* (1993) and differs in a number of ways from YTHS-A outlined in section 4.3. For example, the DNA binding and activation domains are both derived from the yeast Gal4 transcription factor, in pAS (Figure 4.10) and pACT (Figure 4.11), respectively. The various transcription factors used in the different versions of the YTHS have been found to work comparably (Fields and Sternglanz, 1994) and the sensitivity of a particular assay seems to depend mostly on the nature of the DNA binding sites present in the reporter gene and the level of production of the hybrid protein.

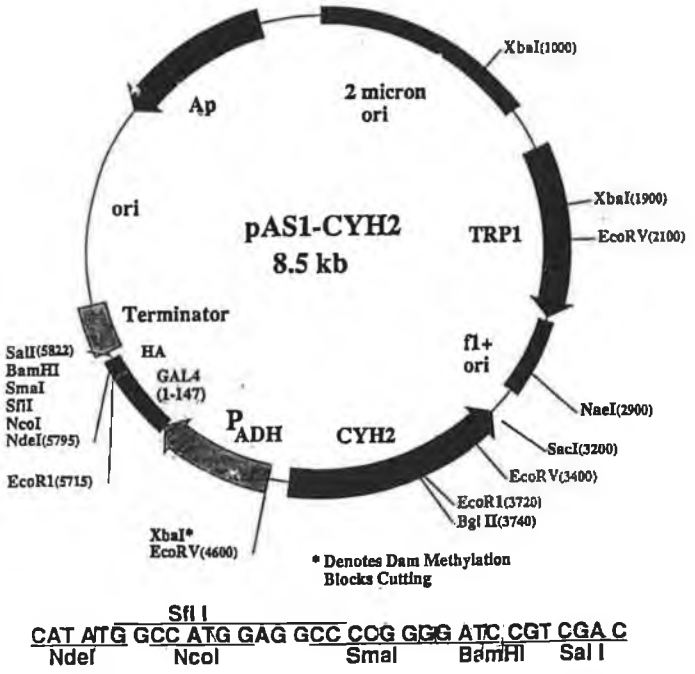


Figure 4.10 pAS-1 expression vector. pAS-1 is shown, containing TRP1, 2 μm origin, and the ADC1 promoter driving expression of the Gal4 DBD (aa1-147, Keegan *et al*, 1986) fused to a polylinker. The Gal4 derivative is tagged with the HA epitope and the polylinker contains several useful cloning sites for insertion of coding sequences.

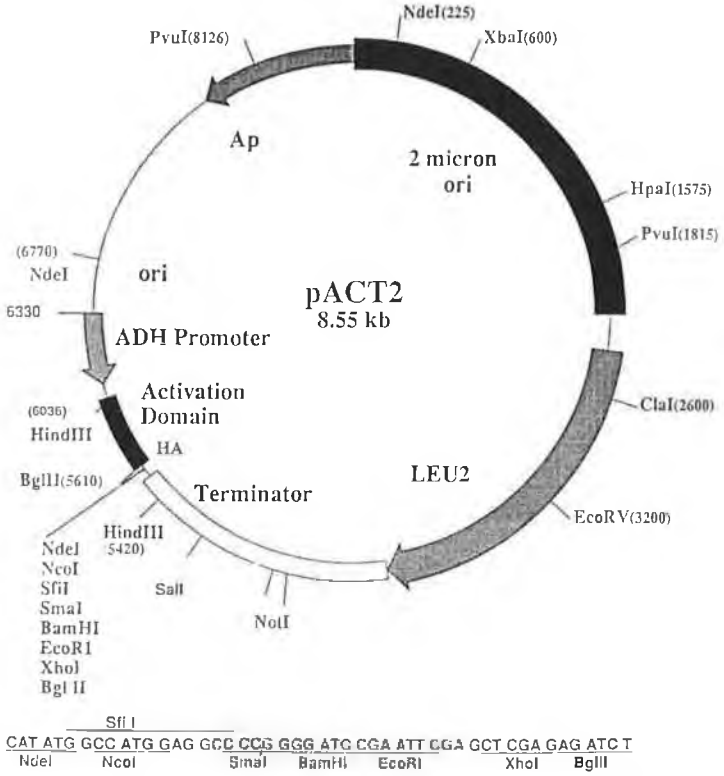


Figure 4.11 pACT library plasmid. pACT contains the ColE1 origin of replication and *bla* gene for replication and selection in *E.coli*, and *LEU2*, 2 μm origin, and the ADC1 (Adh1) promoter sequences for selection, replication and expression in yeast. The ADC promoter drives expression of a hybrid protein consisting of the SV40 large T antigen nuclear localisation signal and sequences encoding the AD II of Gal4.

The yeast strain utilised, Y187, carries two chromosomally located reporter genes whose expression is regulated by Gal4. Firstly, the *E.coli lacZ* gene under the control of the GAL1 promoter (Fields and Song, 1989) and secondly, the selectable *HIS3* gene, where the *HIS3* regulatory sequences have been replaced by the GAL1 UAS_G, to allow Gal4 control. Together, the two reporter genes provide a highly sensitive dual selection system. Because Y187 is deleted for *gal4* (and its negative regulator *gal80*), expression of both reporters should be off in the absence of exogenous Gal4. However, the *GAL1-HIS3* fusion allows production of a low constitutive level of *HIS3* sufficient to allow

growth without exogenous histidine, even in the absence of Gal4. This can be overcome by growing cells in the presence of 3-aminotriazole (3-AT), a chemical inhibitor of IGP dehydratase, which restores histidine auxotrophy (Kishore and Shah, 1988). Thus, incorporation of 25-50 mM 3-AT into the growth media can be used to inhibit the basal level of *HIS3*, preventing growth unless *GAL1-HIS3* expression is activated. The low requirement for His3 protein makes this selection very sensitive such that proteins that only weakly interact can be selected. In order to test the efficacy of the His selection system, SNF1 and SNF4, two proteins known to physically interact in vivo, and whose interaction had previously been detected using the YTH system, were employed (Fields and Song, 1989). Surprisingly, the *HIS3* transcription produced by the *SNF1-SNF4* interaction provided more resistance to 3-AT than the wild-type *HIS3* gene itself. This indicated the potential for interacting hybrids to increase His3 expression above wild type levels, and thus provides a more sensitive selection. Finally, expression of the library cDNA is under the control of the constitutive ADH promoter, in contrast to the conditional galactose-dependent promoter used in YTHS-A.

4.4.2 Preparation of bait constructs

The EBNA3B N-terminal fragment (1575 bp) which was amplified by PCR as per YTHS-A was subcloned into the 8.5 kb pAS-1 bait plasmid at the *Bam*H1 site according to DNA methods described in section 2.3. Due to the presence of an *Nco*1 site in the pACT MCS, a shorter EBNA-3B fragment which lacks the repressor domain could not be derived by enzyme restriction as for YTHS-A. Thus, an 837 bp fragment was amplified by PCR (Figure 4.12). PCR reaction conditions and primer sequences are given in sections 2.1 and 2.3.

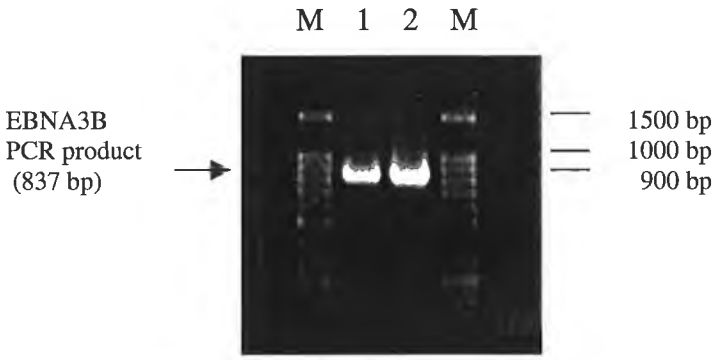


Figure 4.12 EBNA-3B279 PCR product (1.5 % agarose gel).. Lane M: 100 bp DNA markers; lanes 1 and 2: EBNA-3B279 PCR product (837 bp). Arrow indicates PCR product, dashes indicate DNA size markers.

Figures 4.13 and 4.14 show an example of pAS-1/EBNA-3B279 and pAS-1/EBNA-3B525 recombinants, respectively, with inserts in the correct and incorrect orientations.

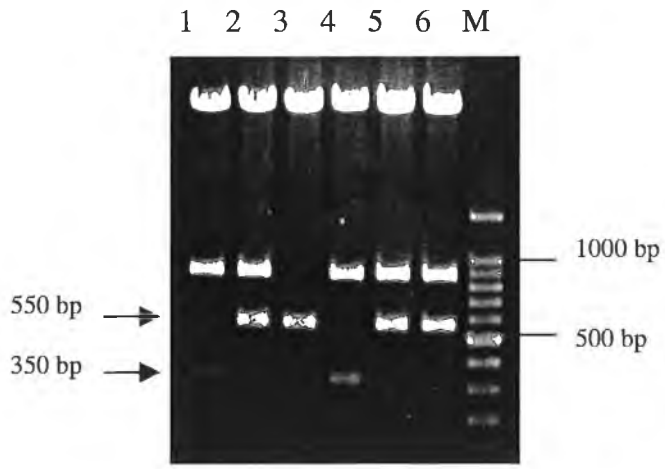


Figure 4.13 pAS-3B-279 orientation in pAS. Digestion of recombinant colonies with *Bst*EII/*Sal*1 give correctly orientated EBNA-3B in lanes 1 and 4 (i.e. 350 bp fragment). Lanes 2, 3, 5 and 6 contain insert but in the wrong orientatinon (i.e. 550 bp fragment). Arrows indicate DNA digest products; dashes indicate DNA size markers.

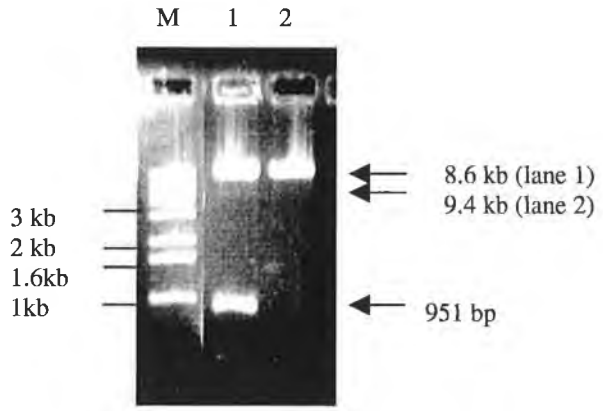


Figure 4.14 pAS-3B-525 orientation in pAS. Digestion of 2 recombinant colonies with *Nco*1 Results show correctly orientated EBNA-3B in lane 2, while clone 1 is incorrectly oriented (see *Nco*1 sites, Figure 4.8A). Arrows indicate DNA digest products; dashes indicate DNA size markers.

4.4.3 Characterisation of baits

The pAS-1/EBNA-3B525 (pAS-3B-525) construct was tested to ensure its suitability as bait in this YTH system. Firstly, it was important to show that baits did not transactivate either of the reporter genes in the absence of library DNA and secondly, the ability to express full-length fusion protein in yeast strain Y187 was confirmed.

4.4.3.1 Transcription activation assay

Constructs which activate transcription in the absence of other constructs cannot be used in the yeast two hybrid assay. For this reason, Y187 strains containing EBNA-3B bait were checked for their growth properties on His- plates containing 3-AT (3-aminotriazole, Sigma, A8056) and for their ability to activate the *lacZ* reporter. These tests were carried out relative to strains carrying pSE1112 (*SNF1* fused to the DNA-binding domain of Gal4 in pAS-1) alone, a bait which is known not to transactivate alone and thus served as negative control. pAS-1 alone is not a good negative control as it can activate *lacZ* weakly. This appears to be lost when genes are cloned into it. It is not understood why pAS-1 is weakly activating alone, but it is likely that it is due to

sequences beyond the polylinker and which are of no consequence once cDNAs are cloned into it. pSE1112 is a better negative control. pSE111/pSE1112 was used as positive control, where pSE111 is the pACT plasmid containing a fusion between *SNF4* and the *Gal4* activation domain. *SNF1* and *SNF4* are known to interact in this yeast strain, thus causing transactivation of both *HIS3* and *lacZ* genes.

Usually 3-AT concentrations of 25 mM to 50 mM are sufficient to select against pAS-1 subclones that fail to activate transcription of the *HIS3* gene on their own. The positive control was observed to give stronger growth at 25 mM 3-AT than on plates containing 50 mM 3-AT. The pAS-3B525 construct failed to grow in the presence of either 50mM or the more transactivation-sensitive 25 mM 3-AT (Table 4.4). X-gal filter lift assays were used to assess transactivation of the *lacZ* gene. Positive control clones produced a positive blue colour within 30 mins, while clones expressing bait protein were found to be negative even after overnight incubation.

	(A) *HIS* Transactivation		**(B) *LacZ* Transactivation**		
pBait	**25mM 3-AT**	**50mM 3-AT**	**30 mins**	**60 mins**	**Overnight**
pSE1111/ pSE1112	+++	++	+	++	+++
pSE1112	-	-	+/-	+	+
pAS-3B	-	-	-	-	-

Table 4.4 Transactivation assay – YTHS-B.

Plasmids used to transform Y187 (given in left-most column) were initially selected on YNB/Glu-T+L+H media. **(A)** Colonies were then firstly streaked onto the indicated media lacking histidine (His) and containing either 25mM or 50 mM 3-aminotriazole (3-AT) (-): no growth; (+): moderate growth; (++): good growth; (+++): very good growth. **(B)** Secondly, colonies from the original plate were patched onto similar YNB/Glu-T+L+H plates and used in X-gal filter lift assays which were left at room temperature for the indicated periods of time. (-): white yeast patches; (+/-): very pale blue colour; (+): pale blue; (++): blue; (+++): intense blue staining. Growth in each case was for 3 days at 30°C.

4.4.3.2 Western immunoblotting

Western immunoblotting was performed in order to detect expression of full-length bait fusion protein. Each bait was transformed into yeast strain Y187 and selected on YNB/Glu-T+L+H plates. The pAS-1 plasmid contains a haemagglutinin (HA) epitope upstream of the multiple cloning site, which is useful for verifying expression of the fusion protein. As specific antisera to EBNA3B were not available, expression of the EBNA3B fusion proteins in pAS-1 were verified by Western blot analysis of yeast protein lysates using anti-HA antibodies (Boehringer Mannheim). Expression of an approximately 60 kD protein was detected, which corresponded to the predicted molecular weight of the Gal4DBD-EBNA3B-525 fusion protein (Figure 4.15). Some smaller bands were also detected of approximately 40 kD and 28 kD, which may represent degradatory products or some incompletely synthesised proteins. Also a band of approximately 120 kD may represent fusion-protein dimers as EBNA3B is known to self-associate. However, it is unlikely that the association is SDS-resistant. Y187 yeast cells containing no foreign DNA were also assayed using HA-antisera in order to rule out non-specific staining due to endogenous yeast proteins. No bands were detected using these cells.

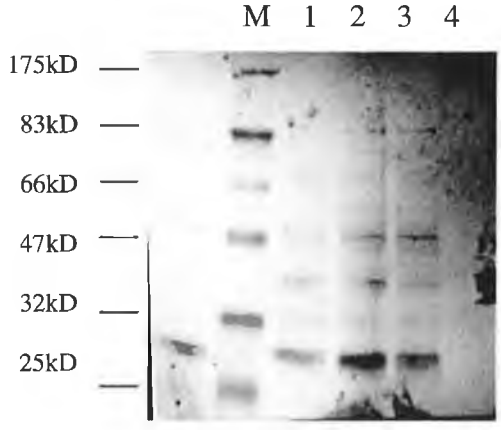


Figure 4.15 Western blot analysis of expression of EBNA-3B fusion protein.

Y187 cells were induced to take up pAS-3B525 using a lithium acetate transformation protocol. The presence of pAS1-3B525 was confirmed by selection on media lacking tryptophan for 3 days at 30°C. Protein extracts were then prepared from yeast colonies (lanes 1-3), fractionated on 10% SDS-PAGE gels, transferred to nitrocellulose and probed with anti-HA antibodies. Mock transformed Y187 cells are shown in lane 4 as negative control.

4.4.4 Detection of interaction of EBNA3B/EBNA2 with RBP-Jk using YTHS-B.

An interaction assay was performed which indicated that the EBNA-3B protein expressed from pAS-3B-525 was functionally intact. YTHS-B was used to investigate the interaction between EBNA-3B and RBP-Jk, using both pAS-3B-525 and pAS-3B-279. The interaction between EBNA2 and RBP-Jk previously detected using this system was included as positive control in these assays. pGBT9-EBNA2 is a yeast expression vector for a fusion protein between EBNA2 (deleted for AD aa 437-472) and Gal4 DBD, while pGAD-RBP expresses a fusion protein between the Gal4 AD and RBP-Jk (aa-487) (both gifts from Evelyn Manet, Ecole Normale Superieure de Lyon). Using 25 mM 3-AT, as before, transformation of pAS-3B-525 and pGAD-RBP in Y187 was found to weakly transactivate the His reporter, while no significant transactivation of His was observed using pAS-3B-279. X-gal filter lift assays were also performed to investigate transactivation of the β-galactosidase reporter, and were consistent with the screen for His transactivation. pAS-3B-525 was found to induce *lacZ* transactivation as efficiently the EBNA2 control. All assays were carried out in duplicate. Results are summarised in Table 4.5 below. These results also suggested that the EBNA3 repressor domain (aa 280-525) included in this bait construct did not prevent transactivation of the reporter genes.

	His +	LacZ+
EBNA-2 / RBP-Jk	++	++
EBNA-3B525 / RBP-Jk	+	++
EBNA-3B279 / RBP-Jk	-	-

Table 4.5 EBNA3B interactions with RBP-Jk. Y187 yeast cells transformed with the indicated plasmids were initially selected on YNB/Glu-T+L+H media. Colonies were then firstly streaked onto the indicated media lacking histidine (His) and containing 25mM 3-AT. (-): no growth; (+): moderate growth; (++): good growth. Secondly, colonies from the original plate were patched onto similar YNB/Glu-T+L+H plates and used in X-gal filter lift assays which were incubated at room temperature. (-): white; (++): blue.

4.4.5 Interactor hunt

The bait and library plasmids can be introduced into yeast either simultaneously or sequentially. Simultaneous transformation has the disadvantage of a lower efficiency of plasmid uptake. Thus, sequential transformation was employed as there was no selective advantage to cells expressing the bait hybrid protein. Y187 clones containing bait plasmid pAS-3B525 were transformed with 40ug EBV-transformed B lymphocyte cDNA library in pACT (Durfee *et al*, 1993) and selected on YNB/Glu-T-L+H plates. The library contains 1.1 x 10^8 total recombinants with >95% inserts. A very high transformation efficiency was required at this stage, so it was important to use an optimised library transformation protocol (modified from Gietz *et al*, 1992) in order to obtain a sufficiently high number of transformants to screen.

Determination of the total number of transformants **:** Transformed cells sampled just after the heat shock step were plated at a range of dilutions (10^{-1}, 10^{-2}, 10^{-3}) on YNB/Glu-T-L+H and incubated at 30°C for 3 days. These results indicated that a total of approximately 100,000 transformants were placed under selection.

Recovery : cells were pooled and added to 100 mls YNB/Glu-T-L-H liquid media and incubated with shaking at 30°C for 1-3 hours. This allowed the transformants to be

established and *HIS3* transcription to be activated. Also, cells in PEG are more fragile and often die when pelleted so the recovery step was useful.

Harvesting of cells **:** cells were spun at 3000 rpm for 4 min and resuspended in 6mls YNB/Glu-T-L-H liquid media. 300 µl cells were spread on YNB/Glu-T-L-H + 25 mM 3-AT media per 150 mm plate.

Storage of transformed cells : cells not plated were aliquoted into 1ml aliquots, spun at 3000 rpm for 4 min, resuspended in 10% DMSO and frozen at –80°C. Cells can be stored frozen indefinitely, and screened as required, once plating efficiency has been estimated.

Determination of plating efficiency : The purpose of this step is to determine the optimal density at which recovered yeast can be plated for screening/selection after thawing from DMSO stocks. Cells can lose some degree of viability when stored at –80°C. To this end, 5 µl neat cells plus dilutions (10^{-1}, 10^{-2}, 10^{-3}) were plated before and after recovery on YNB/Glu-T-L+H plates.

4.4.6 Selection of Interactors

Library-transformed yeast was selected for His+ transformants by plating on YNB/Glu-T-L-H +25mM 3-AT media. As bait plasmid had not activated transcription in the presence of 25 mM 3-AT, the same concentration of 3-AT was used in the selection of His+ clones to increase sensitivity. Selection in the presence of 25 mM 3-AT sometimes behaves like a 100-fold enrichment (Durfee *et al*, 1993; Harper *et al*, 1993) of the total L+T+ colonies. Approximately 1% of transformants grew under these conditions within 3-5 days. However, some of these were microcolonies which failed to grow when streaked on L-T- plates, and thus were ignored. Other reports have indicated that in most cases true positives continue to grow into large colonies while the micro-colonies seem to stop growing. It has been found that the secondary screen for lacZ positive clones usually eliminates these microcolonies. It is likely that the use of higher 3-AT levels would have reduced much of this background; however a lower level of 3-AT was chosen so that weaker interactions would also be detected.

In all, 869 selected colonies were tested for β-galactosidase activity using the X-gal filter lift assay. Of these, four clones were found to be both His+ and lacZ +, and each clone was noted to have given quite large colonies on the original His selection plates. pSE1112 was co-transected with pSE1111 as a positive control for X-gal staining and for 3-AT resistance. pSE1112 was transformed alone as a negative control. Thus, His+, blue colonies were considered positives in the initial screen and were used for additional studies.

4.4.7 Elimination of bait plasmid

For the purpose of verifying the specificity of interactions, it was necessary to eliminate pBait from the positive yeast isolates. As this step generated a strain that contains only the library plasmid, it facilitated plasmid recovery into bacteria which was necessary for more detailed analysis of clones. This step can typically present problems and sometimes different approaches need to be taken. In this case, 3 alternative methods were used before the bait plasmid was finally lost from positive clones.

Method 1 Cycloheximide selection.

Yeast strain Y187 is a derivative of Y153 which is resistant to cycloheximide (2.5 μg/ml) due to a mutation in the *CYH2* gene. This is a recessive drug resistance. The pAS-1 bait plasmid carries the wild type *CYH2* gene which renders cells sensitive to cycloheximide. Thus, loss of the pAS-1-*CYH* plasmid can be achieved by streaking on Leu- media containing 2.5 μg/ml cycloheximide. This may be done directly but it is useful to streak the colonies out on Leu- media before streaking on cycloheximide media to allow plasmid loss and dilution of the *CYH2* gene product. The colonies that grow should be Trp- but to be safe and avoid *CYH2* gene conversion events, it is recommended that loss of the Trp marker is confirmed. However, when this approach was attempted, clones failed to grow in the presence of 2.5 μg/ml CYH. It is possible that perhaps cells needed to be grown for longer on Leu – media in order to dilute the *CYH2* gene product.

Method 2 Plasmid loss on Leu- plates.

Cells were grown overnight in YPD complete liquid media, then grown on Leu- plates, which were then replica plated onto Trp- media to look for loss of the pAS-1 bait plasmid. All clones grew on media lacking Trp, indicating that bait plasmid had been retained.

Method 3 Plasmid loss in Leu- media.

Cells were used to inoculate 5 ml Leu- liquid media and were grown at 30°C for 3 days. DNA was then prepared using a rapid DNA isolation method (described in section 2.8) and used to transform *E.coli DH5α* from which plasmid DNA was prepared by miniprep. Restriction enzyme analysis was then used to check for loss of pBait. Each clone preparation was restricted with *Bam*H1, which should excise a 1.6 kb EBNA-3B525 insert from pAS-1, if pBait was present. An empty pAS vector was included in the digests to control for restriction conditions. As can be seen from figure 4.16, a single band of approximately 8-9 kb was detected in all clones tested. This band probably represents linearised pACT (8.55 kb) which also contains a single *Bam*H1 site. Thus, all clones have apparently lost pBait.

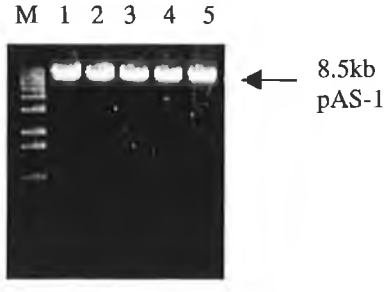


Figure 4.16 Enzyme restriction analysis to detect loss of pBait from positive interactor clones. *Bam*H1 restriction of clones following loss of pAS-3B525 bait plasmid, using methods described above (section 4.4.7). Lane 1: 1 kb DNA size markers; lanes 1-5, *Bam*H1-restricted clones.

4.4.8 Verification of specificity

It was important to verify that any interactions which were detected were dependent on activation of reporter genes by the protein of interest and could not be substituted for by alternative unrelated baits. In this way, specificity of interactions could be confirmed and many false positives could be eliminated. Several individual unrelated pre-characterised fusions in pAS1, i.e. pAS1-*SNF1* (pSE1112), pAS1-*CDK2* and pAS1-*lamin* (a gift from Geraldine Butler, UCD), were each co-transformed with each of the library plasmids isolated from positive clones in method 3 above. The resulting transformants were selected on Trp-Leu- plates and tested for β-galactosidase activity. As a positive control, Y187 containing bait plasmid, pAS-3B525, was also transformed with each of the positive library plasmids isolated. Any clones which were found to transactivate lacZ significantly above background levels in the presence of non-related pAS1 fusions were disregarded (Table 4.6A). These experiments indicated that 2 of the original 4 His+ lacZ+ clones (77, 78) involved specific interactions between the bait protein and a library protein. The other two clones represented false positives which were dependent on the presence of both plasmids but which were not activating transcription as a result of an interaction with the cDNA insert in the bait plasmid. Unfortunately such false positives are a reoccurring problem of the two hybrid system. As can be seen from Table 4.6A, clone 125 transactivated *lacZ* in the presence of just one of the three alternative baits. For this reason, it is important to test against several different unrelated bait proteins in ruling out non-specific interactions. A summary of results of the completed screen illustrates that of 100,000 library transformants which were screened, just two cDNAs were found to specifically interact with EBNA-3B525 in Y187 yeast cells (Table 4.6B).

pBait	Clone 9	Clone 77	Clone 78	Clone 125
PAS1-3B525	+	+	+	+
PAS1-Lamin	+	-	-	-
PAS1-SNF1	+	-	-	+
PAS1-CDK2	+	-	-	-

Table 4.6 (A) Verification of specificity of interactors. Transactivation of the *lacZ* reporter gene was detected using X-gal filter lift assays as described in section 2.8 (-): white; (+): blue.

	Transformants	His +	His+, lacZ+	3B-dependent
No. of cDNA library clones	100,000	869	4	2

Table 4.6 (B) Summary of screening results using YTHS-B

4.4.9 Restriction analysis of plasmids containing positive cDNA interactors.

Restriction enzyme analysis was used to determine the approximate sizes of the putative positive interacting cDNAs. Figure 4.17(A) shows that there is less than a 1kb difference between linearised pACT and linearised pACT containing a positive interactor cDNA (clone 77). In the construction of the library, cDNAs were cloned into a unique Xho1 site (Durfee *et al*, 1993). Therefore, in order to determine the size of inserts, they were excised with Xho1 enzyme. Figure 4.17(B) shows that clone 77 contains a cDNA insert of approximately 800 bp, the 200 bp cDNA in clone 78 was not visible on this gel, while digestion of the non-specific clone 125 showed an insert of approximately 550 bp. The other non-specific clone contained a cDNA of approx. 700 bp (not shown).

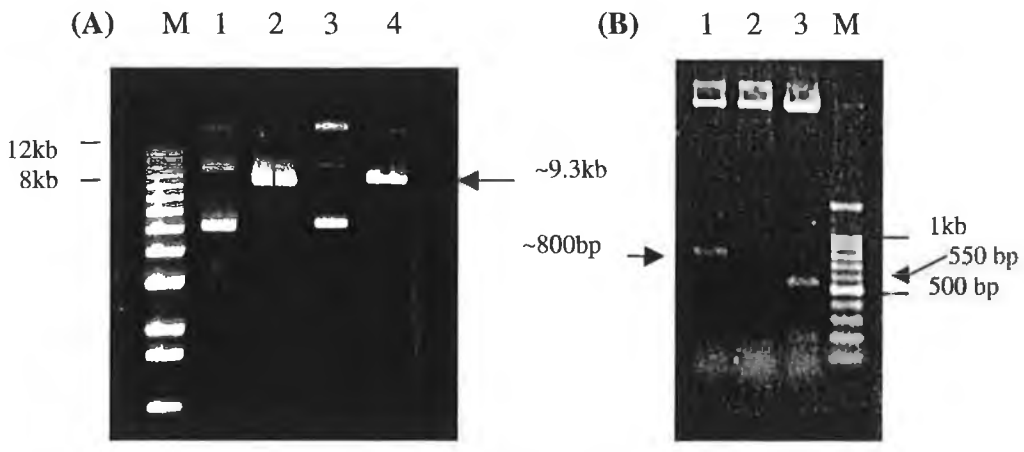


Figure 4.17 Restriction analysis of positive interactors. (A) Lane 1: 1kb DNA size markers; lane 2: pACT; lane 3: *Bam*H1-digested pACT (8.55kb); lane 4: clone 77 (; lane 5: *Bam*H1-digested clone 77. **(B)** Lane 1: *Xho*1-digested clone 77 (~800 bp); lane 2: *Xho*1-digested clone 78; lane 3: *Xho*1-digested clone 125 (~55 bp); lane 4: 100bp DNA size markers.

4.4.10 Sequencing strategy

In order to identify the proteins encoded by the interacting cDNAs, the sequence of each cDNA was determined. To this end, a primer was designed to sequence the cDNA from the activation domain of the pACT plasmid. Use of a T7 sequencing^{TM} kit from Pharmacia as described in section 2.3. failed to produce any sequence from pACT. Therefore, as an alternative approach, cDNA library inserts were subcloned into the Xho1 site of the pTag vector (LigATor MBK-004-40, R&D systems, UK). Insertion of DNA fragments into the MCS of this vector interrupts the lacZ gene which allows the presence of insert to be determined based on white versus blue colonies on media containing X-gal. Use of pTAg permits sequencing using the M13 universal primer. Presence of inserts in white colonies were assessed by restriction analysis i.e. identification of pTAg vectors containing insert was based on loss of the pTAg Xho1 site when Xho1-restricted insert was ligated to the compatible Sal1 site in the pTAg vector. A cDNA which was previously sequenced from pTAg was included as positive control in subsequent sequencing reactions. Despite obtaining a complete sequence from the positive plasmid control, only approx. 40 bases of sequence could be determined from each pTAg plasmid containing library cDNAs. After this short sequence which

corresponded to a portion of the pACT AD, compression (bands in all 4 lanes) was consistently observed, despite variations in protocol (see Table 4.7). Problems with compression can frequently be resolved using adjustments such as these. However, GC-rich regions often corresponding to certain restriction enzymes can also be responsible for poor results. Sometimes Sfi1 or Sma1 sites in the MCS of a plasmid can lead to compression, the only solution for which is to sequence using automated methods. Investigation into the cloning strategy used in the construction of the library revealed an Sfi1 (GGCCTTCG TGGCC) site at the point of compression in the pACT plasmid.

Template	Treatment	Temperature	Enzyme	Result
32 µl	LiCl	37°C	T7	NR
32 µl	LiCl	42°C	T7	NR
64 µl	-	42°C	Taq	NR
32 µl	-	42°C	T7	Compression
32 µl	65°C	42°C	T7	NR
32 µl	DMSO	55°C	T7	Compression
32 µl	DMSO	55°C	T7	NR

Table 4.7 Sequencing of positive interactor cDNAs. A number of variations in the protocol described in section 2.3 were introduced in an attempt to produce sequence data. With higher temperatures increased amounts of T7 enzyme were used.

Sequencing data was generated using commmercially-available automated methods (Oswel Research products, University of Southhampton). Sequences were then analysed using a blast search against DNA databases.

Clone 78 (200 bp cDNA). Database analysis revealed that this clone probably contained "junk" DNA, as it showed closest homology with an E.coli sequence.

Clone 77 represented a fusion of two cDNAs of 200 bp and 600 bp in opposite orientations. In order to obtain sufficient information, this clone was sequenced from both ends of the pACT MCS. Database analysis showed closest homologies as follows:
200 bp cDNA : Human mRNA for proteosome subunit HsN_3 (complete length 925)
600 bp cDNA : Human mRNA for Cyrochrome Oxidase.

On further investigation of pACT sequences, it was found that the correct orientation cDNA was cytochrome oxidase (C.O.). C.O. is a ubiquitous mitochondrial enzyme which has previously been identified as a false positive interactor. Thus, unfortunately, the interaction was meaningless and adds to a growing list of false positive results from the YTHS.

4.5 DISCUSSION

Little is known concerning the functions of the EBNA-3 family of proteins, although the nuclear localisation and predicted structural homologies suggest that they are functionally related (Kerdiles *et al*, 1990.). While a role in the modulation of expression of EBNA2-responsive genes seems likely, much experimental data indicates that other, as yet unidentified, functions exist which probably include activites unique to each member of the EBNA-3 family. To understand the function of a particular protein it is often useful to identify other proteins with which it associates. Using the yeast two hybrid system, this study proposed to investigate protein-protein interactions involving EBNA-3B.

The yeast two hybrid system provides a highly sensitive means to detect an interaction between two proteins in living cells (see section 4.1). In the data presented here, two alternative systems were employed to screen two different cDNA libraries for proteins which may interact with EBNA-3B. To this end, an N-terminal portion of EBNA-3B was expressed as a fusion protein with the relevant DNA binding domain in each case,

and baits were subsequently confirmed as suitable for use in each system. Since the most important consideration was that transcription of reporter genes was not appreciably transactivated by the bait protein, an acidic region of EBNA-3B (including a domain which has been demonstrated in EBNA-3C to behave as an activation domain) was not included in the fragment to be used as bait.

4.5.1 YTHS-A

In the first system described, YTHS-A, the *LEU2* reporter gene in EGY48 is highly sensitive and is activated by even weak transcription activators fused to LexA (or by activation-tagged proteins that interact weakly with LexA fusions.) The high sensitivity is due to 3 high affinity LexA operators from the bacterial *colE1* gene, each of which can potentially bind 2 LexA dimers (Brent *et al*, 1994). If a bait is observed to marginally activate transcription, yeast strains are available which contain fewer operators upstream of *LEU2*. Proteins that are moderate to strong activators will need to be truncated to remove activation domains before they can be used in an interactor hunt (Brent and Ptashne, 1994). If possible, a good way to start is to construct derivatives that lack highly acidic regions which are often responsible for transcription activation in yeast (the obvious disadvantage of this approach is that regions important for interaction with other proteins may be removed).

Despite careful design and testing of bait, only false positive clones were isolated in this study, using YTHS-A. Although significant numbers of Leu+ colonies were observed, none were found to be dependent on galactose. This result implied that activation of *Leu2* expression was not dependent on the expression of library cDNAs, i.e. while bait proteins are constitutively expressed, proteins encoded by library cDNAs are expressed from the yeast conditional *GAL1* promoter. Since the *GAL1* promoter is repressed by glucose, those colonies which grew in glucose medium were classed as false positives, and thus were not further characterised.

In demonstrating galactose-dependence of transactivation of reporter genes, it was necessary to grow the yeast on glucose master plates to shut off cDNA expression before replica plating. Galactose-dependence of Leu+ and lacZ+ phenotypes may sometimes be masked if there is sufficient message and protein product from the activation tagged cDNA protein to allow the yeast to grow on Leu- glucose for several generations and turn blue on glucose X-gal media without further cDNA expression. With regard to selecting colonies for master plate production, generally there will be more galactose-dependent Leu+ and lacZ+ yeast among the colonies that appear sooner and fewer among those that appear later. For stronger interactors, colonies have grown up in two days and are more likely to be interactors that are biologically relevant to the bait protein. Those that appear later may or may not be relevant. However, many parameters can delay the time of colony formation of cells that contain valid interactors, including the strength of the interaction and the level of expression of the library-encoded protein. In the data presented here, Leu-positive colonies were found to appear in 2-4 days incubation. Even those which grew after 2 days were found to be galactose-independent. The reason for these findings is not understood. However, similar results have been documented in other studies and observations such as these appear to be a phenomenon associated with certain baits (Wiley, 1994).

Had galactose-dependent Leu+ clones been isolated, the next step would be to assay for galactose-dependent *lacZ* transactivation. Reporter genes differ in the number and affinity of upstream binding sites (eg. lexA operators) for the bait and in the position of these sites relative to the transcription startpoint (Gyuris *et al*, 1993). They also differ in the number of molecules of the reporter gene product necessary to score the phenotype. These differences affect the strength of the protein interactions the reporters can detect. The second reporter gene used in this version of the method, *lacZ*, is not as sensitive as the *LEU2* reporter in EGY48 (Chien *et al*, 1991; Durfee *et al*, 1993; Gyuris *et al*, 1993; Vojtek *et al*, 1993), so it is possible for a weak interactor not to result in blue colonies on X-gal plates in the *lacZ* transactivation assay. The experiments presented here employed the most sensitive *lacZ* reporter available, pSH18-34 (West *et al*, 1994) (see Table 1). Use of the second reporter gene would normally allow the identification of any false

positives which may have arisen due to a yeast mutation or to binding of the activation-tagged cDNA protein to the *LEU2* promoter (Brent *et al*, 1994).

False positives could also have been due to non-specific interaction with LexA, with the promoters or with some part of the transcription machinery. In practise, the majority of proteins isolated by interaction with a LexA fusion are found to be specific for the fusion domain; very few proteins are isolated that are specific for LexA or that are non-specifically sticky. However, it is generally informative to retest positive clones on more than one LexA bait protein; ideally library-derived clones should be tested against the LexA fusion used for their isolation, several LexA fusions to proteins that are clearly unrelated to the original fusion and, if possible, several LexA fusions that there is reason to believe are related to the initial protein. A rapid genetic test for this has been described (Harper *et al*, 1993). Using this system, library plasmids are rescued from yeast by performing a yeast plasmid miniprep to transform *E. coli* (most yeast miniprep protocols do not provide sufficient clean plasmid DNA for restriction analysis). Thus, if large numbers of positive clones are obtained, it is useful to reduce the number of clones to be rescued by determining which ones contain identical cDNAs. For this purpose, PCR amplification of inserts followed by digested with *Hae*III and *Alu*I (frequent cutter enzymes) may be used to allow comparison of the banding patterns produced on agarose gel electrophoresis.

As part of an investigation into the 100% rate of false positives obtained in this study, a random sample of positive clones were assayed for presence of cDNA insert using PCR amplification. The absence of product indicated that perhaps a Leu-positive contaminant may have contributed to the high numbers of false positives. The cDNA library itself was also used as template in a similar PCR reaction, but again results were negative. A possible PCR technical problem could not be ruled out as no positive control for these primers was available. During construction of the library, cDNA inserts were size selected to give an average insert size of approximately 1400 bp (Brent, 1994). Thus, restriction analysis of the library designed to excise inserts from their vectors should produce a smear of bands around this region, on gel electrophoresis. However, digestion

of library DNA used here, showed no significant banding in this region. It is possible, therefore, that the sub-optimal quality of library DNA may have contributed to the observed results.

4.5.2 YTHS-B

YTHS-B has some disadvantages associated with its use when compared with YTHS-A. For example, because Gal4 is an important yeast transcriptional activator, experiments must be performed in Gal4-negative yeast strains to avoid background from endogenous Gal4 activating the reporter system (Golemis *et al*, 1994). Unfortunately, these strains are frequently less healthy and more difficult to transform than wild-type strains, and either libraries must be constitutively expressed or an alternate inducible system must be used. The library used in this study was expressed from a constitutive ADH promoter. Despite these relatively minor disadvantages, the YTHS-B provided the opportunity to screen a cDNA library which was ideally suited to these investigations i.e. a cDNA library generated from EBV-transformed human B lymphocytes (Durfee *et al*, 1993). As the cDNA library to be used was fused to a Gal4 activation domain (aa768-781, Ma and Ptashne, 1987) in pACT II it was necessary to prepare a bait construct which fused EBNA-3B to a compatible Gal4 DNA binding domain (DBD). To this end, a hybrid was generated between sequences encoding the DBD of the yeast transcription factor Gal4 (aa1-147, Keegan *et al*, 1986) and two amino-terminal portions of EBNA-3B in the pAS1 expression vector (section 4.4.2).

To screen for proteins which interact with EBNA-3B, transformants were subjected to the screening procedure outlined in section 4.4.5. Interacting hybrids were isolated by selecting for growth in the absence of histidine (in media containing 25 mM 3-AT) and subsequently screening for β-galactosidase activity. A relatively low concentration of 3-AT was used to improve sensitivity, although this does produce a greater number of false positives which need to be eliminated by the *lacZ* screen. This secondary screen eliminates His+ revertants and plasmids bearing the *HIS3* gene of the organism from which the library is derived. Thus, using this form of selection, colonies which were

both His+ and blue were considered positives and were isolated for further analysis. An important advantage of the *HIS3* selection/lacZ screen is a large reduction of false positives, i.e from an estimated 100,000 transformants, 869 His+ clones were tested for β galactosidase activity, of which only four were positive. The cDNA library used in this screen was constructed to contain 1.1 x 10^8 total recombinants. Therefore, multiple screens would obviously be required to properly screen the library.

A class of false positives can occur in library screens which seem to depend on the presence of both hybrids, but in the absence of bait plasmid will activate other non-specific fusions bound to a DNA-binding domain. Frequently, these false positives have been identified as transcription factors that are thought to access the promoter DNA adjacent to the target protein when overproduced. For example, in the case of YTHS-B, the *HIS3* and GAL1 promoters share only a small region of DNA sequences in common (150bp) which should mostly be protected by the binding of target fusion proteins. Because of this, use of both screens in the selection process largely eliminated this class of positives. Nonetheless, although the dual His+ selection / β galactosidase screen eliminates many false positives, a low percentage survive the selection at a rate that varies with the target protein (Durfee *et al*, 1993). To help rule out remaining false positives, library plasmids were isolated, by selecting for loss of bait plasmid in the four positive interactor clones and tested against unrelated baits fused to the DBD in pAS-1. Three unrelated baits were employed as the more baits which are used the better the chances of picking up false positives. Of the four His+, βgal+ clones, two of these were found to be specific for interaction with EBNA-3B, that is, *lacZ* was transactivated in the presence of Gal4(1-147) fusions with EBNA-3B but not in the presence of Gal4(1-147) fusions with other non-specific bait proteins.

Unfortunately, however, when the observed interacting library cDNAs were finally isolated and sequenced, neither was found to be biologically relevant. Based on sequence analysis, one cDNA represented *E. coli* DNA, which is most likely to have been incorporated during the preparation of the cDNA library. The other clone contained two cDNAs in opposite orientations, of which the correctly-oriented cDNA encoded the

mitochondrial enzyme, cytochrome oxidase (C.O.) C.O. has been documented as a relatively common false positive of the YTHS. For example, in one survey C.O. was responsible for 5 out of 73 false positive interactions observed (Hengen, 1997).

4.5.3 True and False positives

It has become increasingly evident that the problem of false positives among YTH users is widespread. A recent review revealed that in addition to C.O., the other most common false positives included other mitochondrial proteins, heat shock proteins, ribosomal proteins, proteasome subunits, ferritin, transfer-RNA synthase, collagen-related proteins, zinc finger-containing proteins, vimentin, inorganic pyrophosphatase and proliferating cell nuclear antigen (PCNA) (Hengen, 1997). These findings indicate that many of these false positive interactions involve proteins which are ubiquitous in the life of the cell. Thus, it may also be interesting to sequence the two non-specific interactors which had transactivated both reporter genes (clones 9 and 125). In any given interactor hunt, baits which binds to one or a small subset of related proteins have been found to be more likely to be biologically relevant than baits which interact with many unrelated proteins.

It is important to note that interaction of the target and library-encoded proteins in the YTHS does not necessarily indicate that they normally interact in vivo. While two interacting proteins may produce a signal, they may never normally be present in the same cell type, or cellular compartment or present during the same stage of the cell cycle. Similarly, the YTHS may assay an interaction between domains that are not accessible in the native protein, particularly when an interaction is mediated via a short sequence. To rule out such potentially misleading results, it is essential that any positive signals from this and other interaction assays are confirmed by independent biological or biochemical experiments. This doctrine is supported by a survey of 223 investigators conducted by Ilya Serebriiskii (ilya@scfuzzy.rm.fcc.edu) between October 1994 and January 1996 which revealed that of 100 library searches only 54 revealed biologically relevant interactors, with only 4 reporting that no false positives were found and 13 reporting that no preys worthy of further of investigation were found. It is apparent that

many artifacts exist, thus common practise prescribes that a proposed interaction should be observed by a different technique, such as co-immunoprecipitation of the putative interactors from the appropriate cell or tissue type, which at least may be subject to different artifacts. It is possible to waste a considerable amount of time characterising a cloned gene before realising that the "positive interactor" is a false lead. For this reason, YTH users are recommended to choose the bait very carefully and where possible to use two different baits. Also multiple baiting attempts are recommended with plentiful use of controls.

Once the appropriate measures have been taken to eliminate false positives, the interaction may then be further characterised. The domains/amino acids critical for the interaction can be delineated using the YTHS by carrying out deletion mutations. Further studies are required to determine biological significance and biophysical characteristics of any positive reactions. No YTH technique allows precise quantitation, however some quantitative information does inhere in the data. For example, an idea of the strength of interaction of two proteins may be derived from measuring β-galactosidase activity. This is because the amount of β-gal activity in the cell is proportional to the level of *lacZ* transcription.

4.5.4 True and False negatives

A further problem of the YTHS which is usually given less consideration than the problem of false positives is that of interactions which are not detected. The YTHS is highly sensitive such that even very weak associations should be detected (i.e. any affinity tighter than 10^{-6}). Nonetheless, not all known interactions will be detected by the YTHS. Despite deliberately retaining the RBP-Jk –binding domain in the design of the EBNA-3B bait, this interaction was not detected in library screens. However, the specific interaction of these two proteins was demonstrated using a fusion between a portion of the RBP-Jk gene and the Gal4 A.D in subsequent experiments (see section 4.4.4). In addition, EBNA-3B/EBNA-3B dimerisation may potentially have been detected. On some occasions, the expected interacting protein may simply be absent

from the library used. Using the WI-38 library, it was less likely that relevant specific interactors would be detected than using the library prepared from EBV-transformed B lymphocytes. A number of cases have been documented in which known interactions are either not observed, or are subject to directionality, being observed only when one of the two proteins is a bait and the other a prey (see for example, Estojak *et al*, 1995). The current doctrine for determining that individual interactions do not occur is that full length and truncated putative partners must be tested in all combinations of baits and preys, with the most sensitive reporters, before the investigator can tentatively conclude that the two proteins do not touch. Thus, under the conditions employed in these analyses, it is impossible to rule out false negatives.

4.5.5 The Future of YTH technology

As systems improve, it is likely that many of the false positives highlighted above will be preventable. For example, the potential exists for development of systems in which transcription depends upon protein interactions which occur only at specific phases of the cell cycle, or times during development, or in particular subcellular compartments, or that persist for a restricted length of time, or that depend on particular protein modifications (Brent *et al.*, 1994). In any case, despite it's problems and pitfalls, the YTHS can be a valuable tool in the detection and characterisation of protein-protein interactions.

In recent years, the potential uses for YTH technology have grown with various modifications implying broader applications. For example, the reverse two hybrid system is a modification which enables genetic selection against specific protein/protein interactions (Leanna and Hannink, 1996). This system fulfills a role which is deficient in standard YTH systems in that it can be used to identify mutant proteins that have lost the ability to associate with their partner protein. Furthermore, such a selection scheme might facilitate the identification of genes which encode proteins that interfere with a particular protein/protein complex, for instance, regulators of protein/protein

interactions. Finally, the reverse two hybrid system could be used to screen for drugs that abolish a specific protein/protein interaction.

It has been proposed to apply YTH technology to generate a human protein linkage map (Hua *et al.*, 1998). With a homologous recombination-mediated approach, a modular human expression sequence tag (EST)-derived YTH library in the pACT2 vector has been constructed. This technology provides the extraordinary potential for identification of all human protein-protein interactions, leading to a global human protein linkage map that hopefully will provide important information for functional genomic studies.

The few years since the advent of two-hybrid systems has proven their utility in the study of defined protein interactions, in identification of new interacting proteins, and in the charting of genetic networks of proteins involved in processes from signal transduction to transcription regulation. These tremendous successes suggest that two-hybrid approaches may eventually be used to identify all of the protein-protein contacts made in a cell or an organism.

4.6 GENERATION OF A STABLE CELL LINE IN DG75tTA CELLS

4.6.1 Introduction

In order to study the function of a particular gene, it is often useful to work with a homogeneous population of cells in which expression of the protein of interest is under the control of a regulatable promoter. The aim of this work was to generate a stable derivative of the DG75-tTA cell line in which EBNA-3A expression (as sole EBV protein) would be inducible in a tetracycline-regulated manner. The principal of this gene expression system has been described in section 3.1.8. This approach to the study of gene expression presented several important advantages over relevant alternative methods. For example, the tetracycline (tet)-inducible system guarantees expression of EBNA-3A in every cell, allowing the study of effects on the cell population as a whole. In contrast, transient transfections of B lymphocytes rarely produce efficiencies greater than a few percent, which makes it difficult to look for changes in endogenous cellular gene expression due to expression of a transfected gene. Use of this system eliminates selection for unrepresentative clones which have a growth or survival advantage. This might be particularly relevant in the case of genes associated with the cell cycle or resistance to apoptosis eg. previously published data has been informally criticised on these grounds, with regard to upregulation of Bcl-2 expression by EBNA-3B. Tet-inducible cell lines would permit careful analyses to be carried out in the presence/absence of EBNA-3A in an isogenic background. This approach also makes it possible to study the effects of gene expression over a longer time period than with a transient transfection system. Unlike alternative inducible expression systems, transcription of the heterologous gene is tightly repressed by low, non-toxic concentrations of tetracycline and substantially induced upon removal of drug. This has the advantage of minimal, non-specific pleiotrophic effects on the host cell (reviewed by Schockett and Scatz, 1996).

4.6.2 RESULTS

4.6.2.1 Preparation of pJef-EBNA3 expression constructs.

Constructs were prepared for all three EBNA-3 genes in pJef4, which contains the tTA-responsive element (see section 3.1.8, Figure 3.2 for principal). Thus, pJef-3A, pJef-3B and pJef-3C were prepared as follows: the full-length EBNA-3A (3025 bp), EBNA-3B (3106 bp) and EBNA–3C (3391 bp) genes were excised from pCMV7-EBNA3, pCMV7-EBNA4 and pCMV7-EBNA6 respectively and inserted into the *Bam*H1 site of pJef4, by ligation of *Bam*H1 linkers to each of the EBNA3 genes. This placed expression of each EBNA-3 gene under the control of the tTA-responsive promoter. Each construct was subsequently assessed by restriction enzyme analysis of DNA (Figure 4.18A), and by Western blot analysis for expression of the protein of expected molecular weight (Figure 4.18B). Specific anti-EBNA3A antibodies (supernatant of T2.78 hybridoma cells) and anti-EBNA3C antibodies (E3C.A10.3) (both gifts from Martin Rowe, University of Cardiff) were used to detect protein expression from pJef-3A and pJef-3C, respectively. As no specific antisera was available for EBNA-3B, a panel of 30 high-titre anti-EBNA human sera (a gift from Carol Mongan, UCD) were screened for high-titre anti-EBNA-3B antibodies. B95.8 cell lysates were included as positive control as these cells reliably express high levels of EBNA-3B. However, no antisera were found which showed high reactivity in the absence of background reactivity in the EBNA-3B region. EBNA-3A, -3B and –3C migrate as proteins of molecular weights between 145 and 155kD on SDS-PAGE electrophoresis.

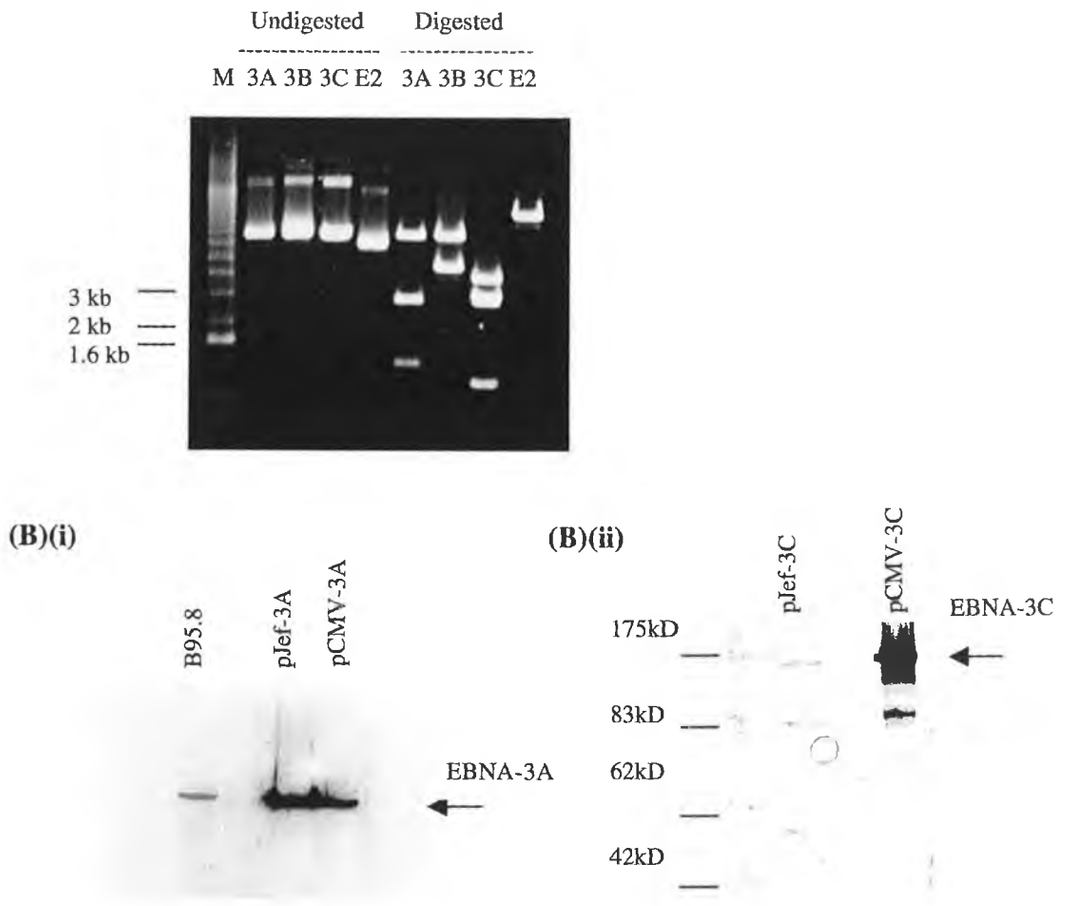


Figure 4.18 Subcloning EBNA-3A, -3B and –3C genes into pJef4. **(A)** DNA agarose gel (1%) for restriction analysis of pJef-EBNA3 recombinant plasmids. **(B)** 8% SDS-PAGE gel electrophoresis of pJef-3A (i) and pJef-3C (ii). Each construct was transiently transfected and grown in tetracycline-free medium for 48 h before harvesting of cells for preparation of protein lysates. Expression of EBNA-3A was detected by ECL, while EBNA-3C expression was detected using an alkaline phosphase conjugate.

Finally, constructs were assessed for functional integrity of the EBNA-3 proteins, based on EBNA3-dependent down-regulation of EBNA2-mediated transactivation (see section 1.5.6). To this end, the LLO-luc construct (a gift from Gerhard Laux, Institut fur

Klinische Molekularbiologie und Tumorgenetik, Munchen) was employed: this plasmid harbours the LMP1 upstream region (+40 to –327) which is inducible by EBNA2. Luciferase assay results, shown in Figure 4.19 below, illustrate an almost 3-fold up-regulation of promoter activity by EBNA-2, which was significantly decreased on addition of pJef-3A or pJef-3B. A relatively low level of transactivation of the LLO plasmid by EBNA2 in DG75 cells is consistent with previous reports (Gerhard Laux, as above, personal communication).

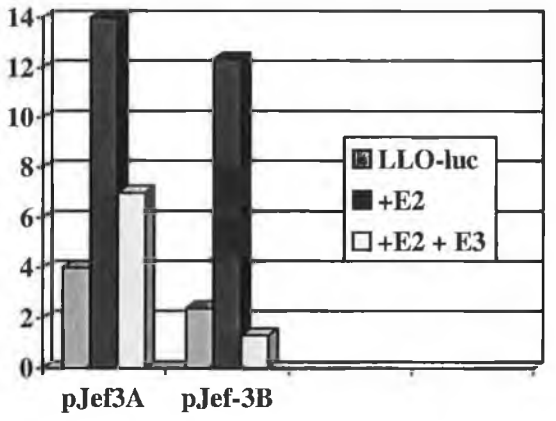


Figure 4.19 Repression of EBNA2-mediated transactivation of the LMP1 promoter by pJef-3A and pJef-3B. DG75 cells were transiently transfected with the indicated plasmids (see section 2.6.6 for methods) to give a total of 30 μg transfected DNA. Cells were harvested after 48 h and used in luciferase assays to determine activity levels from the LLO-luciferase promoter construct. β-galactosidase assays were used to normalise for transfection efficiencies.

4.6.2.1 Stable transfections

pJef-3A was transfected into DG75-tTA cells by electroporation using an optimised protocol for this cell line (Floettmann *et al*, 1996, see section 2.6.6.1). In order to determine the correct concentration of drugs to be used in selection of stably transfected cells, a drug curve was prepared (section 2.6.7.1). A G418 concentration of 2 mg/ml was established for selection of pJef-3A, which was derived from the findings of several independent drug curves. Cells were also maintained in the presence of 500 μg/ml

hygromycin B for selection of the tTA plasmid and 1 μg/ml tetracycline for suppression of the expression of EBNA-3A. A flow chart outlining the main steps used in the generation of the DG75tTA-EBNA3A inducible cell line is given below (Figure 4.20). Clones were assessed for expression of EBNA-3A at 1 week intervals, by withdrawal of tetracycline (as described in section 2.6.5) from a sample of cells. Immunocytochemistry (section 2.6.7.3) was used for this purpose as it was more convenient than Western blotting for screening small numbers of cells from large numbers of clones (results not shown). Clones which were observed to have lost pJef-3A were disgarded, although expression of EBNA-3A protein was found to be detectable up to several weeks post-transfection before being subsequently lost.

Magnetic bead selection (Capture Tec, Invitrogen) was employed as an alternative strategy for selection of cells stably expressing pJef-3A. Using this method according to manufacturers' instructions, DG75-tTA cells were co-transfected with the pHook-1 plasmid, (which expresses a membrane-anchored selection tag, sFv) and pJef-3A. To isolate a homogenous pool of transfected cells, the total cell population was harvested and incubated with magnetic beads that bind to the selection tag displayed on the transfected cells. After exposure to a magnet, bead-bound transfected cells were centrifuged down into a pellet while unbound cells remained in the supernatant and were discarded. Since the pHook-1 sFv tag is expressed from the strong CMV promoter, it offers a very high efficiency selection of pHook-1 vector. Co-transfection of pHook-1 and a vector of interest has previously been found to result in expression of the protein of interest in 95% of selected cells (Chestnut *et al*, 1996). Under microscopic examination, most of the selected cell population were observed to have magnetic beads bound, although it wasn't possible to estimate the fraction of selected cells expressing sFv due to the 2-dimensional viewing of cells by light microscopy. The number of cells that are selected in a particular experiment varies with the transfection efficiency and the ability of the cells to secrete and display the sFv membrane tag, but in general selection efficiencies vary from 2% to 25% in most adherent human cell lines (Mortenson *et al*, 1997). This, however, is greatly dependent on the cell line used. Selection efficiencies were relatively low at 2 to 4% of total cells. As before, clones containing pJef-3A were

slow to expand and plasmid loss was observed again despite maintenance of drug selection in some groups of clones, while drugs were omitted in other cells to encourage expansion of cell numbers.

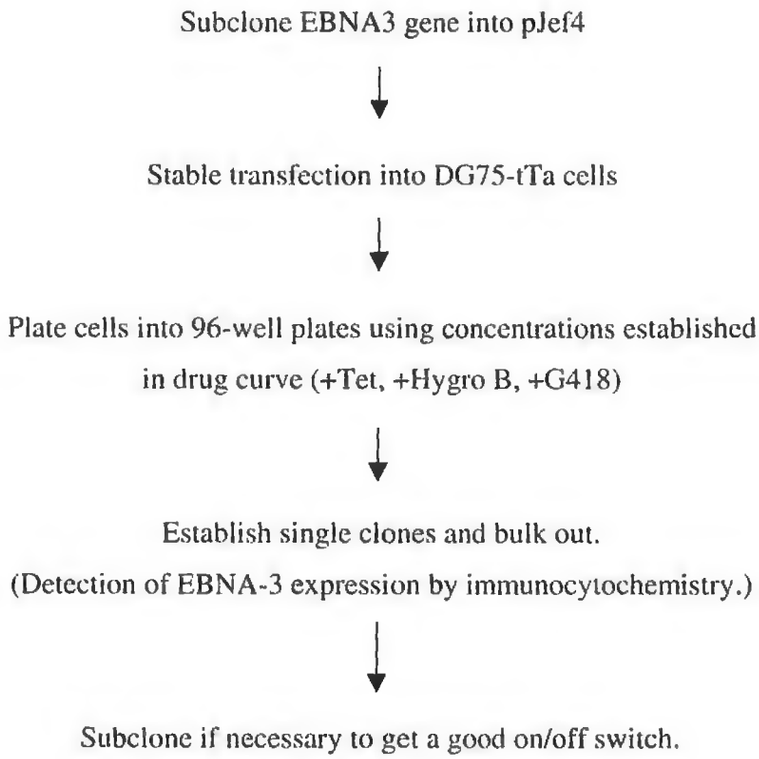


Figure 4.20 Flow chart for generation of tetracycline-inducible cell lines.

4.6.3 Discussion

Generation of stable cell lines for expression studies using inducible promoters have, in the past, met with varied success depending on the cell type and origin of the promoter utilised, so as a result are not always optimal or applicable. Using the approaches

outlined above, expression of EBNA-3A was retained for only a few weeks. The reason for this is not understood, but a number of factors may have had a role to play.

Expansion of cell numbers was found to be difficult in this study. Feeder layers were not used here but are frequently used to aid the growth and expansion of clones from very low cell densities. Feeder layers are not essential and, indeed, can be problematic, but may have improved the growth rate of cells. Expansion should occur from a single cell in the generation of a clone. When this does not occur, mixed populations of cells can be less stable, and cells expressing high levels of the protein of interest may be overgrown by cells expressing lower levels of protein. In order to aid expansion of cells and also to save on expensive drugs, the concentration of selective drugs is frequently reduced during expansion of clones. Application of this approach may have aided outgrowth of non-expressing clones. In addition, spontaneous mutations can sometimes occur, conferring G418 resistance to non-expressing cells, therefore it was important to examine cells for expression of EBNA-3A at intervals during expansion of clones. This was also important to ensure retention of the pJef-3A expression plasmid. Thus, there are several reasons for potential loss of expression in many clones. However, in general, it is usually possible to expand a small number of clones which stably express the plasmid under selection. A low level of leakage is known to be associated with use of the tetracycline-inducible system. Thus, a cumulative toxic effect over several weeks is possible, although unlikely, as no significant cytotoxicity has been associated with this protein. A masking effect of the EBNA-3A protein is also possible. A truncated form of EBNA-3A in pJef may not have the same effects. However, this would be less ideal in that it would only permit the study of effects of expression of part of the EBNA-3A protein.

APPENDIX

SOLUTIONS FOR DNA MANIPULATION

TE buffer

10 mM	Tris-Cl
1 mM	EDTA pH 8.0

Solutions for mini-preparation of plasmid DNA

Solution I

50 mM	Glucose
25 mM	Tris.Cl (pH 8.0)
10 mM	EDTA (pH 8.0)

Solution II (Prepared fresh)

0.2 N	NaOH
1 % (w/v)	SDS

Solution III

60 ml	5 M potassium acetate
11.5 ml	Glacial acetic acid
28.5 ml	Distilled water

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

DNAse-free RNAse

RNAse A (1 mg/ml) in sterile water.

Heat to 100^{o}C for 30 min. Cool slowly and store -20^{o}C

Solutions for Maxipreparations of DNA - Qiagen Buffers

Buffer P1 (Resuspension buffer)

50 mM	Tris-Cl, pH 8.0
10 mM	EDTA
100 µg	RNase A

Store at 4^{o}C after the addition of RNase A.

Buffer P2 (Lysis buffer)

200 mM Sodium Hydroxide

1% (w/v) SDS

Prepared fresh and stored at room temperature.

Buffer P3 (Neutralization buffer)

3.0 M Potassium acetate pH 5.5

Stored at 4°C.

Buffer QBT (Equilibriation buffer)

750 mM NaCl

50 mM MOPS pH 7.0

15% (v/v) Isopropanol

0.15% (v/v) Triton X®-100

Stored at room temperature.

Buffer QC (Wash buffer)

1.0 M NaCl

50 mM MOPS pH 7.0

15% (v/v) Isopropanol

Stored at room temperature.

Buffer QF (Elution buffer)

1.25 M NaCl

50 mM Tris-Cl, pH 8.5

15% (v/v) Isopropanol

Stored at room temperature.

50% (v/v) Glycerol

25 ml Distilled H_2O

25 ml Glycerol

Autoclaved and stored at room temperature.

0.5 M EDTA

186.1 g	EDTA
800 ml	Distilled water
6 g	NaOH pellets

pH to 8.0 with 5 M NaOH. Volume was adjusted to 1 L with water

50X TAE

242 g	Tris
57.1 ml	Acetic acid.
100 ml	0.5 M EDTA pH 8.0

Adjusted to 1L with water

5X TBE

54 g	Tris
27.5 g	Boric acid
20 ml	0.5 M EDTA pH 8.0

Adjusted to 1L with water.

Ethidium bromide

0.1 g/ 10 ml water (10 mg/ml)

Stored in dark at room temperature.

Agarose gel loading dye

40% (w/v) sucrose

0.25% (w/v) bromophenol blue

BACTERIAL GROWTH MEDIA

LB agar

10 g	Tryptone
5 g	Yeast extract
5 g	NaCl
15 g	Agar technical

Autoclaved and plates stored at 4°C.

LB agar plus ampicillin

Ampicillin was added to a final concentration of 100 μg/ml to LB agar (50 °C).

Plates were stored at 4 °C.

LB broth (per L)

10 g	Bacto-tryptone
5 g	Yeast extract
5 g	NaCl

Autoclaved and stored at 4 °C.

LB Ampicillin broth

Ampicillin was added to LB broth to a final concentration of 100 μg/ml from stock solutions (100 mg/ml in dH_2O, stored at –20°C). Stored at 4°C.

SOB medium (per L)

20 g	Tryptone
5 g	Yeast extract
0.5 g	NaCl
10 ml	KCl (250 mM)

Adjusted pH to 7.0 with 5 M NaOH

Autoclaved, cooled to ~5°C and added :

10 ml 1 M $MgCl_2$

Stored at 4 °C.

SOC medium (per L)

1 L	SOB
7.5 ml	50% glucose (filter sterilised)

Stored at 4°C.

IPTG stock solution (100 mM)

24 mg IPTG per ml of sterile H_2O

Filter sterilised and kept on ice until ready to use.

X-Gal stock solution (5%(w/v))

This solution was prepared fresh for each use

50 mg of X-Gal per ml of N,N' dimethyl-formamide in a sterile tube.

Protected from light and stored on ice until ready to use.

Ampicillin stock solution (50 mg/ml)

50 mg of ampicillin per ml of sterile H_2O

Filter sterilised and stored at -20°C.

LB plates with antibiotics and IPTG/X-Gal.

To 1L of autoclaved LB agar (cooled to 50°C) the following were added

0.5 μM	IPTG (5ml IPTG 100mM stock solution)
80 μg/ml	X-Gal (1.6 ml 5% (w/v) X-Gal stock solution)
50 μg/ml	Ampicillin (1 ml of 50 mg/ml solution)

Plates were stored at 4°C protected from light.

CELL CULTURE MEDIA/SOLUTIONS

Supplemented RPMI (200 ml)

176 ml	RPMI 1640
20 ml	Foetal calf Serum (Decomplemented - 50°C for 30 min)
2 ml	200 mM L-glutamine
2 ml	Penicillin/Streptomycin (1000 U/ml-1000 μg/ml)

Supplemented McCOY'S 5A (200 ml)

178 ml	MACOY'S 5A with L-glutamine
20 ml	Foetal calf Serum (Decomplemented; 50°C for 30 min)
2 ml	Penicillin/Streptomycin (1000 U/ml-1000 μg/ml)

Supplemented DMEM High Glucose

178 ml	DMEM high glucose with L-glutamine
20 ml	Foetal calf Serum (Decomplemented; 50°C for 30 min)
2 ml	Penicillin/Streptomycin (1000 U/ml-1000 µg/ml)

10X Phosphate Buffered Saline (PBS)

14.24 g	$Na_2HPO_4.2H_2O$ (8 mM)
2.04 g	KH_2PO_4 (1.5 mM)
80.0 g	NaCl (137 mM)
2.0 g	KCl (2.7 mM)

pH 7.5 and make up to 1 litre.

Diluted 1 in 10 in sterile distilled water and used at a 1X working concentration.

Thiol supplements.

The following were added to 200 ml of supplemented media:

200 µl	α-Thiolglycerol
2 ml	Sodium pyruvate
2 ml	HEPES

Bathocuproine disulfonic acid (BCS - 10 mM stock solution)

36.4 mg	BCS
10 ml	1X PBS

Dissolved by vortexing, filter sterilised using a 0.2 micron filter.

Aliquoted and stored at -20°C.

α-Thiolglycerol

A stock solution of 50 mM in PBS containing 20 µM BCS was prepared.

20 µl	10 mM BCS
10 ml	1X PBS

43.3 µl 100% α-thiolglycerol

Filter sterilised using a 0.2 micron filter, aliquoted and stored at -20°C.

Sodium pyruvate

100 mM stock solution in 1X PBS (Gibco BRL). Stored at 4°C.

HEPES

1 M stock solution pH 7.5 (Gibco BRL). Stored at room temperature.

Microphenolic acid/Xanthine supplements

200 ml	Supplemented RPMI
0.5 µg/ml	Microphenolic acid
50 µg/ml	Xanthine

Microphenolic acid stock solution 2.5 mg/mL

2.5 mg	Microphenolic acid
1 ml	Sterile dH_2O

Two micro litres per ml of media was added giving a final concentration of 0.5 µg/ml.

Xanthine stock solution of 25 mg/mL

25 mg	Xanthine
1 ml	Sterile $d.H_2O$

Twenty micro litres per ml of media was added to give a final concentration of 50 µg/ml.

Geneticin G418 (stock solution 50 mg/ml) for tetracycline inducible cell lines

0.1 g	Geneticin
2 ml	RPMI 1640

Filter sterilised using a 0.2 micron filter, aliquoted and stored at -20°C. 20 µl of the stock solution was added per ml of media to give a final concentration of 1 mg/ml.

Hygromycin B (stock solution 50 mg/ml supplied)

Ten micro litres of the stock solution was added per ml of media to give a final concentration of 500 µg per ml. Stored at 4°C.

Tetracycline (stock solution 5 mg/ml)

5 mg Tetracycline

1 ml 100% Ethanol

Stored at -20°C, 1 µl of tetracycline was added to 5 ml of media to give a final concentration of 1 µg per ml.

Geneticin G418 (stock solution 600 mg/ml) For transfected epithelial cell lines C33A Neo and LMP1

0.6 g Geneticin

1 ml 1 M Hepes pH 7.5

Filter sterilised using a 0.2 micron filter, aliquoted and stored at -20°C. One µl of the stock solution was added per ml of media to give a final concentration of 600 µg per ml.

Solutions for modified DEAE-Dextran Transfection Protocol

T.E.

10 mM Tris (pH 7.8)

1 mM EDTA

Prepared fresh on the day of use using autoclaved stocks of Tris and EDTA. It is important to ensure that the pH of the Tris is at 7.8 at room temperature prior to use.

TBS

25 mM Tris (pH 7.4)

137 mM NaCl

5 mM KCl

0.7 mM $CaCl_2$

0.5 mM $MgCl_2$

0.6 mM Na_2HPO_4

Prepared from autoclaved stocks, aliquoted and filtered before use. Again the pH of the Tris is critical.

DEAE Dextran

1 mg/ml in TBS, prepared fresh and filter sterilised.

SOLUTIONS FOR PROTEIN ISOLATION

Suspension buffer

0.1 M	NaCL
0.01 M	Tris-Cl (pH 7.6)
0.001 M	EDTA (pH 8.0)
1µg/ml	Apoprotinin
100µg/ml	PMSF

Stored at 4°C.

2X SDS gel loading buffer

100 mM	Tris-Cl
200 mM	DTT
4% (w/v)	SDS
0.2% (w/v)	Bromophenol blue
20% (v/v)	Glycerol

Two times loading buffer was prepared without DTT and stored at room temperature. DTT was added just prior to use from a 1 M stock

Protease Inhibitors

2 mg/ml	Leupeptin
0.1 mM	PMSF (phenylmethylsulfonyl flouride)

SOLUTIONS FOR SDS PAGE/WESTERN BLOTTING

1 M	Tris-Cl pH 6.8
1.5 M	Tris-Cl pH 8.8
10% (w/v)	SDS
10% (w/v)	Ammonium persulphate (APS)

Acrylagel

Bis-acrylagel

TEMED

1 M Dithiothreitol

10X Tris glycine running buffer (500 ml)

15.138 g	Tris
71.125 g	Glycine
5.0 g	SDS

Made up to 500 ml with distilled water.

Destain

100 ml	Acetic acid
400 ml	Methanol
500 ml	Distilled water

Coomassie blue stain

1 g	Coomassie blue R
200 ml	Destain

Transfer Buffer (10X stock solution)

30.3g	Tris
144.2g	Glycine

Adjusted to pH 8.3, made up to 1 L with distilled water, stored at room temperature.

Transfer Buffer (1X working Solution)

100 ml	10X Stock solution
200 ml	Methanol
700 ml	Distilled H_2O

Stored at 4°C. Methanol was omitted in transfer of proteins of >120 Kd.

TBS (1X)

6.1 g	Tris
8.8 g	NaCl

Made up to 1 L with distilled water and adjusted to pH 7.5 with HCl.

Autoclaved and stored at room temperature.

TBST (0.1%, v/v)

1 L	TBS (as above)
1 ml	Tween 20

Blotto

50 ml	1X TBS (as above)
25 µl	0.05% (v/v) Tween 20 (0.5 ml/L)
2 g	5% (w/v) non-fat dry milk 50 g/L (Marvel)
0.5g	NaN_3

Sodium azide (5%) (w/v)

50 mg	NaN_3
950 µl	Distilled water

REAGENTS FOR SEQUENCING

Six percent denaturing polyacrylamide gel

Six percent denaturing polyacrylamide gel was prepared for sequence analysis. The following formula was employed to determine the amount of acrylamide and bis-acrylamide required:

Va = volume of acrylamide

Vb = volume of bis-acrylamide

Vt = total volume of gel mix 150 ml

C = % crosslinking 5.2 %

A = % gel 6/8 %

$$Va = \frac{Avt}{30} \qquad Vb = \frac{ACVt}{200}$$

Va = 6*150/30 = 30 ml Vb = 6*5.2*150/ = 24 ml

6 % Denaturing PAG

63 g	Urea
30 ml	Acrylamide
24 ml	Bisacrylamide
15 ml	10X TBE

Made up to 150 ml with UP H_2O

Six hundred and fifty micro litres of 10% (w/v) APS and 150 µl TEMED were added, and mixed briefly, directly before pouring.

10X TBE (per 500 ml)

54 g	Tris base
27.5 g	Boric acid
20 ml	0.5 M EDTA (pH 8.0)

One times concentration was used for polyacrylamide gel preparation.

10% (w/v)Ammonium persulphate

0.1 g APS/ml ultra pure H_2O

Developer (5 L)

1.50 L	H_2O
1.25 L	Developer
2.25 L	H_2O

Stirred for 2 min

Fixer (5.125 L)

3.625 L	H_2O
1.250 L	A fixer
0.250 L	B fixer

Stirred for 2 min

REAGENTS FOR RNA ANALYSIS

RNA sample buffer

50 % (v/v)	Deionized formamide
8.3 % (v/v)	Formaldehyde
0.027 M	MOPS pH 7.0
6.7 mM	Sodium acetate

RNA loading buffer

50%(v/v)	High grade glycerol
1 mM	EDTA (pH 8.0)
0.25% (w/v)	Bromophenol blue
0.25% (w/v)	Xylene cyanol FF

DEPC treated (1ml/L DEPC) overnight, autoclaved and stored at room temperature.

RNA loading buffer (containing ethidium)

50% (v/v)	High grade glycerol
1 mM	EDTA (pH 8.0)
0.4% (w/v)	Bromophenol blue
0.1 µg/ml	Ethidium bromide

Aliquoted and stored at -20°C.

YEAST TWO HYBRID SYSTEM : MEDIA AND REAGENTS

YPD

10g	Yeast extract
20g	Peptone
20g	D-glucose

Made up to 1L with dH_20 and autoclaved.

Supplemented YNB

6.7g	YNB media, without amino acids (Difco)
2 g	Dropout powder (lacking appropriate amino acids)

Made up to 800 ml with dH_2O and autoclaved.

100 ml	Carbon source (20% stock solutions, filter sterilised)*

Amino acids added as required from stock solutions.

Made up to 1 L with sterile dH_2O.

* YNB/Glu media : Glucose added to a final concentration of 2% (w/v).

YNB/Gal media : Galactose/Raffinose at 2% (w/v) and 1% (w/v) respectively.

Dropout Powder

Nutrient	Quantity (g)	Final Conc. (µg/ml)
Adenine	2.5	40
L-arginine (HCl)	1.2	20
L-aspartic acid	6.0	100
L-glutamic acid	6.0	100
L-isoleucine	1.8	30
L-lysine	1.8	30
L-methionine	1.2	20
L-phenylalanine	3.0	50
L-serine	22.5	375
L-threonine	12.0	200
L-tyrosine	1.8	30
L-valine	9.0	150

All ingredients were combined and ground in a clean dry mortar and pestle until homogeneous. Stored at room temperature.

Amino Acid Supplements (Stock Solutions)

1 g/100 ml	Tryptophan (500X)
1.5 g/100 ml	Leucine (500X)
1 g/100 ml	Histidine (500X)
200 mg/100 ml	Uracil (100X)

YPD/supplemented YNB agar plates

20 g agar plus 1 pellet NaOH (0.1 g) was added per 1L YPD/supplemented YNB medium.

Z buffer (1X)

16.1 g	Na_2HPO_4. $7H_2O$ (60 mM final)
5.5 g	NaH_2PO4. H_2O (40 mM final)
0.75 g	KCl (10 mM final)
0.246 g	$MgSO_4.7H_2O$ (1 mM final)

Adjusted to pH 7.0 and brought to 1 liter with dH_2O. Do not autoclave.

2X FSB

2 ml	Glycerol
4 ml	10% SDS
2.5 ml	Buffer (0.5 M Tris pH 6.8, 0.4% SDS)

Made up to 10 ml with dH_2O. β-mercaptoethanol (1 µl/20 µl) added just before use.

PEG solution

40% (w/v)	Polyethyleneglycol (PEG) 3350*
0.1 M	Lithium acetate
10 mM	Tris-Cl, pH 8.0
1 mM	EDTA

*PEG was fully dissolved in dH_2O by mixing on a magnetic stirrer. The remaining constituents were added, volume was adjusted and the solution was filter sterilised.

Glycerol solution

65 % (v/v)	Glycerol (sterile)
0.1 M	$MgSO_4$
25 mM	Tris-Cl, pH 8.0

Stable for at least 1 year when stored at room temperature.

CHAPTER 5

SUMMARY

SUMMARY

This thesis consists of two main parts, the results of which are detailed and discussed in chapters 3 and 4. In conclusion overall, the most important findings of the work presented here are described in chapter 3, which outlines investigations into EBV-associated deregulation of cell cycle progression. Using a multi-template probe specific for a subset of genes associated with cell cycle control in RPA assays, the level of mRNA transcripts of each of these genes was assessed in the context of a range of EBV-related cell lines. Of the genes under study, which included *p130, pRb, p107, p53, p57, p21, p19, p18, p16* and *p14/p15*, the most significant change was seen in the level of *p21* mRNA which was markedly upregulated on transition from EBV latency type I to type III BL cells. Elevated levels were also observed in EBV-immortalised LCL cell lines and a similar pattern of p21 expression was found at the protein level. Consistent with this data, previous reports have described a rise in p21 protein levels as a result of EBV infection of B lymphocytes (Chen and Cooper, 1996; Cannell *et al* 1996), while another report described high levels of p21 in a number of LCLs (Pokrovskaja *et al*, 1999).

A tetracycline-regulated gene expression system in DG75tTA-LMP1 and DG75tTA-EBNA2 cells was used to express either LMP1 or EBNA2 as sole EBV protein. In this context, LMP1 was found to contribute to upregulation of *p21* mRNA whereas EBNA2 had no effect on *p21* mRNA levels. Levels of p21 mRNA were barely detectable or undetectable in a T cell line (Jurkat-tTA-LMP1) and in epithelial cells (C33A) and expression of LMP1 in either cell context had no effect on *p21* mRNA levels. Based on these observations, LMP1-mediated *p21* upregulation would seem to be a B cell-specific effect. Moreover, no increase in *p21* mRNA was detected in BJABtTA-LMP1 cells, which lack the characteristic BL *c-myc* translocation. The increase in *p21* levels is likely to be a p53-independent effect, as DG75 cells harbour a mutant *p53* gene and *p53* mRNA and protein levels did not significantly change as a result of LMP1 expression.

Further investigations into the mechanism of upregulation revealed that stabilisation of *p21* mRNA was important in the observed LMP1-mediated effect. No transactivation of

the p21 promoter was detected as a result of LMP1 expression, but a high basal activity was detected, which is characteristic of this promoter. These results suggested that the LMP1-mediated increase in *p21* mRNA is controlled through a post-transcriptional mechanism that probably results from an increase in p21 mRNA stability. Thus, even a modest enhancement of mRNA stability (2-fold) in the presence of a high p21 promoter activity would result in the rapid accumulation of *p21* mRNA. A similar scenario of low basal cellular levels, high promoter activity in the absence of transactivation of the promoter, coupled with a 3-fold increase in *p21* mRNA stability has been shown elsewhere in response to Et_2Mal-induced oxidative stress in HeLa cells (Esposito *et al.*, 1997). Also, an almost 2-fold increase in *p21* mRNA stability was observed in response to PMA stimulation in SKOV-3 human ovarian carcinoma cells (Akashi *et al.*, 1999). Preliminary investigations into the role of the PKC and/or MAPK pathway in LMP1-mediated p21 upregulation were inconclusive, but it will be important in future studies to elucidate the signal transduction pathway involved. The level to which p21 protein is induced can be critical in the determination of cell fate (Chen *et al.*, 1998). Thus, it may also be interesting in future studies to assess the effect on cdk-associated kinases in LMP1-expressing cells in order to determine whether or not the level of induced p21 protein is sufficient to inhibit kinase activity. It is unlikely that the modest increase observed here would reach the levels required to induce growth arrest. In a previous study where elevated p21 protein expression was observed following outgrowth of EBV-infected cells into LCLs, the level detected was below the threshold required to prevent kinase activity and to cause growth arrest. LMP1-mediated upregulation of p21 expression is more likely to be linked to the previously documented cytostatic effect of LMP1 (Floettmann *et al*, 1996).

The second part of this dissertation describes two different approaches taken in the study of the function of the EBNA3 proteins. Firstly, the yeast two hybrid system was employed to detect potential binding partners for EBNA3B. This system is a widely-used, highly sensitive method which depends on the reconstitution of transactivation activity of a transcription factor by the interaction of two proteins, which are each fused to either a transactivation domain or a DNA binding domain (DBD). To this end, two

independent systems were used, based on the LexA DBD (referred to as YTHS-A) or the Gal4 DBD (YTHS-B) to screen two different cDNA libraries. False positives remain a significant problem with respect to use of these systems and in the data presented here no true positives were detected using either system, despite confirmation of two EBNA3B-specific interactions. However, it is generally recommended that any given library should be screened several times using two alternative bait constructs before safely ruling out the possibility of true interactors being encoded by the cDNA library.

As an alternative approach to the study of EBNA3 protein function, a tetracycline-regulatable gene expression system was used in an attempt to generate a stable cell line harbouring an inducible EBNA3A gene. For reasons that are not clear, clones were observed to express inducible EBNA3A protein for several weeks, but not thereafter.

CHAPTER 6

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APPENDIX

SOLUTIONS FOR DNA MANIPULATION

TE buffer

10 mM	Tris-Cl
1 mM	EDTA pH 8.0

Solutions for mini-preparation of plasmid DNA

Solution I

50 mM	Glucose
25 mM	Tris.Cl (pH 8.0)
10 mM	EDTA (pH 8.0)

Solution II (Prepared fresh)

0.2 N	NaOH
1 % (w/v)	SDS

Solution III

60 ml	5 M potassium acetate
11.5 ml	Glacial acetic acid
28.5 ml	Distilled water

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

DNAse-free RNAse

RNAse A (1 mg/ml) in sterile water.

Heat to 100^{o}C for 30 min. Cool slowly and store -20^{o}C

Solutions for Maxipreparations of DNA - Qiagen Buffers

Buffer P1 (Resuspension buffer)

50 mM	Tris-Cl, pH 8.0
10 mM	EDTA
100 µg	RNase A

Store at 4^{o}C after the addition of RNase A.

Buffer P2 (Lysis buffer)

200 mM Sodium Hydroxide

1% (w/v) SDS

Prepared fresh and stored at room temperature.

Buffer P3 (Neutralization buffer)

3.0 M Potassium acetate pH 5.5

Stored at 4°C.

Buffer QBT (Equilibriation buffer)

750 mM NaCl

50 mM MOPS pH 7.0

15% (v/v) Isopropanol

0.15% (v/v) Triton X®-100

Stored at room temperature.

Buffer QC (Wash buffer)

1.0 M NaCl

50 mM MOPS pH 7.0

15% (v/v) Isopropanol

Stored at room temperature.

Buffer QF (Elution buffer)

1.25 M NaCl

50 mM Tris-Cl, pH 8.5

15% (v/v) Isopropanol

Stored at room temperature.

50% (v/v) Glycerol

25 ml Distilled H_2O

25 ml Glycerol

Autoclaved and stored at room temperature.

0.5 M EDTA

186.1 g	EDTA
800 ml	Distilled water
6 g	NaOH pellets

pH to 8.0 with 5 M NaOH. Volume was adjusted to 1 L with water

50X TAE

242 g	Tris
57.1 ml	Acetic acid.
100 ml	0.5 M EDTA pH 8.0

Adjusted to 1L with water

5X TBE

54 g	Tris
27.5 g	Boric acid
20 ml	0.5 M EDTA pH 8.0

Adjusted to 1L with water.

Ethidium bromide

0.1 g/ 10 ml water (10 mg/ml)

Stored in dark at room temperature.

Agarose gel loading dye

40% (w/v) sucrose

0.25% (w/v) bromophenol blue

BACTERIAL GROWTH MEDIA

LB agar

10 g	Tryptone
5 g	Yeast extract
5 g	NaCl
15 g	Agar technical

Autoclaved and plates stored at 4°C.

LB agar plus ampicillin

Ampicillin was added to a final concentration of 100 μg/ml to LB agar (50 °C).

Plates were stored at 4 °C.

LB broth (per L)

10 g	Bacto-tryptone
5 g	Yeast extract
5 g	NaCl

Autoclaved and stored at 4 °C.

LB Ampicillin broth

Ampicillin was added to LB broth to a final concentration of 100 μg/ml from stock solutions (100 mg/ml in dH_2O, stored at –20°C). Stored at 4°C.

SOB medium (per L)

20 g	Tryptone
5 g	Yeast extract
0.5 g	NaCl
10 ml	KCl (250 mM)

Adjusted pH to 7.0 with 5 M NaOH

Autoclaved, cooled to ~5°C and added :

10 ml 1 M $MgCl_2$

Stored at 4 °C.

SOC medium (per L)

1 L	SOB
7.5 ml	50% glucose (filter sterilised)

Stored at 4°C.

IPTG stock solution (100 mM)

24 mg IPTG per ml of sterile H_2O

Filter sterilised and kept on ice until ready to use.

X-Gal stock solution (5%(w/v))

This solution was prepared fresh for each use

50 mg of X-Gal per ml of N,N' dimethyl-formamide in a sterile tube.

Protected from light and stored on ice until ready to use.

Ampicillin stock solution (50 mg/ml)

50 mg of ampicillin per ml of sterile H_2O

Filter sterilised and stored at -20°C.

LB plates with antibiotics and IPTG/X-Gal.

To 1L of autoclaved LB agar (cooled to 50°C) the following were added

0.5 µM	IPTG (5ml IPTG 100mM stock solution)
80 µg/ml	X-Gal (1.6 ml 5% (w/v) X-Gal stock solution)
50 µg/ml	Ampicillin (1 ml of 50 mg/ml solution)

Plates were stored at 4°C protected from light.

CELL CULTURE MEDIA/SOLUTIONS

Supplemented RPMI (200 ml)

176 ml	RPMI 1640
20 ml	Foetal calf Serum (Decomplemented - 50°C for 30 min)
2 ml	200 mM L-glutamine
2 ml	Penicillin/Streptomycin (1000 U/ml-1000 µg/ml)

Supplemented McCOY'S 5A (200 ml)

178 ml	MACOY'S 5A with L-glutamine
20 ml	Foetal calf Serum (Decomplemented; 50°C for 30 min)
2 ml	Penicillin/Streptomycin (1000 U/ml-1000 µg/ml)

Supplemented DMEM High Glucose

178 ml	DMEM high glucose with L-glutamine
20 ml	Foetal calf Serum (Decomplemented; 50°C for 30 min)
2 ml	Penicillin/Streptomycin (1000 U/ml-1000 µg/ml)

10X Phosphate Buffered Saline (PBS)

14.24 g	$Na_2HPO_4.2H_2O$ (8 mM)
2.04 g	KH_2PO_4 (1.5 mM)
80.0 g	NaCl (137 mM)
2.0 g	KCl (2.7 mM)

pH 7.5 and make up to 1 litre.

Diluted 1 in 10 in sterile distilled water and used at a 1X working concentration.

Thiol supplements.

The following were added to 200 ml of supplemented media:

200 µl	α-Thiolglycerol
2 ml	Sodium pyruvate
2 ml	HEPES

Bathocuproine disulfonic acid (BCS - 10 mM stock solution)

36.4 mg	BCS
10 ml	1X PBS

Dissolved by vortexing, filter sterilised using a 0.2 micron filter.

Aliquoted and stored at -20°C.

α-Thiolglycerol

A stock solution of 50 mM in PBS containing 20 µM BCS was prepared.

20 µl	10 mM BCS
10 ml	1X PBS

43.3 µl 100% α-thiolglycerol

Filter sterilised using a 0.2 micron filter, aliquoted and stored at -20°C.

Sodium pyruvate

100 mM stock solution in 1X PBS (Gibco BRL). Stored at 4°C.

HEPES

1 M stock solution pH 7.5 (Gibco BRL). Stored at room temperature.

Microphenolic acid/Xanthine supplements

200 ml	Supplemented RPMI
0.5 μg/ml	Microphenolic acid
50 μg/ml	Xanthine

Microphenolic acid stock solution 2.5 mg/mL

2.5 mg	Microphenolic acid
1 ml	Sterile dH_2O

Two micro litres per ml of media was added giving a final concentration of 0.5 μg/ml.

Xanthine stock solution of 25 mg/mL

25 mg	Xanthine
1 ml	Sterile $d.H_2O$

Twenty micro litres per ml of media was added to give a final concentration of 50 μg/ml.

Geneticin G418 (stock solution 50 mg/ml) for tetracycline inducible cell lines

0.1 g	Geneticin
2 ml	RPMI 1640

Filter sterilised using a 0.2 micron filter, aliquoted and stored at -20°C. 20 μl of the stock solution was added per ml of media to give a final concentration of 1 mg/ml.

Hygromycin B (stock solution 50 mg/ml supplied)

Ten micro litres of the stock solution was added per ml of media to give a final concentration of 500 μg per ml. Stored at 4°C.

Tetracycline (stock solution 5 mg/ml)

5 mg Tetracycline

1 ml 100% Ethanol

Stored at -20°C, 1 µl of tetracycline was added to 5 ml of media to give a final concentration of 1 µg per ml.

Geneticin G418 (stock solution 600 mg/ml) For transfected epithelial cell lines C33A Neo and LMP1

0.6 g Geneticin

1 ml 1 M Hepes pH 7.5

Filter sterilised using a 0.2 micron filter, aliquoted and stored at -20°C. One µl of the stock solution was added per ml of media to give a final concentration of 600 µg per ml.

Solutions for modified DEAE-Dextran Transfection Protocol

T.E.

10 mM Tris (pH 7.8)

1 mM EDTA

Prepared fresh on the day of use using autoclaved stocks of Tris and EDTA. It is important to ensure that the pH of the Tris is at 7.8 at room temperature prior to use.

TBS

25 mM Tris (pH 7.4)

137 mM NaCl

5 mM KCl

0.7 mM $CaCl_2$

0.5 mM $MgCl_2$

0.6 mM Na_2HPO_4

Prepared from autoclaved stocks, aliquoted and filtered before use. Again the pH of the Tris is critical.

DEAE Dextran

1 mg/ml in TBS, prepared fresh and filter sterilised.

SOLUTIONS FOR PROTEIN ISOLATION

Suspension buffer

0.1 M	NaCL
0.01 M	Tris-Cl (pH 7.6)
0.001 M	EDTA (pH 8.0)
1µg/ml	Apoprotinin
100µg/ml	PMSF

Stored at 4°C.

2X SDS gel loading buffer

100 mM	Tris-Cl
200 mM	DTT
4% (w/v)	SDS
0.2% (w/v)	Bromophenol blue
20% (v/v)	Glycerol

Two times loading buffer was prepared without DTT and stored at room temperature. DTT was added just prior to use from a 1 M stock

Protease Inhibitors

2 mg/ml	Leupeptin
0.1 mM	PMSF (phenylmethylsulfonyl flouride)

SOLUTIONS FOR SDS PAGE/WESTERN BLOTTING

1 M	Tris-Cl pH 6.8
1.5 M	Tris-Cl pH 8.8
10% (w/v)	SDS
10% (w/v)	Ammonium persulphate (APS)

Acrylagel

Bis-acrylagel

TEMED

1 M Dithiothreitol

10X Tris glycine running buffer (500 ml)

15.138 g Tris

71.125 g Glycine

5.0 g SDS

Made up to 500 ml with distilled water.

Destain

100 ml Acetic acid

400 ml Methanol

500 ml Distilled water

Coomassie blue stain

1 g Coomassie blue R

200 ml Destain

Transfer Buffer (10X stock solution)

30.3g Tris

144.2g Glycine

Adjusted to pH 8.3, made up to 1 L with distilled water, stored at room temperature.

Transfer Buffer (1X working Solution)

100 ml 10X Stock solution

200 ml Methanol

700 ml Distilled H_2O

Stored at 4°C. Methanol was omitted in transfer of proteins of >120 Kd.

TBS (1X)

6.1 g Tris

8.8 g NaCl

Made up to 1 L with distilled water and adjusted to pH 7.5 with HCl.

Autoclaved and stored at room temperature.

TBST (0.1%, v/v)

1 L	TBS (as above)
1 ml	Tween 20

Blotto

50 ml	1X TBS (as above)
25 µl	0.05% (v/v) Tween 20 (0.5 ml/L)
2 g	5% (w/v) non-fat dry milk 50 g/L (Marvel)
0.5g	NaN_3

Sodium azide (5%) (w/v)

50 mg	NaN_3
950 µl	Distilled water

REAGENTS FOR SEQUENCING

Six percent denaturing polyacrylamide gel

Six percent denaturing polyacrylamide gel was prepared for sequence analysis. The following formula was employed to determine the amount of acrylamide and bis-acrylamide required:

Va = volume of acrylamide

Vb = volume of bis-acrylamide

Vt = total volume of gel mix 150 ml

C = % crosslinking 5.2 %

A = % gel 6/8 %

$$Va = \frac{Avt}{30} \qquad Vb = \frac{ACVt}{200}$$

Va = 6*150/30 = 30 ml Vb = 6*5.2*150/ = 24 ml

6 % Denaturing PAG

63 g	Urea
30 ml	Acrylamide
24 ml	Bisacrylamide
15 ml	10X TBE

Made up to 150 ml with UP H_2O

Six hundred and fifty micro litres of 10% (w/v) APS and 150 µl TEMED were added, and mixed briefly, directly before pouring.

10X TBE (per 500 ml)

54 g	Tris base
27.5 g	Boric acid
20 ml	0.5 M EDTA (pH 8.0)

One times concentration was used for polyacrylamide gel preparation.

10% (w/v)Ammonium persulphate

0.1 g APS/ml ultra pure H_2O

Developer (5 L)

1.50 L	H_2O
1.25 L	Developer
2.25 L	H_2O

Stirred for 2 min

Fixer (5.125 L)

3.625 L	H_2O
1.250 L	A fixer
0.250 L	B fixer

Stirred for 2 min

REAGENTS FOR RNA ANALYSIS

RNA sample buffer

50 % (v/v)	Deionized formamide
8.3 % (v/v)	Formaldehyde
0.027 M	MOPS pH 7.0
6.7 mM	Sodium acetate

RNA loading buffer

50%(v/v)	High grade glycerol
1 mM	EDTA (pH 8.0)
0.25% (w/v)	Bromophenol blue
0.25% (w/v)	Xylene cyanol FF

DEPC treated (1ml/L DEPC) overnight, autoclaved and stored at room temperature.

RNA loading buffer (containing ethidium)

50% (v/v)	High grade glycerol
1 mM	EDTA (pH 8.0)
0.4% (w/v)	Bromophenol blue
0.1 μg/ml	Ethidium bromide

Aliquoted and stored at -20°C.

YEAST TWO HYBRID SYSTEM : MEDIA AND REAGENTS

YPD

10g	Yeast extract
20g	Peptone
20g	D-glucose

Made up to 1L with dH_20 and autoclaved.

Supplemented YNB

6.7g	YNB media, without amino acids (Difco)
2 g	Dropout powder (lacking appropriate amino acids)

Made up to 800 ml with dH_2O and autoclaved.

100 ml	Carbon source (20% stock solutions, filter sterilised)*

Amino acids added as required from stock solutions.

Made up to 1 L with sterile dH_2O.

* YNB/Glu media : Glucose added to a final concentration of 2% (w/v).

YNB/Gal media : Galactose/Raffinose at 2% (w/v) and 1% (w/v) respectively.

Dropout Powder

Nutrient	Quantity (g)	Final Conc. (µg/ml)
Adenine	2.5	40
L-arginine (HCl)	1.2	20
L-aspartic acid	6.0	100
L-glutamic acid	6.0	100
L-isoleucine	1.8	30
L-lysine	1.8	30
L-methionine	1.2	20
L-phenylalanine	3.0	50
L-serine	22.5	375
L-threonine	12.0	200
L-tyrosine	1.8	30
L-valine	9.0	150

All ingredients were combined and ground in a clean dry mortar and pestle until homogeneous. Stored at room temperature.

Amino Acid Supplements (Stock Solutions)

1 g/100 ml	Tryptophan (500X)
1.5 g/100 ml	Leucine (500X)
1 g/100 ml	Histidine (500X)
200 mg/100 ml	Uracil (100X)

YPD/supplemented YNB agar plates

20 g agar plus 1 pellet NaOH (0.1 g) was added per 1L YPD/supplemented YNB medium.

Z buffer (1X)

16.1 g	Na_2HPO_4. $7H_2O$ (60 mM final)
5.5 g	NaH_2PO4. H_2O (40 mM final)
0.75 g	KCl (10 mM final)
0.246 g	$MgSO_4.7H_2O$ (1 mM final)

Adjusted to pH 7.0 and brought to 1 liter with dH_2O. Do not autoclave.

2X FSB

2 ml	Glycerol
4 ml	10% SDS
2.5 ml	Buffer (0.5 M Tris pH 6.8, 0.4% SDS)

Made up to 10 ml with dH_2O. β-mercaptoethanol (1 µl/20 µl) added just before use.

PEG solution

40% (w/v)	Polyethyleneglycol (PEG) 3350*
0.1 M	Lithium acetate
10 mM	Tris-Cl, pH 8.0
1 mM	EDTA

*PEG was fully dissolved in dH_2O by mixing on a magnetic stirrer. The remaining constituents were added, volume was adjusted and the solution was filter sterilised.

Glycerol solution

65 % (v/v)	Glycerol (sterile)
0.1 M	$MgSO_4$
25 mM	Tris-Cl, pH 8.0

Stable for at least 1 year when stored at room temperature.