Early Events in the Replication Cycle of Human Immunodeficiency Virus

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Chapter 2 Materials and Methods

Chapter 4 Further characterization of HIV DNA and RNA synthesis early after cell-to-cell transmission of infection

Chapter 5 Kinetics and pattern of HIV DNA integration during cell-to-cell transmission infection

Abstract

A one-step cell-to-cell transmission model of HIV infection was used to study viral RNA expression in the early phase of viral replication. In this model, H3B viral donor lymphoid cells were co-cultured with CD4+ Hut78 recipient cells in a ratio of 1:4. Co-culturing of these cells produced a synchronous, one-step replication cycle with *de novo* synthesis of unintegrated viral DNA within 4 h p.i. The persistently infected (HIV) H3B cell line does not contain detectable levels of unintegrated viral DNA but constitutively produces mainly the multiply spliced (2 kb) and singly spliced (4.3 kb) species of HIV RNA. Minimal levels of genomic-length viral RNA (9.2 kb) were detected in some batches of H3B cells by Northern blot hybridization analysis. However, a small amount of 9 kb HIV RNA is presumably made because the cells release 0.01 $TCID_{50}$ of virus/cell/hr. When H3B and Hut78 cells were co-cultured in a synchronous one-step infection cycle two distinct phases of HIV RNA synthesis were observed. The first phase (4h - 12h p.i.) was marked by a significant increase in only the full-length 9.2 kb RNA, while the second phase (24h p.i. onwards) comprised a dramatic increase in the levels of all three species of viral RNA. In the presence of reverse transcriptase inhibitors, such as azidothymidine (AZT), the first phase but not the second phase of viral RNA synthesis was abolished in the co-culture. Actinomycin D (AmD) binds to double-stranded DNA irreversibly and inhibits RNA transcription. When H3B cells were pre-treated with AmD, washed free of the drug and mixed with untreated recipient Hut78 cells, normal amounts of full length, linear, unintegrated viral DNA were produced and the first phase of induced viral RNA transcription was unaffected. The continual presence of AmD at 50 μg/ml, while having minimal effect on reverse transcriptase activity when tested *in vitro*, abolished all detectable viral nucleic acid synthesis *in vivo*. The virus in H3B donor cells is Vpr defective in entering interphase nuclei. When both the virus donor cells and recipient cells were arrested in the late G_1 phase of the cell cycle by aphidicolin, the first phase of induced viral RNA synthesis was unaffected whereas cytoplasmic linear unintegrated viral DNA was the only viral DNA species produced. When AZT was added at 2h or 4h after cell-cell mixing, the level of viral DNA detected was reduced significantly. This was accompanied by a corresponding reduction in the level of genomic length HIV RNA. These results indicated that the template for the first phase

of viral RNA synthesis was likely to be newly synthesized, linear unintegrated viral DNA and not the pre-existing proviral DNA present in the H3B donor cells or newly integrated viral DNA. *De novo* reverse transcription of genomic length viral RNA in the cell-to-cell transmission infection model yields unintegrated viral DNA which subsequently integrates in the host genome to form provirus. Extensive electrophoresis was used to remove unintegrated viral DNA from chromosomal DNA - extracted from the co-culture mix of H3B and Hut78 cells - in studies of viral DNA integration. The kinetics of HIV DNA integration suggested an incomplete integration 'intermediate', although the structure of this intermediate has not been proven experimentally. The results in this thesis suggest that there exists a yet to be fully characterized pathway of concurrent viral DNA and RNA synthesis that leads to production of viral genomic RNA early after cell to cell transmission of HIV infection and with simultaneous integration of viral DNA.

Declaration of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference has been made in the thesis.

In accordance with the University of Adelaide regulations I consent to this thesis, when deposited in the University Library, being made available for loan or photocopying.

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"...the best teaching can be done only when there is a direct individual relationship between a student and a good teacher - a situation in which the student discusses the ideas, thinks about the things, and talks about the things. It is impossible to learn very much by simply sitting in a lecture, or even by simply doing problems that are assigned." *Richard Feynman (1918-1988), Nobel Laureate for Physics, 1965.*

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"... It may seem unreasonable, impractical, call it counter-intuitive, even to scientists, to try to solve an urgent problem like a disease by pursuing apparently unrelated questions in basic biology or chemistry. And yet the pursuit of curiosity about basic facts of nature has proven throughout the history of medical science to be the most practical, most cost-effective route to successful drugs and devices. We probe the inexhaustable mysteries of nature in a variety of directions. We use different styles, and these probings are determined by our emotions, our moods, our cultural heritage, much as these feelings influence the artist. And the major discoveries in science are more often intuitive and serendipitous than they are the result of logical analysis." *Arthur Kornberg, Nobel laureate for Medicine, 1959.*

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Abbreviations

>	greater than
<	less than
AIDS	acquired immune deficiency syndrome
bp	base pairs
ca.	circa
°C	degrees, Celsius
CD	cluster of differentiation
Ci	Curie
cpm	counts per minute
CTL	cytotoxic T-lymphocytes
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytidine-5'-triphosphate
dGTP	deoxyguanosine-5'-triphosphate
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine-5'-triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetate.dihydrate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gp	glycoprotein
h	hours
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus type 1
kb	kilobases
kD	kilodaltons
LTR	long terminal repeat
min	minutes
m.o.i	multiplicity of infection
mRNA	messenger ribonucleic acid

M.W.	molecular weight
NSI	non-syncytium inducing
p	protein
PBMC	peripheral blood mononuclear cells
pbs	primer binding site
p.i.	post infection
pol	polymerase
PCR	polymerase chain reaction
PPT	polypurine tract
Pr	precursor (protein)
RNA	ribonucleic acid
rRNA	ribosomal RNA
rNTP	ribonucleotide triphosphate
RE	restriction enzyme
Rnase H	ribonuclease H
RRE	Rev-responsive element
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SI	syncytium inducing
Tris	Tris(hydroxymethyl)methylamine
tRNA	transfer RNA
VSB	virus solubilization buffer

Manuscripts and Presentations related to the work in this thesis

Manuscripts:

Kok, T.-W., Li, P. & Burrell, C. J. (1998). Further Characterization of HIV RNA Synthesis Early After Cell-to-Cell Transmission Infection. *Archives of Virology* **143**, 1-16.

Benovic, S., Kok, T., Stephenson, A., McInnes, J., Burrell, C. & Li, P. (1998). *De novo* reverse transcription of HTLV-1 following cell-to-cell transmission of infection. *Virology* **244**, 294-301.

Fan, J., Li, P., Kok, T.-W. & Burrell, C. J. (1997). AZT blocks down-regulation of IL-2 and IFN-g gene expression in HIV acutely infected cells. *Arch Virol* **142**, 1035-1043.

Li, P., Stephenson, A. J., Brennan, P. A., Karageorgos, L., Kok, T., Kuiper, L., Swift, J. & Burrell, C. J. (1994). Initiation of reverse transcription during cell-to-cell transmission of human immunodeficiency virus infection uses pre-existing reverse transcriptase. *J Gen Virol* **75**, 1917-1926.

Kok, T., Li, P. & Burrell, C. (1993). Cell-to-cell transmission of human immunodeficiency virus infection induces two distinct phases of viral RNA expression under separate regulatory control. *J Gen Virol* **74**, 33-38.

Presentations:

T-W Kok, P. Li and C J Burrell (1998)
HIV RNA transcription patterns (seminar)
Institute of Veterinary Virology
University of Bern, Switzerland.

T-W Kok, P. Li and C J Burrell (1997)
Further characterization of mutually dependent viral DNA and RNA synthesis early after cell-to-cell transmission of HIV infection
Australian Society for HIV Medicine Annual Conference, Adelaide, South Australia.

T-W Kok, P. Li and C J Burrell (1994)
HIV RNA transcription patterns (seminar)
Department of Haematology-Oncology
University of California, Los Angeles, USA.

T-W Kok, P. Li and C J Burrell (1993)
Mutually dependent, concurrent RNA and DNA synthesis during early phase HIV replication
(i) Oral presentation at the 5th Annual Conference of the Australasian Society for HIV Medicine, Melbourne
(ii) Poster presented at the 1994 Keystone Symposia on Treatment and Prevention of AIDS, South Carolina, USA.

T-W Kok, P Li and C J Burrell (1991)
Kinetics and characteristics of human immunodeficiency virus (HIV) DNA integration. Oral presentation: Annual Meeting of the Australian Society for Microbiology, Queensland, Aust.

Chapter 1

Nature uses only the longest threads to weave her pattern, so each small piece of the fabric reveals the organization of the entire tapestry.
Richard Feynman (1918-1988), Nobel Laureate for Physics 1965.

Introduction and Literature Review

1.1.A Acquired Immune Deficiency Syndrome (AIDS) - Historical notes:

On 5th June, 1981 a report entitled "*Pneumocystis* pneumonia - Los Angeles" was published in the journal Morbidity and Mortality Weekly Report (MMWR) (Gregg, 1981b). Five cases of this rare pneumonia (caused by *Pneumocystis carinii*) were confirmed in young homosexual men within the Los Angeles county. On 3rd July, 1981 the MMWR (Gregg, 1981a) reported a rare type of skin cancer - called Kaposi's sarcoma - amongst twenty-six young homosexual men and four of this group also presented with biopsy-confirmed *Pneumocystis* pneumonia. The MMWR editorial reported that the occurrence of this high number of Kaposi's sarcoma in young men was unusual and there had been no previous association between this sarcoma and sexual preferences. All these patients showed a decreased T-lymphocyte response to stimulation by antigens and mitogens (Gottlieb *et al.*, 1981). Subsequently, a link between *Pneumocystis* pneumonia and Kaposi's sarcoma was confirmed. A year later, in 1982, it was reported that the immune status of these patients was dramatically deficient and various manifestations of opportunistic infections were present. Initially this syndrome was called "Gay Related Immune Deficiency Syndrome (GRIDS)" but was later renamed by the Centers for Disease Control (CDC), Atlanta, Georgia to "Acquired Immune Deficiency Syndrome (AIDS)". Since this recognition and formal description, the number of AIDS has become a global epidemic which has involved not just homosexual men and intravenous drug users but in addition sexual partners of infected patients, babies born to at-risk mothers, sexual workers and blood-transfusion recipients (Royce *et al.*, 1997). In 1996, the Joint United Nations Programme on HIV/AIDS estimated that there were 28 million people with AIDS (Quinn, 1996).

It was suspected at that time that the aetiological agent was infectious and transmitted via blood and body secretions. A retrovirus was considered because feline leukemia virus (a retrovirus)

was known to induce immune deficiency as well as leukemia/lymphoma in cats (Hardy, 1980, Hardy, 1982). In addition, the previously discovered Human T-cell Leukaemia/Lymphotropic Virus types I and II (HTLV-I & II) (Poiesz *et al.*, 1980, Kalyanaraman *et al.*, 1982) showed a similar cell tropism and modes of transmission.

In 1983, Barre-Sinoussi and colleagues detected reverse transcriptase activity, cytopathic effects and viral particles in phytohaemagglutinin - and interleukin-2 - stimulated lymphocytes from a patient with lymphadenopathy, a common manifestation of AIDS (Barre-Sinoussi *et al.*, 1983). The virus was named lymphadenopathy-associated virus (LAV). Subsequently, two separate laboratories reported the propagation of viruses from AIDS patients in CD4+ T-cell lines (Gallo *et al.*, 1984, Montagnier *et al.*, 1984, Popovic *et al.*, 1984). The isolate was named as HTLV-III by Gallo and colleagues. Similar viruses, designated as AIDS-related viruses (ARV), were isolated by another team led by Levy in San Francisco (Levy *et al.*, 1984). LAV, HTLV-III and ARV were later shown to be variants of the same virus (Ratner *et al.*, 1985a) and in 1986 an international committee assigned the name - Human Immunodeficiency Virus (HIV) for the causative agent of AIDS (Coffin *et al.*, 1986). This was identified as a new member of the Retroviridae family.

1.1.B HIV and AIDS in Australia - Epidemiology

In Australia, AIDS was first reported in 1982 and in April, 1997, 20548 people have been identified with this infection. Within this group, 5338 (26%) have died of the infection and 7416 (36%) have been reported with AIDS-defining conditions (Kaldor *et al.*, 1993, Harvey, 1997). Patients who are antibody positive to HIV and who present with one or more other specified clinical conditions such as candidiasis of the respiratory tract, cytomegalovirus retinitis, Kaposi's sarcoma, lymphomas, infection with *Mycobacterium tuberculosis/Mycobacterium avium* complex, *Pneumocystis carinii* pneumonia, Toxoplasmosis of the brain, etc. fall into the current definition of AIDS. In Australia, these syndromes form part of the 23 AIDS-defining conditions (Kaldor & Crofts, 1996). However, in the United States, an HIV antibody-positive patient with a CD4 cell count of <200/µl, regardless of clinical condition, is included in the definition of AIDS (Castro *et al.*, 1992).

1.2.A Retroviridae - General Properties

Members of the Retroviridae family have been intensely studied for many decades due to their importance as human and animal pathogens, causing significant morbidity and mortality. Retroviruses have also been studied for their usefulness as genetic engineering tools, for example as gene therapy vectors in proliferating (Peng *et al.*, 1996) as well as in non-proliferating cells (Naldini *et al.*, 1996, Miyoshi *et al.*, 1997).

One of the earliest retroviral diseases to be studied was that caused by Rous sarcoma virus of fowls (Rous, 1911). Subsequently, diseases caused by other retroviruses have been recognized e.g. equine infectious anaemia (McGuire & Henson, 1973), maedi-visna in sheep (Haase, 1975), adult T cell lymphoma/leukaemia (ATLL) (Poiesz *et al.*, 1980, Miyoshi *et al.*, 1981) and acquired immunodeficiency syndrome (AIDS) in humans (Gottlieb *et al.*, 1981). It is the latter disease which has attracted immense study due to this virus family. The principal features of a retroviral disease are chronic infection and degenerative pathology.

Retroviruses are unique in that they are the only viruses with a diploid positive strand RNA genome. Three main genes code for the structural proteins, viz. core or group antigens (*gag*), polymerase and other virally coded-enzymes (*pol*) and envelope (*env*) proteins. After infection, reverse transcription of the viral genome results in the formation of a double-stranded DNA molecule which subsequently integrates into the host DNA to form the provirus - first described by Temin with his studies of the Rous sarcoma virus (Temin, 1963, Temin, 1976). The enzyme - reverse transcriptase - needed for this process is carried by the mature virion. The viral DNA contains repeated sequences called long terminal repeats (LTR) at the 5' and 3' ends of the molecule. The LTR contains the enhancer and promoter sequences which regulate viral transcription (see Fig. 1.6.Ei). The DNA form of the retroviral genome has a common arrangement viz. LTR-gag-pol-env-LTR (5' - 3').

1.2.B Retroviridae - Classification

The Retroviridae family has traditionally been divided into three subfamilies viz. Oncovirinae, Lentivirinae and Spumavirinae (Teich, 1984) based broadly on pathogenicity, transmission and epidemiology. The oncoviruses are associated with malignant tumours, lentiviruses with slow, progressive disease and spumaviruses with foamy degeneration of infected cells *in vitro*. The oncoviruses can be further subdivided into endogenous and exogenous groups. The endogenous oncoviruses are those that are transmitted as the integrated viral DNA in the germline only between parent and offspring. The exogenous oncoviruses can be transmitted horizontally between members of the host species. In 1992, Coffin proposed a new classification of retroviruses into seven genera - based on nucleotide and amino acid sequence similarities as well as virion structures (Coffin, 1992). The proposed seven genera, with the common strains, are :

(i) Avian C-type (e.g. Avian leukosis virus, Rous sarcoma virus)

(ii) Mammalian B-type (e.g. Mouse mammary tumour virus)

(iii) Mammalian C-type (e.g. Moloney murine leukaemia virus, Spleen necrosis virus)

(iv) Mammalian D-type (e.g.Mason-Pfizer monkey virus)

(v) Human T-cell Leukaemia/Lymphotropic Virus & Bovine leukaemia virus (HTLV & BLV)

(vi) Spumavirus (e.g. Simian foamy virus)

(vii) Lentivirus (e.g. Human immunodeficiency virus 1 & 2, Visna virus, Equine infectious anaemia virus)

The avian C-type and mammalian B, C & D-type retroviruses are members of the Oncovirinae subfamily. Retroviruses have also been classified into Types A to D according to their morphology (by electron microscopy) and budding charateristics (Bernhard, 1960, Teich, 1984). The size of a retrovirus ranges from 80-120 nm in diameter. The A type structure is observed only intracellularly within the cell cytoplasm or cisternae and the particles are not known to be infectious. Similar structures have been observed in certain mammalian tumour cells, likely a product from endogenous, integrated defective proviral DNA (Kuff & Leuders, 1988, Wilkenson *et al.*, 1994). The B-type particles, unlike A-type, are extracellular and possess an envelope. Characteristic features of the B-type particles are the fact that the virion core is located eccentrically within the envelope and the presence of external spikes. The

majority of retroviruses are of the C-type structure and the mature virions bud from the cell membrane. Unlike the B-type particle, the C-type particle has a centrally located electron-dense core within the viral envelope. C-type viruses are rarely observed within the cell cytoplasm, but can be seen budding from the plasma membrane, when observed under the electron microscope. Bovine leukemia (BLV) and human T-cell leukaemia (HTLV) viruses are type C retroviruses. D-type particles are seen in both intra (immature form) as well as extracellular locations (mature form). Extracellular D-type particles contain a centrally located core within the envelope which may contain spikes - usually shorter than those in B-type viruses. The basic characteristic of lentiviruses (prototype maedi-visna virus) is their slow chronic course of infection. Unlike the majority of retroviruses which have spherical cores, lentiviruses have characteristic cylindrical or cone-like cores within the viral envelope.

1.2.C Scope of this introduction

This part of the thesis briefly reviews the structure, genomic organization, replication and pathogenesis of the human immunodeficiency virus type 1. HIV-2, mainly found in West Africa, also causes a similar syndrome, but with a longer period of disease development than HIV-1 (Markovitz, 1993). Each of the introductory sections will discuss the relevant literature in regard to original and representative reports on specific topics. Where relevant, reports of experiments with other retroviruses would be compared and discussed.

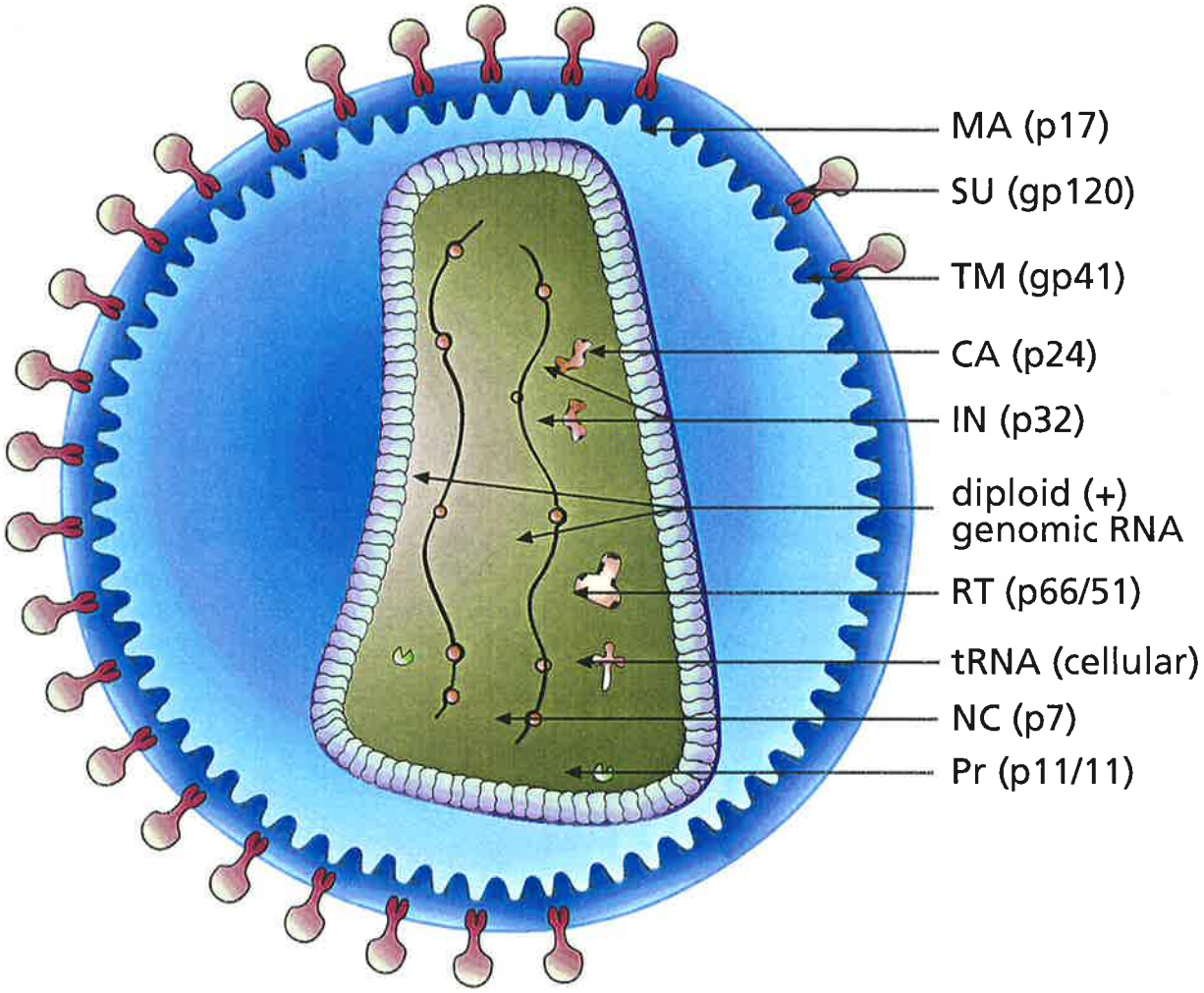
1.3.A Structure of the human immunodeficiency virus (HIV)

Figure 1.3.Ai shows a representative diagram of the human immunodeficiency virus. The enveloped virion is *ca.* 110 nm in diameter. The envelope surrounds the viral matrix which is composed of matrix protein (MA) p17. The matrix contains a capsid or core in the shape of a cone or trapezium as observed in electron micrographs (Gelderblom *et al.*, 1988). This core contains the genomic RNA (diploid, positive strand) associated with viral nucleocapsid protein (NC) p7, integrase (IN) p32, reverse transcriptase (RT), protease p11 and tRNA molecules. (The convention of using a two-letter mnemonic is used when referring to virion proteins (Leis *et al.*, 1988) and three-letter names in italics and lower-case to refer to the genes (Baltimore, 1975))

Fig. 1.3.Ai

Diagrammatic representation of Human Immunodeficiency Virus

MA	Matrix
SU	Surface glycoprotein
TM	Transmembrane glycoprotein
CA	Capsid
IN	Integrase
RT	Reverse transcriptase
tRNA	transfer RNA (cellular)
NC	Nucleocapsid
Pr	Protease


MA (p17)
SU (gp120)
TM (gp41)
CA (p24)
IN (p32)
diploid (+)
genomic RNA
RT (p66/51)
tRNA (cellular)
NC (p7)
Pr (p11/11)

1.3.B The envelope proteins

The viral envelope is a lipid bilayer derived from the host plasma membrane when the virus buds from the cell. The ability of certain strains of retroviruses to induce syncytium formation by infected cells is mediated via the viral envelope proteins (Rowe *et al.*, 1970). There are two major envelope, viz. a transmembrane glycoprotein (gp41, designated TM) with a molecular weight of 41 kD and an external protein (gp120, designated SU, for surface) of M.W. 120 kD. These two envelope proteins, non-covalently associated in the mature virion, are cleaved from a glycosylated polyprotein precursor of 160 kD (gp160). The spikes on HIV are composed of oligomers of these two glycoproteins (Fig. 1.3.Ai). As with all retroviruses and indeed with many mammalian enveloped viruses (e.g. ortho- and paramyxoviruses), cleavage of the HIV envelope gp160 precursor protein is necessary for expression of viral infectivity to allow fusion between the virus and the target cell (McCune *et al.*, 1988). It has been reported that eighty-five percent of the initially synthesized gp160 is degraded in lysosomes and only the cleaved proteins reach the cell surface (Willey *et al.*, 1988). The precursor and cleaved proteins are extensively glycosylated as nearly half of the molecular weight of the cleaved proteins are attributable to asparagine-linked carbohydrate (Allan *et al.*, 1985a, DiMarzo-Veronese *et al.*, 1985). This extensive glycosylation suggests that intracellular processing proceeds from the rough endoplasmic reticulum to the Golgi complex and then to the plasma membrane.

The gp120 glycoprotein of the human immunodeficiency viral (HIV-1) envelope contains three major antigenic domains - viz. the variable loops V1/V2 and V3 - which elicit strain-specific neutralizing antibodies. In addition, these variable loops determine viral tropism, in conjunction with other cell surface co-receptors (see section 1.6.A) for entry and infection of lymphocytes (Hwang *et al.*, 1991, Weiss, 1996), monocytes (Westervelt *et al.*, 1991) and macrophages (Koito *et al.*, 1994). Genetic changes, with single point mutation, in the variable V1/V2 domains of gp120 can alter cell tropism (Tsai *et al.*, 1992, Boyd, 1993). Mutation in the V3 loop resulted in a change of tropism from neural cell infection to macrophage infection (McKnight *et al.*, 1995). After seroconversion HIV sequence variants with different replication capacities, changes in syncytium-inducing properties and cellular tropism are seen (Evans *et al.*, 1987, Roos *et al.*, 1992, Schuitemaker *et al.*, 1992). Such mutations in the hypervariable V3 domain may then lead to escape from neutralization by anti-V3 antibodies as has been

observed *in vivo* (McKeating *et al.*, 1989, Arendrup *et al.*, 1992). Antibodies to the two major envelope proteins are detectable in the sera of HIV infected patients and are the main markers used in diagnostic tests.

1.3.C The core proteins

The core proteins are synthesized as a 55kD Gag precursor protein which is cleaved into the p24 capsid (CA), p17 matrix (MA), p7 nucleocapsid (NC) and p6 proteins by the viral protease (aspartyl proteinase). The hydrophobic capsid is the major protein component of the virion core. This can be detected in the sera of patients during the early and late stages of infection and is also used as a marker of viral replication in cell cultures. The matrix protein, located on the inner face of the viral envelope, is modified at the amino terminus by the addition of myristic acid. It is this p17 matrix protein which provides structural integrity to the virion shell (Gelderblom *et al.*, 1989). Mutations in the MA protein have been reported to cause substantial reduction in the levels of the viral envelope proteins and it was shown that incorporation of the envelope proteins into mature virions required the p17 matrix protein (Yu *et al.*, 1992). One of the two possible nuclear localization signals in HIV may be located within amino-acids 25-33 in the basic region of the matrix protein (Bukrinsky *et al.*, 1993b, Gallay *et al.*, 1995, Nadler *et al.*, 1997). However, recent studies have provided contrasting evidence that a nuclear localization signal within the MA protein is unlikely to be essential as mutations in the amino-terminal basic region of this protein failed to yield a virus that was replication-impaired in non-dividing or dividing cells (Fouchier *et al.*, 1997). An earlier report using similar mutants (amino-acid substitutions in the basic domain of the matrix protein) showed that infectivity in monocyte-derived macrophages was retained (Freed *et al.*, 1995). The use of experimental systems with different fusion proteins and cell types (as well as the likely weak nuclear localization signal, if present, of the MA protein) may contribute to conflicting reports (Bukrinsky & Haffar, 1997).

1.3.D Nucleocapsid protein

The nucleocapsid (NC p7) protein of HIV is found within the virion core and is tightly associated with the genomic RNA. This small basic protein of 72 amino acids is processed from the NC p15 protein (DiMarzo-Veronese *et al.*, 1987). Other retroviruses, such as murine

leukemia virus, have a similar NC protein and are associated with the viral RNA (Henderson *et al.*, 1981). Comparisons of this p7 protein with those of other retroviruses and retrotransposons show a highly conserved amino-acid sequence of the form Cys-X_2-Cys-X_3-His-X_3-Cys which binds zinc ions (Schiff *et al.*, 1988, South *et al.*, 1990). The Cys-His motif sequence (also called cysteine array) is similar to the zinc-finger domains in DNA-binding proteins. The NC p7 protein is necessary for viral RNA packaging, as deletions within the Cys-His motif sequence lead to loss of viral genomic RNA packaging during virus assembly as well as loss of infectivity despite formation of intact virion particles (Gorelick *et al.*, 1990, DeRocquigny *et al.*, 1992). In addition, the NC p7 protein is necessary for dimerization of the viral genomic RNA and binding of the replication primer tRNA onto the viral RNA (Darliz *et al.*, 1990).

1.4.A Genomic Organization

HIV and other retroviruses are the only virus family with a diploid genome. Although the genome of the retrovirus is generally diploid, deviations from this number have been reported (Coffin, 1979). Like other retroviral genomes, the 5' end of the genomic RNA is capped with 7-methylguanosine and the 3' end is polyadenylated (Guntaka, 1993). Although capping is usually associated with translation, the retroviral plus-strand genomic RNA does not serve as mRNA. To date there is no known function for this 5' capping of the retroviral genomic RNA. Polyadenylation, *ca.* 200 adenosine residues, like those of other viral mRNA as well as eukaryotic mRNA serves to facilitate translation. This is unlike the case with eukaryotic mRNA where the cap and poly (A) tail act as mutually dependent regulators of translational efficiency (Gallie, 1991). However, with the HIV and indeed other retroviral genomic RNA, the function of polyadenylation is not fully resolved. A recent study reported the inhibition of the HIV 5' LTR poly (A) site by the interaction of small nuclear ribonucleoproteins with the major splice donor site which is invariably used whenever the HIV transcript is spliced (Ashe *et al.*, 1997). This is in contrast to the report that the enhanced use of the HIV-1 poly (A) site is a direct result of the splicing reaction (Scott & Imperiale, 1996).

The HIV genomic RNA is 9181 bases in length (Myers *et al.*, 1996). Figure 1.4.Ai shows the genomic organization of HIV-1. Unlike the simpler retroviruses (e.g. avian and murine retroviruses) the HIV genome is more complex and has six regulatory and accessory genes in

Fig. 1.4.Ai

Landmarks on HIV-1 Genomic RNA

Structural elements:

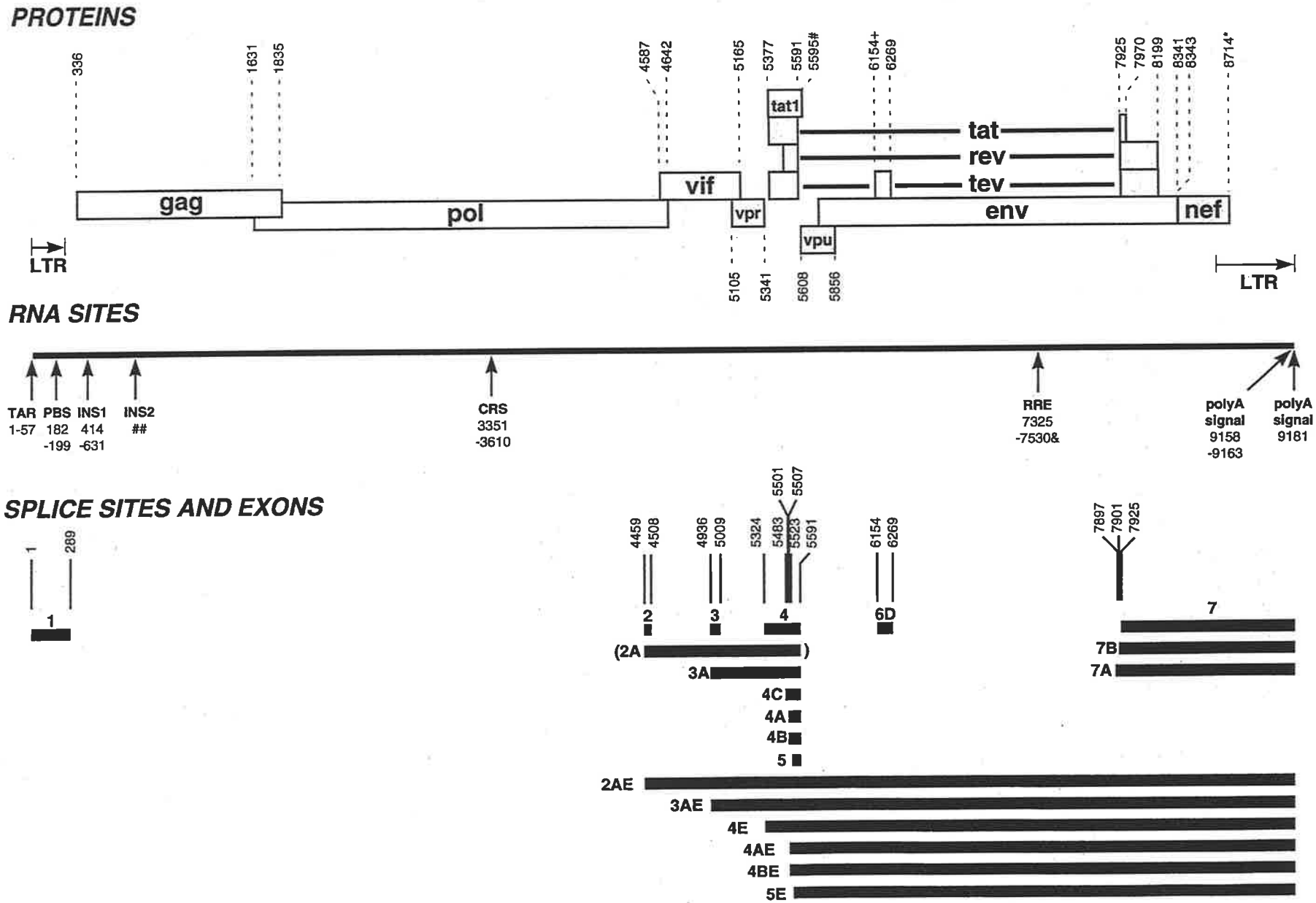
LTR	long terminal repeat
TAR	target sequence for viral transactivation, the binding site for Tat and cellular proteins
RRE	Rev responsive element
CRS	cis-acting repressive sequences
INS	inhibitory/instability RNA sequences

Genes:

GAG	Genomic region encoding the capsid proteins (group specific antigens)
POL	genomic region encoding the viral protease, reverse transcriptase and integrase
ENV	Envelope gene
TAT	Transactivator of HIV gene expression
REV	Regulatory factor for HIV expression
VIF	Viral infectivity factor
VPR	Viral protein R
VPU	Viral protein U
NEF	Negative factor which regulates viral replication *in vivo*

(adapted from Myers *et al.* 1996)

LANDMARKS ON THE HIV-1 GENOMIC RNA



addition to the *gag, pol* and *env* genes common to all retroviruses. The *gag, pol* and *env* are classed as structural genes; *tat, rev* and *nef* as regulatory genes and *vif, vpr* and *vpu* as accessory genes.

1.4.B Structural genes and their products (Gag-Pro-Pol and Env)

The *gag* gene codes for the group specific antigens which are cleaved from a 55 kD gag polyprotein precursor Pr55 [NH2 - p17 (MA) - p24 (CA) - p7 (NC) and p6 - COOH]. The viral protease cleaves this polyprotein into the p17 matrix, p24 capsid, p7 nucleocapsid proteins and a small p6 polypeptide. The three main viral enzymes - protease, reverse transcriptase and integrase - are initially produced as a Gag-Pro-Pol precursor fusion protein (Pr160) which is subsequently cleaved by the viral protease to generate the mature enzymes found in the infectious virion. As the 3' end of the *gag* gene overlaps the 5' end of the *pol* gene by 241 nucleotides (Ratner *et al.*, 1985b, Wain-Hobson *et al.*, 1985) the synthesis of this Gag-Pro-Pol precursor has been shown to be a result of translational suppression by ribosomal frameshift and not due to mRNA processing (Jacks *et al.*, 1988, Wilson *et al.*, 1988). Translational suppression is the continual synthesis of the protein by the ribosomes extending beyond the termination codon. Indeed all retroviruses use translational suppression in *pol* gene expression and only differ in the particular mechanism of this supression - either 'inframe'/'readthrough' suppression or ribosomal frameshift. Only the mammalian type C retroviruses use readthrough suppression for *pol* gene expression (Panganiban, 1988, Jacks, 1990).

The viral protease is carried within the virion core and performs proteolytic cleavage of the Gag and Gag-Pol fusion proteins into the active components essential for viral assembly to form the mature virions. It thus offers an attractive anti-viral target for therapeutic intervention. (A class of the recently used anti-HIV drugs inhibits this viral protease, see (Bartlett & Moore, 1998).) The HIV protease is a small protein of 99 amino acids (11 kD) and belongs to the aspartyl-protease family of enzymes which is characterized by the conserved Aspartic acid-Threonine-Glycine sequence (Pearl & Taylor, 1987). It is one of the four categories of proteolytic enzymes classified on the nature of the catalytic mechanism viz. serine proteases, sulfhydryl proteases, metalloproteases and aspartyl proteases (Fitzgerald & Springer, 1991).

Crystallographic studies show this protease to be a dimer of two identical subunits and dimerization is essential for proteolytic activity (Lapatto *et al.*, 1989, Navia *et al.*, 1989, Wlodawer *et al.*, 1989). The catalytic site is at the centre between the monomers and contains the aspartic acid residue. This protease may not only function during budding and maturation; but may have a role during the early steps of viral infection (Nagy *et al.*, 1994).

The HIV reverse transcriptase is translated as a component of the Gag-Pro-Pol polyprotein precursor which is then cleaved by the viral protease to yield the active forms of this and other enzymes (Farmerie *et al.*, 1987, Mous *et al.*, 1988). The active form of RT is a heterodimer of 66-kD and 51-kD polypeptides which have identical N-termini (Lightfoote *et al.*, 1986, DiMarzo-Veronese *et al.*, 1989). The p66 subunit contains the two separate domains of DNA polymerase and RNaseH activities (Tanese & Goff, 1988), whereas the p51 subunit is a carboxy-terminal truncation (by the viral protease) of the larger subunit and lacks RNase H activity (Hostomsky *et al.*, 1991, LeGrice *et al.*, 1991). Though structurally distinct, these two domains are functionally interdependent. The RNaseH domain is located within the C-terminal portion of RT and its absence leads to a decrease of DNA polymerase activity (Hostomsky *et al.*, 1991). The reverse transcriptase lacks proof-reading functions and consequently is error-prone (Patel & Preston, 1994); in particular the HIV RT has been reported to have a nucleotide misincorporation frequency of 1:1700 to 1:4000 (Preston *et al.*, 1988, Roberts *et al.*, 1988). This rate of misincorporation is more than ten fold higher than avian myelobastosis or murine leukemia virus RT. This may contribute to the high mutation rates observed during replication of the HIV. When compared to the high fidelity eukaryotic DNA polymerases γ and δ (which contain proof-reading exonuclease activity (Kunkel *et al.*, 1987, Kunkel & Soni, 1988)), misincorporation by the HIV RT is greater by 16 to 100 fold respectively (Roberts *et al.*, 1988). These earlier mutation rate studies of the HIV RT used purified enzyme with cell-free systems. A lower mutation rate of 3.4×10^{-5} per bp per cycle was reported for the HIV RT with *in vivo* studies (Mansky & Temin, 1995) compared to *ca.* 5 to 6.7×10^{-4} with a DNA template in an *in vitro* system (Roberts *et al.*, 1988). The RT has four major enzymatic activities viz. RNA-directed DNA synthesis, degradation of RNA in

RNA-DNA hybrids (RNase H activity), DNA-directed DNA synthesis and specific cleavage of RNA at the 5' of U3 (see reverse transcription section 1.6.C).

The integrase protein is carried within the virion core. The 3' end of the *pol* gene encodes the integrase protein which is essential for a major step in the replication of retroviruses - integration of the reverse-transcribed viral DNA into the host DNA. The integrase has been shown to be necessary and sufficient for integration of the viral DNA into the host DNA (Katz *et al.*, 1990, Goodarzi *et al.*, 1995, Wiskerchen & Muesing, 1995, Leavitt *et al.*, 1996). This protein has two enzymic functions - viz. cleavage (Craigie *et al.*, 1990, Pauza, 1990) and joining (Bushman *et al.*, 1990) of DNA molecules to insert the viral DNA to the host DNA. The viral integration process is discussed in section 1.6.D.

1.4.C Regulatory genes and proteins (Tat, Rev and Nef)

In addition to the genes described above that code for the structural proteins, the HIV genome contains three main regulatory genes (Cullen, 1998, Emerman & Malim, 1998). These genes regulate viral replication and are located between the *pol* and *env* regions, with some overlapping each other (see Fig. 1.4.Ai). The products of the three main regulatory genes of HIV are Tat, Rev and Nef which are translated from more than 25 mRNAs (Purcell & Martin, 1993). The mRNAs that encode these proteins all have a common leader sequence (the first 289 bases from 5' end of genomic RNA) as well as a common 3' terminal exon (Exon 7, 1256 bp) (Muesing *et al.*, 1985, Myers *et al.*, 1996) and are produced by alternative splicing of overlapping genes in the HIV genome (Arrigo *et al.*, 1990, Guatelli *et al.*, 1990, Schwartz *et al.*, 1990a, Purcell & Martin, 1993). These regulatory genes and their proteins are essential for viral replication and each will be discussed in the following sections.

1.4.C.i Tat

HIV and other complex retroviruses (e.g. lentiviruses, HTLV, spumaviruses and bovine leukaemia viruses) use viral encoded activators to regulate their transcription. In HIV the *trans*-acting Tat protein is a potent activator of viral transcription. This protein (16 kD, 86-101 amino acids) is encoded from two exons within the central region and *env* gene of the HIV genome

(Purcell & Martin, 1993) and is produced from eight doubly or triply spliced mRNAs (Schwartz *et al.*, 1990a, Purcell & Martin, 1993). However, the two-exon Tat predominates in infected cells (Felber *et al.*, 1990, Rosen & Pavlakis, 1990) and is located within the nucleus/nucleolus of infected cells (Ruben *et al.*, 1989). Tat increases the steady state levels of all HIV mRNAs (Wright *et al.*, 1986, Sadaie *et al.*, 1988) by increasing the rate of transcription from the HIV LTR (Hauber *et al.*, 1987) as well as decreasing the level of premature transcription termination; this has been shown by the use of LTR-driven reporter gene plasmids (Kao *et al.*, 1987, Laspia *et al.*, 1989) and nuclear run-on assays that used HIV proviruses with mutations in the Tat protein (Feinberg *et al.*, 1991). *In vitro* studies have also shown that Tat enhances by two to three orders of magnitude in transcription driven by the viral LTR (Laspia *et al.*, 1989). This protein is essential for HIV replication as Tat-defective mutants do not produce virus unless it is provided *in trans* (Dayton *et al.*, 1986, Fisher *et al.*, 1986).

The Tat protein functions by binding to the *cis*-acting Tat-responsive sequence (TAR - Transactivation response element) (Berkhout *et al.*, 1989) at the site of the viral RNA bulge-loop structure formed at the 5' end of nascent viral RNA (Dingwall *et al.*, 1990, Roy *et al.*, 1990, Gait & Karn, 1993). The TAR element consists of a stem, a 3-nucleotide bulge and a six-nucleotide loop (hairpin) at the end of the stem (Jeang, 1996) (Fig. 1.4.Ci). The arginine residues within the basic domain of Tat interact with the uridine residues at the base of the stem-loop bulge (Frankel, 1992). These arginine residues play a similar role in other sequence-specific RNA-binding proteins (Gait & Karn, 1993). The secondary structure of the TAR RNA stem-loop is important for its function and forms the 5' end of all HIV mRNA species (Cullen, 1990). The highly conserved hairpin structure of TAR and the specific uridine residues are essential for transcription activation by Tat. The direct binding of Tat to the TATA-binding protein of TFIID basal transcription factor suggests that this viral protein has strong association with cellular transcription complexes (Kashanchi *et al.*, 1994, Veschambre *et al.*, 1995). In addition to RNA binding, the Tat protein binds to cellular proteins which may enhance HIV LTR trans-activation (Desai *et al.*, 1991, Shibuya *et al.*, 1992, Yu *et al.*, 1995). It is also likely that Tat remains associated with the elongating cellular RNA polymerase II transcription complexes (Keen *et al.*, 1996). Recent studies have shown that the transactivation domain of

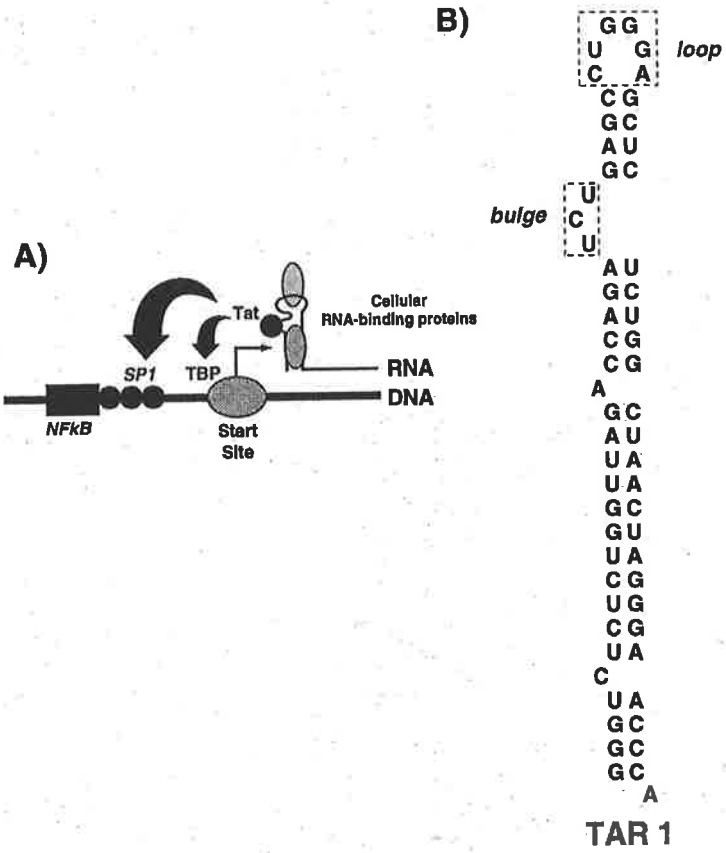
Fig. 1.4.Ci

Interaction of Tat with DNA and RNA targets in HIV-1 LTR

A A schematic representation of the functional interactions between Tat, TAR-RNA-binding proteins and promoter elements. Biochemical evidence exists that Tat contacts directly SP1 and TATAA-binding protein (TBP).

B Secondary structure of TAR RNA. The crucial trinucleotide bulge and hexanucleotide loop elements are boxed.

(From Myers *et al*, 1996)


A)
Tat
Cellular
RNA-binding proteins
SP1
TBP
RNA
DNA
NFkB
Start
Site
B)
loop
bulge
TAR 1

Tat interacts strongly with a nuclear Tat-associated kinase (Jones, 1997) which has identical sequence to the kinase sub-unit of P-TEFb (Mancebo *et al.*, 1997, Zhu *et al.*, 1997). The latter is a positive-acting transcription elongation factor which acts with RNA polymerase II for transcript elongation (Marshall & Price, 1995).

1.4.C.ii Rev

There are twelve doubly or triply spliced mRNAs which code for the Rev protein of 116 amino acids (Schwartz *et al.*, 1990a, Purcell & Martin, 1993). The Rev protein contains a series of highly charged basic amino acids which serve as nuclear localization signal (Cochrane *et al.*, 1990). This protein is essential for the transport of unspliced and singly-spliced HIV RNA species from the nucleus to the cytoplasm (Felber *et al.*, 1989, Malim *et al.*, 1989). Unspliced mRNAs may need to be polyadenylated in order to be transported by the Rev protein from the nucleus to the cytoplasm (Huang & Carmichael, 1996). Rev functions by binding with high affinity to a *cis*-acting sequence (234 nt in length) called the Rev-responsive element (RRE) which is located within the HIV *env* gene (Rosen *et al.*, 1988, Malim *et al.*, 1989). The Rev protein was first located in the infected cell's nucleus/nucleolus (Cullen *et al.*, 1988); however this protein has since been shown to accumulate passively in the cytoplasm and may shuttle between the cytoplasm and the nucleus (Fischer *et al.*, 1994, Kalland *et al.*, 1994, Meyer & Malim, 1994, Richard *et al.*, 1994). The Rev protein has two main functional domains - the amino terminal and the activator/effector domains (Venkatesh & Chinnadurai, 1990, Malim *et al.*, 1991). The amino terminal domain is necessary and sufficient for RRE binding, Rev multimerization and nuclear translocation (Berger *et al.*, 1991, Malim & Cullen, 1991). The leucine-rich activator or effector domain of Rev is believed to interact with cellular factors required for Rev function. Many cellular RNAs require particular proteins for active nucleocytoplasmic transport through the nuclear pores (Izaurralde *et al.*, 1997, Ullman *et al.*, 1997). One of these nuclear transport proteins - Ran - has been reported to be required for Rev to function in nucleocytoplasmic transport (Izaurralde *et al.*, 1997).

Rev increases the stability of the unspliced HIV mRNA which contains the RRE, but not the multi-spliced viral mRNAs (which do not contain the RRE) as shown by the cytoplasmic

expression of a non-spliceable HIV-1 mutant *env* gene sequence (Felber *et al.*, 1989, Malim *et al.*, 1989). A similar observation has also been reported with equine infectious anemia virus - another lentivirus (Martarano *et al.*, 1994). As Rev promotes the transport of RRE-containing unspliced HIV mRNA from the nucleus to the cytoplasm, it could be expected that this would decrease the levels of unspliced mRNA available for splicing and accordingly result in lower levels of the multi-spliced viral small mRNAs in the nucleus. In this regard, Rev may be an important protein in the regulation of viral mRNA splicing as well as nucleocytoplasmic transport (Chang & Sharp, 1989). The nuclear export of unspliced HIV RNA (as well as those of visna and equine infectious anemia viruses) into the cytoplasm is determined by the nuclear export signal in the effector domain (carboxy-terminal region) of Rev (Meyer *et al.*, 1996, Whittaker & Helenius, 1998).

In addition to the regulation and nucleocytoplasmic transportation of singly spliced and unspliced viral mRNAs, the stability of HIV mRNAs and translation are two other viral processes that have been associated with Rev. The stability of HIV mRNAs is influenced by the presence of distinct cis-acting elements present in unspliced RNAs within the gag, pol and env regions (Cochrane *et al.*, 1991, Maldarelli *et al.*, 1991, Schwartz *et al.*, 1992). These elements are known as inhibitory sequences (INS) or cis-acting regulatory sequences (CRS). In the absence of Rev-RRE interactions, it has been reported that the INS decrease the expression and stability of the viral mRNAs by inhibition of cytoplasmic transport and increasing RNA degradation respectively. The latter observations were shown by mutagenesis studies of the INS in the gag-coding region, without alteration of the expressed Gag protein. The mechanism of how INS influences the stability and nuclear export of of Rev-dependent viral mRNAs is not known.

In the absence of functional Rev, HIV mRNAs were shown to have minimal binding to poly(A)-binding protein 1 (PAB1) (Arrigo & Chen, 1991, D'Agostino *et al.*, 1992, Campbell *et al.*, 1994). The binding of PAB1 to the poly(A) tail of mRNAs is essential for RNA stability and translation. The binding of eukaryotic protein synthesis initiation factor 5A (eIF-5A) to the activation domain of Rev has been reported to be associated with preferential translation of RRE-containing mRNAs (Ruhl *et al.*, 1993).

The regulatory protein - Rex - found in HTLV-1 has similar properties to Rev in HIV, viz. the transport of unspliced RNA from the nucleus to the cytoplasm (Inoue *et al.*, 1991), mRNA splicing (Itoh *et al.*, 1989) and binding to the RRE of HIV as well as the Rex-responsive element (RXRE) of HTLV-1 (Unge *et al.*, 1991). These similar properties indicate a common solution by different retroviruses to the need for regulation of mRNA splicing, nucleocytoplasmic translocation, stability and possible translational capacity.

1.4.C.iii Nef

The *nef* gene is located in a single open reading frame at the 3'end of the *env* gene and extends into the U3 domain of the 3' LTR (see Fig. 1.4.Ai). More than 80% of the multi-spliced regulatory transcripts encode Nef (Robert-Guroff *et al.*, 1990). Antibodies to this protein are found in AIDS patients early after the onset of infection (Allan *et al.*, 1985b). The *nef* gene product is a myristoylated phosphoprotein of 27 kD (210 amino acids) which is located at the inner face of the cytoplasmic membrane in expressing cells (Kan *et al.*, 1986). When this protein was reported to have sequence similarity with guanine nucleotide-binding proteins (G proteins) and guanosine triphosphatase activity, it was suggested that Nef may be phosphorylated by protein kinase C and may be associated with signal transduction (Guy *et al.*, 1987, Guy *et al.*, 1990). The use of Nef antisera in immunoprecipitation studies showed that this protein associates with cellular serine kinase suggesting its involvement in cell activation (Sawai *et al.*, 1994). In addition, Nef contains repeats of a proline motif which is a feature of cellular signal transduction proteins (Saksela *et al.*, 1995).

One of the more significant functions of Nef is its downregulation of CD4 which would be expected to decrease multiple rounds of subsequent reinfection by the released virus. Expression of this gene in CD4+ cells was associated with reduction in the levels of CD4 receptors by endocytosis (Benson *et al.*, 1993, Aiken *et al.*, 1994, Rhee & Marsh, 1994). However, the mechanism of how this endocytosis of CD4 is triggered by Nef has not been established. Nef has also been reported to cause rapid internalization of surface MHC-1 molecules followed by accumulation in endosomal vesicles and degradation (Schwartz *et al.*,

1996). This stimulation of MHC class I endocytosis by Nef was associated with resistance of HIV-infected primary T cells to CTL killing (Collins *et al.*, 1998).

Although it has been reported that Nef inhibits HIV LTR-specific gene expression and NF-kappa B transcription factor induction as well as viral replication (Ahmad & Venkatesan, 1988, Niederman *et al.*, 1989, Niederman *et al.*, 1992) these observations have not been confirmed by others (Hammes *et al.*, 1989, Kim *et al.*, 1989a). (The earlier reports of Nef acting as an inhibitor of gene expression at the LTR led to this acronym for negative factor.) This is further complicated by the report that Nef-deficient mutants do not affect viral replication in cultured cells, although this protein has been shown to be required for production of high viral loads in rhesus monkeys infected with a pathogenic molecular clone of simian immunodeficiency virus (Kestler *et al.*, 1991). In other animal studies, *nef* mutant virus did not induce depletion of differentiating human thymocytes in severe combined immunodeficient (SCID) mice unlike the parental viral strain (Jamieson *et al.*, 1994). Some viral isolates from HIV-infected patients who were considered long-term survivors/non-progressors have been reported to have major deletions in the *nef* and *nef*-LTR overlap regions (Deacon *et al.*, 1995, Kirchhoff *et al.*, 1995). These HIV patients are clinically healthy and show both intact humoral and cellular responses (Levy, 1993, Schrager *et al.*, 1994, Cao *et al.*, 1995). However, the *nef* genotype and phenotype from other analyses of long term survivors were shown to be intact (Huang *et al.*, 1995a, Huang *et al.*, 1995b). More recently, it was reported that *nef* mutant viruses are 4 - 40 times less infectious than the wild type in a single-round infection system (Aiken & Trono, 1995). When the Nef protein was provided in a *trans* complementation assay to *nef*-defective virus producer cells, the viral infectivity was enhanced in the co-culture; suggesting a possible stimulation of proviral DNA synthesis (Aiken & Trono, 1995). These conflicting findings indicate that the true functions of Nef in viral replication and in pathogenesis of infection *in vivo* remains unclear.

1.5.A Accessory genes and proteins (Vpr, Vif and Vpu)

In addition to the structural (*gag, pol* and *env*) and the major regulatory genes (*tat, rev* and *nef*), the HIV contains three accessory (or non-essential) genes viz. *vpr, vif* and *vpu*. The latter were

termed 'accessory' because these genes were not absolutely required for viral replication *in vitro*. Subsequent *in vivo* studies and analyses of viral isolates from patients have identified functions that require the protein products of these genes.

1.5.A.i Vpr

The viral protein R - Vpr protein (15 kD, 96 amino acids) - is found within the virion and sera from HIV-infected patients show specific antibodies to this protein (Wong-Staal *et al.*, 1987, Cohen *et al.*, 1990a, Cohen *et al.*, 1990b). Incorporation of Vpr within the virion requires the interaction of this protein with the carboxyl-terminal domain of the Gag precursor. This protein enhances viral cytopathicity (Ogawa *et al.*, 1989) and replication by *trans*-activation of the HIV LTR (Cohen *et al.*, 1990b). An intact *vpr* sequence has been reported to increase viral load and progression to AIDS (Hattori *et al.*, 1990). Two major functions have been identified for Vpr - viz. nuclear targeting of the viral pre-integration complex and arrest of cell cycle at the G_2/M phase. One of the nuclear localization signals in the HIV is located within the Vpr. This protein is required for translocating the preintegration or viral replication complex - formed after reverse transcription (see section 1.6.C) - from the cytoplasm to the nucleus in nondividing cells (Emerman *et al.*, 1994, Freed & Martin, 1994, Heinzinger *et al.*, 1994). In virus-infected cells Vpr is detected largely within the nucleus (Lu *et al.*, 1993), although punctate cytoplasmic staining has also been reported (Zhou *et al.*, 1998). This protein has recently been shown to regulate the nuclear import of HIV pre-integration complex by association with karyopherin α (Gallay *et al.*, 1996), which results in the increased binding of this cellular receptor for basic-type nuclear localization signals (Popov *et al.*, 1998). The nuclear targeting function of Vpr has been reported to be associated with both N-terminal α-helix region (Mahalingam *et al.*, 1997, Nie *et al.*, 1998) and the C-terminus (Zhou *et al.*, 1998). As nuclear localization signals usually consists of basic residues within the C-terminus of the protein, these latter reports on the associations of the amino and carboxy termini of Vpr with nuclear localization may reflect differences in experimental systems and possible interactions of this protein with those containing similar nuclear targeting functions (e.g. MA) (Heinzinger *et al.*, 1994).

Vpr was first reported to arrest cell growth and induce differentiation in rhabdomyosarcoma cell line (Levy *et al.*, 1993). Subsequent studies have shown that this protein can cause the accumulation of cells in the G_2/M phase of the cell cycle by dephosphorylating the inactive p34cdc2 cyclin B kinase (present at the G1/S phase) to the active form at the G_2/M phase (He *et al.*, 1995, Jowett *et al.*, 1995, Re *et al.*, 1995). A recent study showed that the arrest of infected, dividing cells at the G_2/M phase of the cell cycle by Vpr (Conti *et al.*, 1998) leads to a reduction in apoptosis or programmed cell death (Shen & Shenk, 1995, Vaux & Strasser, 1996). By prolonging survival of infected cells, this effect might help maintain persistence of HIV infection.

1.5.A.ii Vif

The viral infectivity factor (Vif) is a highly basic protein of 23 kD (Lee *et al.*, 1986) and is found predominantly in the cytoplasm of infected cells as well as being membrane-associated (Goncalves *et al.*, 1994, Simon *et al.*, 1997). In cell-free viral infection, viruses which lack the Vif protein are 1000-fold less infectious than wild type virus (Fisher *et al.*, 1987, Strebel *et al.*, 1987). However, with cell-to-cell transmission of infection there was no significant reduction in infectivity with Vif^- mutants. These observations suggest that released HIV virions may need to be modified by this protein (likely prior to release) in order for infection by the cell-free viral route. Vif^- mutants of HIV-1 show assembly or maturation defects and lack the characteristic cone-like structure of the mature HIV-1 virion core (Hoglund *et al.*, 1994, Borman *et al.*, 1995). A latter report with vif^- mutants in cell-free viral infection showed that these virions were defective in their ability to synthesize viral DNA in an endogenous reverse transcriptase reaction (Goncalves *et al.*, 1996).

In addition Vif may assist to transport incoming virions to the nucleus as this protein was shown to interact with vimentin - a principal intermediate filament protein in the cytoskeleton which connect the plasma and nuclear membranes (Karczewski & Strebel, 1996). Another role of Vif has been suggested by the rapid degradation of reverse transcripts in Vif^- mutant cell-free

viral infection but not with wild-type virus (Simon & Malim, 1996). Thus defects in assembly, reverse transcription or nuclear transport may explain the reduced infectivity with these mutants in cell-free viral infection.

Although the mechanism of how Vif modulates viral infectivity and other functions of this protein remain to be elucidated, the observations that this gene is conserved among all lentiviruses (except equine infectious anemia virus) (Oberste & Gonda, 1992), and that an intact *vif* gene is invariably present in viral isolates from infected patients (Wieland *et al.*, 1994) suggest the importance of this protein in the replication cycle of primate lentiviruses (Simon *et al.*, 1995).

1.5.A.iii Vpu

The Vpu protein (16 kD, 81 amino acids) is unique as it is found only in HIV-1, but not in HIV-2 or other lentiviruses (Strebel *et al.*, 1988, Strebel *et al.*, 1989). The Vpu is a transmembrane phosphoprotein consisting of 27 hydrophobic amino acids at the N-terminal domain and 54 amino acids at the hydrophilic cytoplasmic domain. The hydrophobic amino terminal domain acts as membrane anchor as well as endoplasmic reticulum targeting signal. The 3' end of the *vpu* gene overlaps the 5' end of the *env* gene (see Fig. 1.4.Ai) and both of these proteins are translated from the bicistronic mRNA (Schwartz *et al.*, 1990b). The Vpu protein has been shown to have two distinct functions in the life cycle of the HIV - the enhancement of viral particle release from the cytoplasmic membrane and induction of CD4 degradation in the endoplasmic reticulum. Although this protein is not essential for viral replication in cell cultures and is not present in the virion, there is a 5-10 fold reduction in progeny virus production with Vpu^- mutant infection of T-cells and is associated with a defect in virion release (Strebel *et al.*, 1988). Vpu^- mutant viral infection of T-cells show an increased accumulation of cell-associated virions (Terwilliger *et al.*, 1989) which can be subsequently released by vigorous shaking of the cultures to yield infectious virions (Klimkait *et al.*, 1990). The structural similarity of Vpu to M2 ion channels of influenza A virus (Strebel *et al.*, 1988, Klimkait *et al.*, 1990) and the formation of cation-selective channels with *in vitro* studies have led to suggestions that this protein may enhance virus release (Ewart *et al.*, 1996).

Although the importance of the Influenza A virus M2 ion channels for virion uncoating has been established, the mechanism of how Vpu acts an an ion channel during HIV replication requires further studies (Lamb & Pinto, 1997). However, the studies to date suggest the importance of the Vpu protein for virion assembly/release.

The other major function of Vpu is the degradation of CD4 from complexes of CD4 and viral Env formed at the endoplasmic reticulum (Willey *et al.*, 1992b, Willey *et al.*, 1992a). The cytoplasmic domain of Vpu interacts closely with the cytoplasmic domain of CD4 (Margottin *et al.*, 1996) leading to the suggestion that the interaction may cause the release of Env proteins from the above complexes, as well as a decrease in the steady state levels of CD4 (Kimura *et al.*, 1994). Vpu does not target cell surface CD4 or CD4 that has left the endoplasmic reticulum. The induction of CD4 degradation is dependent on phosphorylation by the ubiquitous casein kinase of two conserved seryl residues (Ser52 and Ser56) in the cytoplasmic domain of Vpu (Schubert & Strebel, 1994).

Other *in vivo* functions of Vpu have been obtained from studies of macaques infected with Vpu-negative simian-human immunodeficiency virus chimeras which showed lower viral loads than with Vpu-positive virus (Li *et al.*, 1995). Deletion of the *vpu* gene reduced infectivity of the virus in severe combined immune deficient -hu mice (Aldrovandi & Zack, 1996). Although initially recognized as an accessory or non-essential protein in virus-infected cell cultures, the Vpu protein has multiple roles in the infectious process.

1.6 HIV Replication

A virus may infect a susceptible cell by the cell-free route or by cell-to-cell transmission of infection. In the cell-free route, HIV infects a susceptible cell by initial interaction of the virion envelope protein (gp120) with the CD4 cellular receptor and co-receptors. Alternatively, HIV infection can be initiated by cell-to-cell transmission in which an infected cell fuses with or otherwise transmits infection to an adjacent uninfected cell (Li & Burrell, 1992, Sato *et al.*, 1992, Barbosa *et al.*, 1994).

After entry into the cell the viral genomic RNA is reverse transcribed into the complementary double-stranded DNA molecule. The viral DNA (unintegrated form) is associated with various viral as well as cellular proteins as the pre-integration complex. This complex enters the cell nucleus where the viral DNA integrates into the host chromosome to form the proviral DNA. The integrated proviral DNA may persist as a stable component of the host chromosome for the lifetime of the cell. Under appropriate conditions, transcription from the provirus yields HIV genomic RNA and mRNAs; subsequent translation to produce viral proteins, followed by processing and assembly, forms viral particles which are released by budding from the cell membrane. Integration of the viral DNA into the host chromosome may lead to three possible outcomes of HIV infection as observed *in vitro*. One outcome is a lytic infection which leads to cell death. The second is a latent infection in which the proviral DNA is not activated and progeny virus is not produced (Zack *et al.*, 1990, Zack, 1995). The third outcome is the establishment of a persistent infection in which the proviral DNA is activated to yield mature virions but does not kill the infected cell.

1.6.A Attachment

As described earlier (section 1.3.B) the HIV envelope glycoprotein (gp 120) binds to the CD4 cellular receptor upon viral attachment, which induces the phosphorylation of CD4 (Fields *et al.*, 1988). This binding causes conformational changes in the viral envelope (Moore *et al.*, 1990), leading to virus-cell fusion. The CD4 is a member of the immunoglobulin superfamily (Roitt, 1997). The first implication that the CD4 receptor is necessary for HIV attachment was reported with the use of anti-CD4 monoclonal antibody to inhibit viral infection (Dalgleish *et al.*, 1984, Klatzmann *et al.*, 1984). However, presence of this principal receptor alone was not sufficient for viral replication, as it was subsequently shown that non-human cells which express CD4 receptor were not sensitive to HIV infection (Maddon *et al.*, 1986, Ashorn *et al.*, 1990, Clapham *et al.*, 1991). With CD4-expressing non-human cells HIV binds, but virus-host membrane fusion does not occur. This inability to support replication could be overcome by formation of heterokaryons between human and non-human cells, thus suggesting that the latter cell types lacked other co-factors or co-receptors which are necessary for HIV infection (Dragic *et al.*, 1992, Broder *et al.*, 1993). In addition, HIV-1 strains show distinct cellular tropisms (Cheng Mayer *et al.*, 1988, Fenyo *et al.*, 1988, Fauci, 1996) as macrophage tropic

(M-tropic) strains do not enter CD4 positive T cell lines. Similarly, T-cell tropic (T-tropic) strains do not generally infect macrophages. This preference for certain cell types with different HIV strains suggested the requirement for different co-receptors (Alkhatib *et al.*, 1996). Subsequently, fusin (Feng *et al.*, 1996) and CCR5 (Deng *et al.*, 1996) transmembrane proteins were identified as co-receptors for HIV infection. Fusin (previously also called LESTR or HUMSTR) or CXCR4 is used by the syncytium-inducing (SI) HIV-1 strains which are frequently found in late-stage disease (Bleul *et al.*, 1996, Oberlin *et al.*, 1996). The CCR5 co-receptor is used by the non-syncytium-inducing (NSI) macrophage-tropic HIV-1 strains which are found throughout the course of infection in the patient (Alkhatib *et al.*, 1996, Bleul *et al.*, 1997) although transmission of the virus involves mainly the latter phenotype (Roos *et al.*, 1992, Zhu *et al.*, 1993). More recent studies with the use of freshly isolated primary target cells (Langerhans and macrophages) have suggested the importance of these co-receptors in the transmission of macrophage-tropic/non-syncytium inducing HIV isolates (Zaitseva *et al.*, 1997). Other co-receptors viz CCR3 for neurotropic virus (He *et al.*, 1997), CCR2b and CCR8 have also been implicated in HIV infection (Clapham & Weiss, 1997). These transmembrane proteins are members of the chemokine (chemoattractant)-receptor superfamily which are involved in controlling activation and migration of various leukocytes to inflammation sites and lymphoid organs (Premack & Schall, 1996, Luster, 1998). Three of these chemokines - RANTES (Regulated on Activation Normal T cell Expressed and Secreted), MIP-1α and MIP-1β (Macrophage Inflammatory Protein) which binds CCR5 – were initially shown to inhibit replication of primary HIV isolates in macrophages but not T-cell line adapted strains (Cocchi *et al.*, 1995).

Recently, a new classification of HIV was proposed based on viral phenotype and co-receptor usage (Berger *et al.*, 1998). This proposed that the term R5 be used for those viruses which use CCR5, X4 for those which use CXCR4 and R5X4 for the dual co-receptor users.

1.6.B Penetration and Uncoating

The external protein (gp 120) binds to the CD4 receptor on the host cell (Kowalski *et al.*, 1987, Sodroski *et al.*, 1991) (see section 1.6.A) and subsequent fusion of the viral envelope with the

host cell membrane is mediated by the gp41 glycoprotein. The binding of gp120 and CD4 induces conformational changes which lead to increased exposure of the variable V3 loop (see below) in the gp120 subunit. One proposed mechanism suggests that this then enables the viral envelope to interact with the corresponding co-receptor on the cell (e.g. chemokine receptors - CXCR4 for T lymphocytes or CCR5 for macrophages) (see section 1.6.A) which provide the 'trigger' that may complete the fusion-inducing conformational changes between the virus and target cell (Doms & Peipert, 1997). The hydrophobic amino-terminal domain of gp41 has homology to fusion peptides (F protein) of ortho- and paramyxoviruses (Gallagher, 1987, Gonzalez-Scarano *et al.*, 1987). The 'spring-loaded mechanism' described for influenza hemagglutinin (Carr & Kim, 1993) has been proposed for the fusion of the HIV envelope and target cell membrane. It is suggested that the 'sprung' conformation of gp41 displaces its hydrophobic amino-terminal domain towards the target cell membrane; and this is believed to be the stable state after virus-cell fusion (Chan *et al.*, 1997, Weissenhorn *et al.*, 1997). Mutation or insertion of hydrophobic amino acids in the amino-terminal domain of gp41 causes >95% reduction in syncytium formation with CD4+ cells (Kowalski *et al.*, 1987), although virion production and release in a cell-to-cell transmission infection route were comparable with the wild-type virus-infected cultures (Kowalski *et al.*, 1991).

Following attachment of the virus to the cell, the HIV envelope gp120 glycoprotein binds the CD4 cellular receptor as well as with the CCR5 or CXCR4 co-receptors to release the virion core into the cytoplasm. Unlike influenza viruses in which the viral core is internalized by endocytosis, requiring low pH 5-6 within the endosomes (White *et al.*, 1982, White, 1992), the HIV and cell membrane binding process is pH-independent but requires prior activation of the T-cell (Stein & Engleman, 1991). The binding process occurs on the cell surface and was shown by the use of cells expressing the viral Env protein fusing directly with uninfected, CD4-positive cells (Broder & Berger, 1993).

1.6.C Reverse transcription of the genomic RNA

After penetration and uncoating of the virion core in the host cytoplasm the HIV diploid genomic RNA is reverse transcribed to produce the linear, double-stranded viral DNA. This process is catalyzed by the multifunctional, viral-coded reverse transcriptase (Baltimore, 1970,

Temin & Mizutani, 1970). The viral complementary DNA copy is longer than the genomic RNA owing to the addition of unique sequences called U3 and U5 at the 5' and 3'ends respectively of the DNA molecule. Thus the reverse transcription of the HIV genomic RNA produces direct repeat sequences - U3, R and U5 - called the long terminal repeat (LTR) at each end of the viral DNA.

Reverse transcription begins with the binding of a specific host-encoded transfer RNA (tRNA) to a complementary sequence - to the viral primer binding site (pbs) - at the 5' end of the viral RNA (Haseltine *et al.*, 1976, Taylor, 1977) (see Fig. 1.6.Ci). The HIV RT has a specific affinity for the $tRNA^{Lys-3}$ replication primer (Barat *et al.*, 1989, Barat *et al.*, 1991). Upon annealing of the tRNA to the pbs, a short segment of minus strand DNA (minus-strand strong-stop DNA) is synthesized from the 3' end of the primer to the 5' terminus of the viral RNA. In order to synthesize a full length minus strand DNA, the nascent minus DNA (minus strong-stop DNA), RNA primer and RT must translocate to the 3' end (r sequence) of the same (intrastrand transfer) or different viral RNA molecule (interstrand transfer). This process is referred to as the first jump or first-strand transfer reaction. Simultaneously, the 5' end (r and u5) of the viral RNA in the RNA-DNA hybrid is degraded by the RNase H activity of RT (Molling *et al.*, 1971, Jacobo-Molina & Arnold, 1991, Champoux, 1993), which allows the translocation. After translocation and annealing of the R sequence of the newly-made minus strand DNA to the complementary 3' r sequence of the genomic RNA, minus strand DNA synthesis continues to the pbs sequence at the 5' end of the genomic RNA. Similarly, the RNA template of the newly-made RNA-DNA hybrid is degraded by RT after the first strand transfer reaction.

The plus strand synthesis is initiated from short conserved, purine-rich sequences - polypurine tract (ppt) - which resists the concurrent RNase H degradation of the viral RNA template (Pullen & Champoux, 1990). This results in the formation of plus strong-stop DNA which consists of the U3, R, U5 and PBS sequences. According to one model of reverse transcription (Gilboa *et al.*, 1979, Telesnitsky & Goff, 1993) the second strand-transfer reaction (the second jump) then occurs when the complementary copies of PBS sequences at the 3' ends of plus-strand strong-stop DNA and the nascent minus-strand DNA base pair to form a partially circular molecule.

Fig. 1.6.Ci

A reverse transcription model for HIV

A Synthesis of minus-strand strong-stop DNA.

B First template switch (only intermolecular transfer shown).

C Synthesis of plus-strand DNA from the right PPT site.

D Synthesis of plus-strand DNA from the central PPT site.

E The plus-strand strong-stop DNA (from the right PPT site) is being displaced.

F Displaced plus-strand strong-stop DNA.

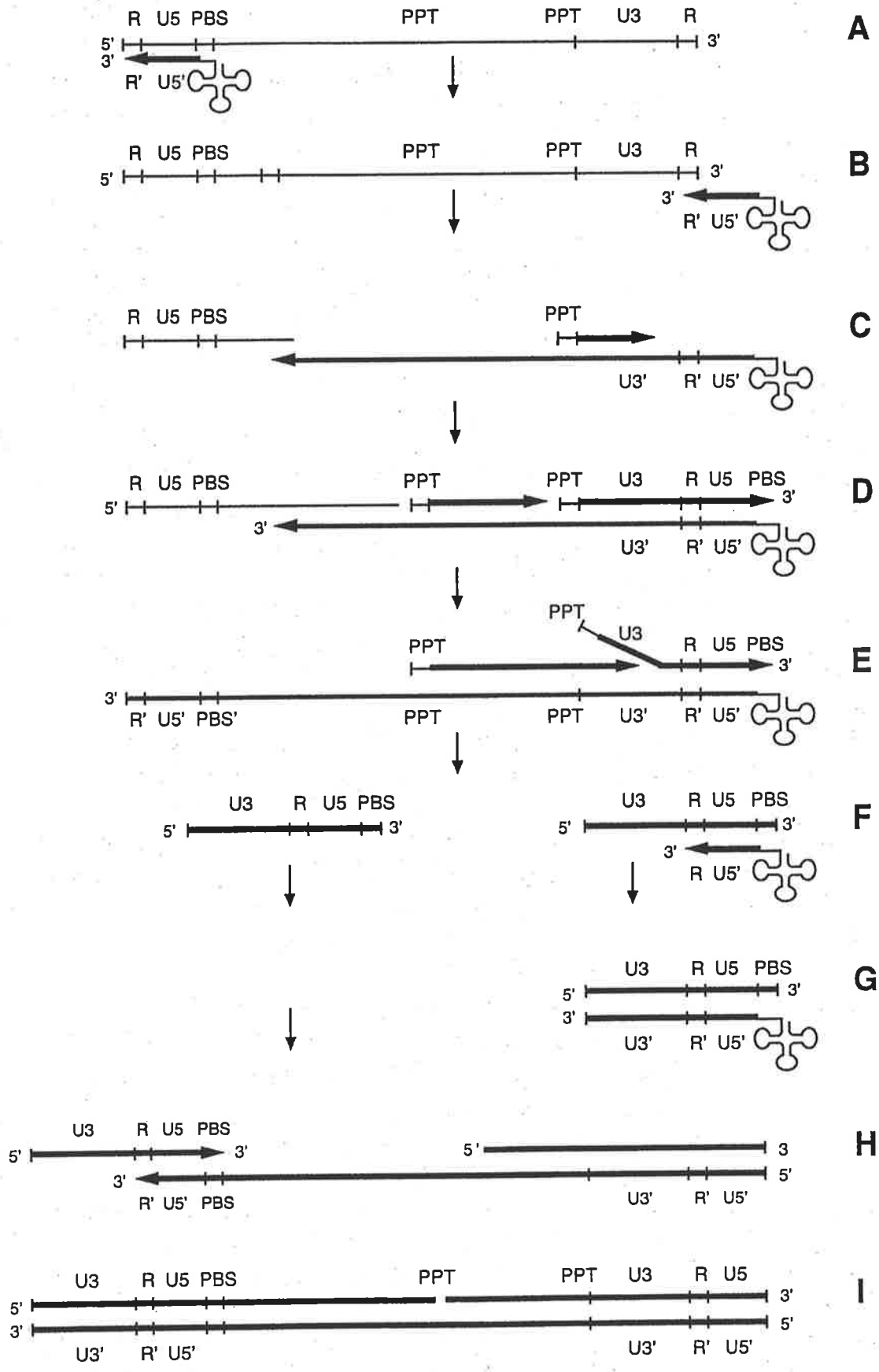
F - G Synthesis of ds strong-stop DNA.

F - H Second template switch.

I Full length ds linear viral DNA with a gap at the central PPT site.

(The thin lines represent RNA, thick lines represent DNA.)

(adapted from Li *et al.*, 1993)


A
R U5 PBS
PPT
PPT U3 R
5'
3'
R' U5'
B
C
U3' R' U5'
D
E
F
G
H
I

Minus and plus strand DNA synthesis continue with each strand using the other as template; thus resulting in the production of the complete linear, double-stranded viral DNA.

The proposed partially circular double-stranded DNA molecule formed after the second jump has to date not been detected. Subsequently, an alternative model was proposed for the second-strand transfer reaction using cell-to-cell transmission infection (Li *et al.*, 1993b). The latter model showed that the plus-strand strong-stop DNA is displaced by a growing plus-strand DNA initiated at the right or the central ppt site (D and E of Fig. 1.6.Ci) resulting in the formation of a stable double-stranded strong-stop DNA (E and F of Fig. 1.6.Ci). In addition, unlike minus-strand strong-stop DNA, the persistence of the plus-strand strong-stop DNA and its presence in abundance within the cytoplasm of infected cells (Swanstrom *et al.*, 1982, Lee & Coffin, 1991) may likely contribute to the formation and detection of the double-stranded strong-stop DNA reported by Li et al. This strand-displacement synthesis on double stranded DNA is likely catalyzed by RT (Huber *et al.*, 1989, Boone & Skalka, 1993). A second ppt sequence located at the centre of the HIV genome can potentially serve as an origin of plus-strand DNA synthesis (Charneau & Clavel, 1991, Charneau *et al.*, 1992). The displaced plus-strand strong-stop DNA can base pair with the 5' end of the nascent minus-strand DNA, at the R, U5 and PBS sites. Upon annealing, the minus and plus strand DNA can thus complete the synthesis of the full length, double stranded DNA using each as a template (G and H in Fig. 1.6.Ci). This alternative model of reverse transcription has been further supported by the observation of a distinct time lag between formation of the minus-strand strong-stop DNA (initiation of reverse transcription) and plus-strand strong-stop DNA (Karageorgos *et al.*, 1995).

The presence of the diploid RNA genome within the retrovirion has led to much discussion on intrastrand and interstrand transfer reactions during reverse transcription (Telesnitsky & Goff, 1993). Studies using two different retroviral vectors with genetic differences in both their U3 and U5 regions suggested that the first-strand transfer reaction always occurs between one copackaged RNA and the other (Panganiban & Fiore, 1988). However, reverse transcription studies from the same laboratory of recombination between two copackaged genetically distinguishable RNA molecules in heterozygous particles showed that template switching

between the two genomic RNA occurs frequently during the first strand transfer reaction (Hu & Temin, 1990b, Hu & Temin, 1990a, Katz & Skalka, 1990). This discrepancy may likely be a result of different experimental approaches as well as using retroviral vectors that have different expression levels. However, studies by these same researchers showed that only intrastrand transfer was observed in the second jump. This may be explained by the observation that two intact minus-strand DNAs are seldom produced in a virion (Telesnitsky & Goff, 1993) and one minus-strand DNA, once completed, would then serve as target for the second jump as an intrastrand transfer. If two intact minus-strand DNAs were produced, then either inter- or intra-strand second transfer would be possible.

Why does the retrovirus carry two identical copies of the genomic RNA when both the first and second jump can occur as intrastrand transfers within one RNA molecule (Jones *et al.*, 1994)? As retroviruses replicate with frequent errors due to the high mutation rate observed with the RT (see section 1.4.B) and frequent recombination (10-25%) during reverse transcription (Vogt, 1971, Kawai & Hanafusa, 1972, Blair, 1977, Clavel *et al.*, 1989, Hu *et al.*, 1993), it is likely that the encapsidation of two genomic RNA molecules within the virion would enhance the availability of intact templates for synthesis of minus-strand and plus-strand DNA (Temin, 1991, Hu *et al.*, 1993, Pathak & Hu, 1997)(S.P.Goff 1997, *pers. comm.*). When a break occurs in one of the genomic RNA molecules, recombination would allow the completion of the minus strand DNA by crossing over (Temin, 1991).

The reverse transcription process thus yields a linear, double-stranded viral DNA molecule which is longer than the genomic RNA due to the addition of the LTRs at both ends. This viral DNA does not exist as a 'naked' double-stranded DNA molecule but instead is associated with viral as well as cellular proteins. The product of this reverse transcription - the replication or preintegration complex - has been reported to occur in structures of different sizes depending on the route of infection. HIV infection by the cell-free route yielded a 160S replication complex (Farnet & Haseltine, 1990, Farnet & Haseltine, 1991, Karageorgos *et al.*, 1993), whereas cell-to-cell transmission infection yielded a larger complex of 320S (Karageorgos *et al.*, 1993). The latter was shown to be associated with viral-coded proteins viz. RT, integrase, protease and matrix as well as cellular histones. A similar replication complex (also called

nucleoprotein complex) has been isolated in murine leukemia virus-infected cells (Bowerman *et al.*, 1989).

1.6.D Integration of the viral DNA

The replication complex is then transported into the nucleus for integration of the viral DNA, with the host DNA, to form the provirus. Like the reverse transcription process, integration is one of the major steps in the life cycle of retroviruses. The nuclear localization signal (NLS) is important for the active transportation, between the cytoplasm and nucleus, of molecular complexes which are larger than the nuclear pores (*ca.* 50 kDa) (Gorlich & Mattaj, 1996). The NLS is usually a short peptide sequence of basic amino acids of which the simian virus (SV40) large T antigen has been one of the more intensely studied (Dingwall & Laskey, 1991, Dingwall, 1996, Greber & Kasamatsu, 1996). This is likely important for the nucleocytoplasmic transport of the viral pre-integration or replication complex (Farnet & Haseltine, 1991, Karageorgos *et al.*, 1993) formed after reverse transcription from the cytoplasm to the nucleus. The large size of the HIV replication complex reported earlier (Farnet & Haseltine, 1990, Farnet & Haseltine, 1991, Bukrinsky *et al.*, 1992, Karageorgos *et al.*, 1993) suggests that its entry into the nucleus must use an active transport process as the nuclear pores (9 nm diameter, (Ohno *et al.*, 1998)) are not sufficiently large to allow passive diffusion. Alternatively, the replication complex can enter the nucleus during mitosis as this cellular process is essential for murine retroviral integration (Brown, 1990, Roe *et al.*, 1993) (see section 5.5). After nuclear entry, the replication complex has a lower sedimentation coefficient (80S) than that in the cytoplasm and two of the viral proteins viz. RT and matrix protein (p17) were no longer associated with the nuclear replication complex (Karageorgos *et al.*, 1993).

The products of reverse transcription are linear, double-stranded DNA molecules with identical LTRs at each end and in addition two circular forms of viral DNA, viz. the two-LTR and one-LTR molecules (Luciw *et al.*, 1983, Li & Burrell, 1992). Most of the retroviral LTRs have similar short inverted repeat sequences (ATT at the 3' end of the right LTR) (Brown, 1990), except for HIV which has CAG at the 3' end of the right LTR. Hence the corresponding inverted repeat sequence (at the 5' end of the left LTR) for HIV is CTG. The two-LTR circle is likely a result of blunt-end ligation between the ends of the DNA molecule, as was initially

shown with the murine leukemia viral DNA (Shoemaker *et al.*, 1980). The one-LTR form is likely a result of homologous recombination between the two LTRs of the linear DNA or a product of incomplete reverse transcription (Goff, 1990, Zack *et al.*, 1992). The function of the circular viral DNA is not known although it may be presumed not to integrate into the host genome as its incorporation into replication complex is not associated with integrase (Bukrinsky *et al.*, 1993a).

The presence of the three forms of viral DNA as well as those formed by autointegration (Shoemaker *et al.*, 1980, Shoemaker *et al.*, 1981, Saag *et al.*, 1988, Pauza & Galindo, 1989, Goff, 1990, Zack *et al.*, 1992) make it difficult to identify the immediate precursor to viral DNA integration into the host DNA in infected cells. (Autointegration is intramolecular integration of viral DNA molecules into themselves (Lee & Coffin, 1991).) Using an *in vitro* model of murine leukemia virus integration into the bacteriophage μ (Fujiwara & Mizuuchi, 1988) or λ DNA as target (Brown *et al.*, 1989), the blunt end, linear viral DNA was shown to be the immediate precursor to viral integration. The two 3' termini of each viral DNA are covalently linked to the 5' ends of the host DNA prior to the linkage of the 5' termini of each viral DNA strand to the host 3' DNA ends. In all retroviral DNA integration, the proviral DNA is colinear with unintegrated linear viral DNA (Hughes *et al.*, 1978, Brown, 1990, Goff, 1990).

Two characteristic features of retroviral DNA integration are the removal of one or two bases from the viral DNA termini to produce recessed 3' ends (Bushman *et al.*, 1990, Pauza, 1990, Whitcomb *et al.*, 1990) and the duplication of cellular DNA bases at the integration sites prior to the covalent linkage (Ellison *et al.*, 1990). In the case with HIV two bases are removed. Removal of the terminal nucleotides of the blunt end, linear, viral DNA by the endonucleolytic activity of viral integrase is invariably adjacent to the CA dinucleotide (Brown *et al.*, 1989, Katzman *et al.*, 1989, Roth *et al.*, 1989). Mutants without the viral integrase cannot produce processed viral DNA 3' ends and no proviruses are formed. This CA dinucleotide at the 3' viral DNA end is then covalently linked to the target DNA (Brown, 1990, Goff, 1990). The number of duplicated host DNA bases is characteristic of the retrovirus species viz. 4 bp for murine

leukemia virus, 5 bp for HIV and avian retroviruses and 6 bp for mouse mammary tumour virus (Goff, 1990). The requirement of the viral integrase for the retroviral integration reaction has been well confirmed by studies with HIV (Bushman *et al.*, 1990), murine leukemia virus (Craigie *et al.*, 1990) and avian sarcoma-leukosis virus (Katz *et al.*, 1990). Thus the integrase has at least three major enzymic activities viz. processing of the viral DNA ends, target DNA cleavage and joining of the viral and target DNA molecules.

Although not requiring dNTPs or rNTPs (Ellison *et al.*, 1990) *in vivo* experiments showed that integration occurs preferentially in cells with actively replicating DNA (Varmus *et al.*, 1977, Humphries *et al.*, 1981) and at transcripitonally active sites (Scherdin *et al.*, 1990). Integration of murine leukemia virus tends to be located near transcribed and nucleosome-free DNaseI hypersensitive sites (Vijaya *et al.*, 1986, Rohdewohld *et al.*, 1987). Hot spots or highly preferred integration regions in the avian genome were described with a million fold higher frequency of integration of Rous sarcoma viral DNA (Shih *et al.*, 1988). However, subsequent reports from the same laboratory showed that preferred integration sites were determined by local structural features of the target DNA (Withers-Ward *et al.*, 1994). More recently, the HIV DNA was shown to integrate at a higher frequency near *Alu* repeat elements in the human genome (Stevens & Griffith, 1996). These different observations may likely be due to use of *in vitro* as against *in vivo* experiments as well as the particular clone of provirus or cell type selected (Carteau *et al.*, 1998).

Unless integrated into a critical site on the target DNA which affects cellular function, the proviral DNA becomes an integral part of the host and the provirus is maintained from one cell generation to the next. Thus the proviral DNA replicates as part of the cellular DNA during cell division. Activation of the integrated provirus, for example during cellular DNA transcription, can lead to production of viral specific RNA transcripts which then leads to production of infectious virions.

Although integration is one of the more significant steps in the replication cycle of the retrovirus integrase-deficient murine leukemia viral mutants have been shown to produce viruses but with reduced infectivity (Schwartzberg *et al.*, 1984). Unintegrated DNA of spleen necrosis virus

was shown to be sufficient not only for transcription of viral RNA but also for progeny virus production (Panganiban & Temin, 1983).

1.6.E HIV RNA synthesis and regulation

The initiation and regulation of HIV transcription, splicing and export of the viral RNA from the nucleus are dependent on an array of *cis*-acting sequences and *trans*-acting factors. In addition, host cell factors interact with the viral transcription elements during the complex regulation and expression of HIV genes (Barry *et al.*, 1991). The viral promoter and *cis*-acting control elements are located within the U3 region of the LTR, upstream of the transcription start site. Similar to the eukaryotic transcriptional machinery, the HIV promoter element consists of the core, enhancer and modulatory domains (see Fig. 1.6.Ei).

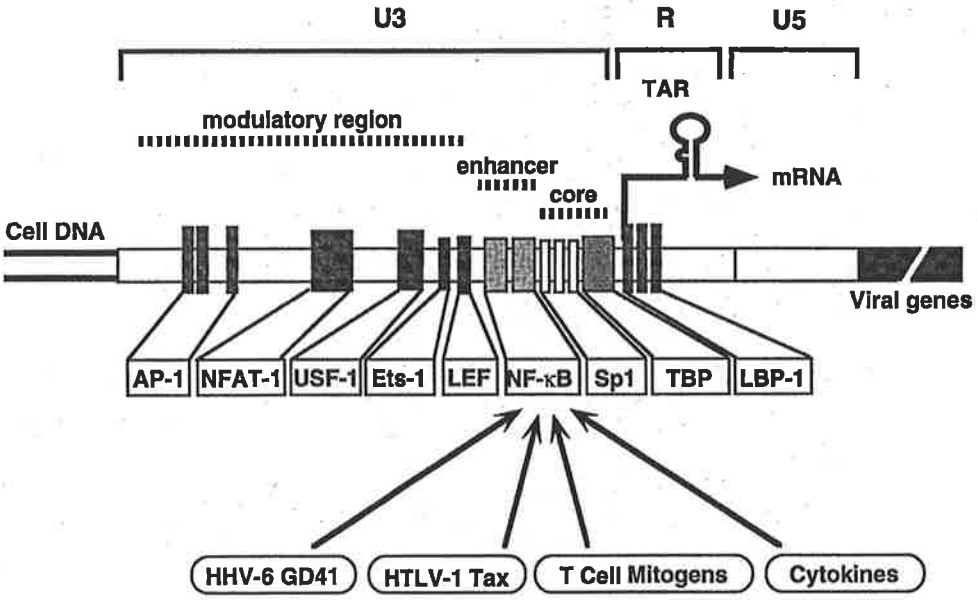
HIV transcription is initiated at the cap site - U3/R junction - located in the left end LTR (Jones & Peterlin, 1994). There are three types of eukaryotic RNA polymerases (I, II and III) which vary in their degrees of sensitivities to α-amanitin (Kornberg & Baker, 1992). RNA polymerase II is the most sensitive (<0.1 μg/ml) to α-amanitin and HIV transcription is sensitive to very low concentrations of this drug (Laspia *et al.*, 1989). This suggests that HIV transcription from the proviral DNA, as with cellular messenger RNA production (Orphanides *et al.*, 1996), is mediated by RNA polymerase II. The host cell RNA polymerase II and cellular transcription factors (e.g. TATAA binding protein and Sp-1) bind to the core promoter (TATAA box) within the U3 region of the LTR to initiate and regulate viral RNA synthesis. Proximal to the core promoter is the enhancer domain which binds NF-κB - an important cellular transcription factor with wide tissue specificity. NF-κB activates several cellular genes leading to increased production of cytokines, immunoglobulins or major histocompatibility antigens (Lenardo & Baltimore, 1989). NF-κB has significant effects on transcriptional regulation of HIV infection of generally resting T cells and macrophages (Nabel *et al.*, 1988, Pazin *et al.*, 1996). Indeed one of the more potent HIV-inducing cytokines is tumor necrosis factor (TNF)-α which activates NF-κB (Fauci, 1996). The binding of other transcription factors (e.g. Ap-1, LEF, USF, etc.) to the modulatory domain of the viral promoter may

Fig. 1.6.Ei

Transcription elements in HIV-1 LTR

The viral LTR is divided into three functionally distinct regions designated U3 (-453 to +1), R (+1 to +98) and U5 (+99 to +185). Three transcriptional domains constitute the viral promoter in the U3 region; the core or basal domain, the enhancer and the modulatory domain. Transcription initiates at the U3/R border in the 5' LTR; the core promoter contains binding sites for cellular transcription factors including TATAA-binding protein (TBP) and SP-1. Regulatory proteins of other viruses, T-cell mitogens and cytokines enhance HIV transcription and replication through activation/induction of NF-κB and other cellular transcription factors. The modulatory domain in the U3 portion of the LTR contains binding sites for several cellular factors. The R region also encodes the RNA sequence which forms the trans-activation response element (TAR). The R/U5 boundary in the 3' LTR defines the 3' end of all viral transcripts; this boundary is determined by the polyadenylation signal AAUAAA in R.

(adapted from Luciw, 1996).


U3
R
U5
TAR
modulatory region
enhancer
core
mRNA
Cell DNA
Viral genes
AP-1
NFAT-1
USF-1
Ets-1
LEF
NF-κB
Sp1
TBP
LBP-1
HHV-6 GD41
HTLV-1 Tax
T Cell Mitogens
Cytokines

contribute to the activation and regulation of HIV gene expression, but these have not been conclusively characterized (Luciw, 1996).

Following synthesis in the nucleus, the capping and polyadenylation of the HIV RNA are post-transcriptionally modified by cellular enzymes. The spliced and unspliced HIV transcripts are then translocated from the nucleus to the cytoplasm. The nuclear export of doubly spliced viral RNA is constitutive while that of the unspliced and singly spliced viral RNA is regulated by the Rev protein (see section 1.4.C.ii). One unusual feature of this observation is the localization of Rev in the nucleolus (Cochrane *et al.*, 1990). The only RNA polymerase located within the nucleolus is RNA pol I, whereas RNA pol II and III are located within the nucleoplasm (Kornberg & Baker, 1992). These observations may suggest that there are other pathways in which HIV RNA processing occurs, in addition to that using RNA polymerase II.

The activation of HIV gene expression is regulated by viral as well as cellular encoded transcriptional factors. One of these is the *trans*-acting Tat which is a potent HIV gene activator (see section 1.4.C.i). Upon activation of transcription to produce the viral RNA, like other retroviruses, the HIV primary transcripts are capped at the 5' end and polyadenylated at the 3' end (see section 1.4.A). There are more than forty-six species of unspliced and multi-spliced mRNAs encoded by the HIV genome from complex splicing events (Purcell & Martin, 1993). These mRNA species can be grouped into three main classes viz. the unspliced 9 kb mRNA; the singly spliced 4 kb mRNAs and the multi-spliced 2 kb mRNAs. All of the HIV mRNAs contain a common leader sequence of 289 bases at the 5' end (Fig. 1.4 Ai). The unspliced, genomic length mRNA codes for the Gag and Gag-Pol polyproteins and ~~are~~ is also packaged as genome into virions. The singly spliced mRNAs (after excision of gag/pol as a single intron) comprise the *env, vif, vpu* and *vpr* genes. These are produced by alternative splicing mechanisms with the different acceptor splice sites determining the particular transcripts (Malim *et al.*, 1988, Schwartz *et al.*, 1990b, Schwartz *et al.*, 1991). Multi-spliced transcripts viz. *tat*, *rev* and *nef* are generated by removal of *gag/pol* and regions of the *env* (Guatelli *et al.*, 1990, Robert-Guroff *et al.*, 1990, Schwartz *et al.*, 1990a). The ratio of unspliced to spliced transcripts is determined by the regulation of viral gene expression through the regulatory protein - Rev - which acts on the Rev-responsive element (RRE) to increase the stability of

unspliced mRNA (Malim & R., 1993) (see section 1.4.C.ii). Rev regulates the amounts of unspliced and spliced viral RNA by translocating RRE-containing unspliced mRNA from the nucleus to the cytoplasm, with less unspliced viral RNA available for splicing in the nucleus.

HIV RNA transcription and splicing processes are complex and influenced by a number of cellular (RNA polymerase II, transcription factors and cytokines) as well as viral components. These cellular and viral processes together have the potential to regulate HIV gene activation and transcription during the course of infection.

1.6.F Viral protein synthesis and processing

After export to the cytoplasm, the spliced HIV transcripts serve as mRNAs for the Env and regulatory proteins. The unspliced mRNA can serve as genomic RNA which is packaged into the virion or is translated on free polyribosomes to generate the p160 Gag-Pol precursor fusion protein. Due to the termination codon at the 3' end of the *gag* gene, it is necessary to bypass this in order to generate the Gag-Pol fusion protein. This process requires translational suppression by ribosomal frameshifting from the *gag* gene to the *pol* gene prior to the termination codon at the end of the *gag* gene (Jacks *et al.*, 1988). Ribosomal frameshift requires the ribosome to pause, slip back one nucleotide prior to the termination codon and then continue the translation process. This is different from the other mechanism of translational suppression - readthrough suppression - which represents perturbation of the ribosome at a termination codon (Hatfield *et al.*, 1992). Ribosomal frameshifting may occur at a considerable distance on the mRNA from the termination codon and is not affected by mutation at the latter site. Translational suppression in the synthesis of the Gag-Pol polyprotein offers two benefits to the HIV. Firstly, this process facilitates regulation of the ratio of viral enzymes to structural proteins (Jacks, 1990). It can be assumed that more structural proteins are required than viral enzymes as the latter function catalytically during viral replication. The other benefit is to ensure viral enzymes are included in the virion during assembly by incorporation of Gag and Gag-Pol polyproteins (Jones *et al.*, 1990, Weldon *et al.*, 1990).

The p55 Gag protein contains the sequences for MA (p17), CA (p24), NC (p7) proteins and a small polypeptide (p6). The latter p6 protein may be involved with viral budding as viruses

with mutations in this protein remain tethered to the plasma membrane (Gottlinger *et al.*, 1991). More recently deletion of the p6 domain showed that viral particles did not package HIV RNA (Zhang & Barklis, 1997). The relative locations of the matrix, capsid and nucleocapsid proteins on the virion correspond to the order of these sequences in the Gag polyprotein (see Fig. 1.3.Ai). Prior to cleavage of the Gag and Gag-Pol polyproteins by the viral protease the Gag protein is modified by myristoylation and phosphorylation. These modifications are necessary for viral replication. Myristoylation of Gag is necessary for binding of this protein to the plasma membrane. A myristic acid is added to the glycine residue next to the methionine at the amino terminus of Gag. This is essential as mutation at this site prevents formation of the viral particle (Wilcox *et al.*, 1987, Schultz *et al.*, 1988, Gottlinger *et al.*, 1989, Bryant & Ratner, 1990).

The Gag-Pol precursor protein is cleaved during and after viral assembly to generate the p10 aspartyl-protease, p66/51 reverse transcriptase and p32 integrase proteins. These three virion enzymes, together with the RNaseH activity located at the carboxyl end of the p66 RT, are discussed in section 1.4.B.

The *env* gene codes for the gp160 Env polyprotein which contains the gp120 surface (SU) and gp41 transmembrane (TM) proteins. The synthesis and processing (glycosylation, maturation, proteolysis and oligosaccharide modification) of this polyprotein involves cellular enzymes. Translation of the polyprotein is initiated on free ribosomes and shortly after chain initiation a small stretch of hydrophobic signal sequence at the amino terminus of the newly synthesized Env polyprotein binds to a signal recognition protein (SRP). This interaction of the signal sequence and the SRP leads to cotranslational transport of the nascent protein from the ribosome through the membrane and into the lumen of the rough endoplasmic reticulum (ER) (Singer & Berg, 1991, Walter & Johnson, 1994, Lutcke, 1995). Upon entry into the ER lumen, a cellular signal sequence peptidase cleaves the signal sequence from the Env polyprotein. The remaining Env protein is then glycosylated, oligomerized, folded and transported through the Golgi apparatus to the plasma membrane. Although these processes are not well characterized, they are thought to be driven by cellular mechanisms (Swanstrom & Wills, 1997, Vogt, 1997). Within the Golgi apparatus, the Env polyprotein is cleaved by a cellular protease (likely furin) to

yield the mature surface (gp 120) and transmembrane proteins (gp 41) found in the virions (Stein & Engleman, 1990). Proteolytic cleavage of the Env glycoprotein generates the hydrophobic fusion peptide located at the amino terminus of the TM protein.

1.6.G Viral assembly and release

The dynamic and ordered assembly of the viral proteins and nucleic acids to form the immature viral particles occurs adjacent to the plasma membrane (Hunter, 1994). Initially, Gag and Gag-Pol polyproteins interact (Gelderblom, 1991, Wills & Craven, 1991) and are directed to the cytoplasmic membrane by myristoylation of their amino termini (see section 1.6.F). The interactions between the Gag precursor polyproteins are crucial for the targeting and binding of these complexes to the plasma membrane as well as budding of the viral particles (Craven & Parent, 1996). These viral proteins interact with the genomic RNA and tRNA primers to form assembly complexes which are observed as electron-dense crescents in the plasma membrane (Gelderblom, 1991).

The packaging of the viral genome is determined by the *cis*-acting packaging signal (psi) (Mann *et al.*, 1983), which is located at the leader sequence of the genomic RNA between the *u5* and *gag* regions (Linial & Miller, 1990). It is likely that the HIV genomic RNA to be packaged into the viral particles is from a different pool to that used for Gag-Pol translation (Berkowitz *et al.*, 1996). In an earlier report with murine leukemia virus, translated RNA was shown not to be packaged as virion RNA (Levin & Rosenak, 1976). These observations may be similar to that of hepatitis B viral assembly in which binding of ribosomes to the RNA prevents packaging (Nassal *et al.*, 1990). The packaging of HIV genomic RNA involves a conserved cysteine array (Cys--His motif) located within the NC protein that functions as a nucleic acid binding domain (Aldovini & Young, 1990) (see section 1.3.D).

Although it may be expected that packaging of the tRNA primer into the viral particle is a result of its 3' end binding to the pbs at the 5' end of the genomic RNA, this association is unlikely as the total pool of tRNAs in the virion is unchanged even with genomic RNA mutated at the pbs (Jiang *et al.*, 1993). In addition, the pool of tRNAs found within virions with RT mutations is largely that of cellular tRNAs and not those characteristic of wild type virions. With the HIV-1,

the Gag-Pol precursor was shown to be important for the packaging of the characteristic $tRNA^{Lys3}$ primer and the RT molecule was considered likely to be responsible for this process (Mak *et al.*, 1994).

After translocation of the Gag and Gag-Pol polyproteins to the sites on the plasma membrane, the assembly complexes can be observed by (electron microscopy) being budded through the membrane. It has not been established how these polyprotein precursors are transported to the plasma membrane but the likely mechanism may be via the cytoskeleton (Edbauer & Naso, 1983, Rothman, 1989, Hartl *et al.*, 1994) (see section 1.5.A.ii, Vif and association with cytoskeleton). During the budding process viral protease is activated to cleave the Gag and Gag-Pol polyproteins (which are intact within the viral particle prior to budding) to form the electron dense crescents observed in budding viral particles (Skalka, 1989, Kaplan & Swanstrom, 1991, Kaplan *et al.*, 1994) and to generate the mature proteins found within the released virions (Swanstrom *et al.*, 1990). The functions of other HIV proteins viz. Vif, Vpr and Vpu during viral maturation are discussed in section 1.5.A. Although the ordered assembly of viral proteins at the plasma membrane and subsequent processing and budding may give the impression that virions contain only defined viral components; other viral RNAs (Bishop *et al.*, 1970a, Bishop *et al.*, 1970b), viral DNAs (Lori *et al.*, 1992, Arts *et al.*, 1994) (Coffin, J. M. *pers. comm.*) and cellular mRNAs and proteins (Aronoff & Linial, 1991) are found within the mature particles. The functions of these fortuitously packaged viral and cellular components within the virion are not known but may explain in part that not all virions within a population are homogenous and infectious.

1.7 HIV Pathogenesis

The principal features of HIV infection, like other lentiviral infections, are chronic, persistent and degenerative pathology. Studies of HIV pathogenesis have shown a complex interaction of the host's immune response and viral replication. The tropism of this lentivirus for macrophages and CD4+ cells, its integration into the host DNA and the clinically "latent/quiescent" state of infection but with continuous production of infectious virions all add to the eventual breakdown of the host defense mechanisms. Unlike studies of the pathogenesis of other human viral infections which have relied heavily on animal models for host-viral

interactions, animal models of this infection are not particularly satisfactory and clinical specimens (sera, lymph node biopsies, PBMC, etc.) from documented patients have provided much of the valuable materials for studies of this disease.

1.7.A Routes of transmission

The main routes of HIV transmission, like many other blood-borne and sexually-transmitted diseases, are via sexual contacts (Winkelstein *et al.*, 1987, Anderson & May, 1988), parenteral inoculations (DesJarlais *et al.*, 1989) and perinatal transmissions (Lapointe *et al.*, 1985). Although infected blood is highly efficient in transmitting the infection (Ward *et al.*, 1987) the virus is detected in other body fluids such as semen (Ho *et al.*, 1984) and genital secretions (Vogt *et al.*, 1986, Wofsy *et al.*, 1986) as well as cervical cells (Pomerantz *et al.*, 1988). When infection occurs via the mucosal epithelia, the dendritic cells (Langerhans) are the usual targets of HIV. These infected cells in turn travel to other target cells in particular those in lymphoid tissues (Cameron *et al.*, 1992, Cameron *et al.*, 1996, Spira & al., 1996).

1.7.B Viraemia, Viral Reservoir and Viral Load

Following seroconversion, plasma viral levels decline but are nevertheless detectable by quantitative competitive polymerase chain reaction (Piatak *et al.*, 1993). Virus-infected cells are disseminated throughout the lymphoid organs with actively-replicating viruses in infected CD4+ lymphocytes, macrophages and large amounts of virus on the surface of follicular dendritic cells (Fox *et al.*, 1991, Embretson *et al.*, 1993, Pantaleo *et al.*, 1993). During the asymptomatic period (clinical latency), infected cells are still detected within the lymphoid tissues where they act as reservoirs for viral replication and contribute to persistence of this infection (Pantaleo *et al.*, 1993, Pantaleo *et al.*, 1998). The infected cells in the lymphoid tissues can also contribute to viral replication by cell-to-cell spread (see section 1.8.B). Most importantly, production of virus and turnover of infected cells occur at high levels during this phase (Lineberger *et al.*, 1995). Studies of viral loads in HIV infected patients have estimated that there are 10^{10} virions produced per day (with an average equivalent to 10^5 virions/ml of plasma) (Ho *et al.*, 1995, Wei *et al.*, 1995, Perelson *et al.*, 1996, Perelson *et al.*, 1997). In the same studies, the kinetics of CD4+ lymphocyte turnover (produced and destroyed) were estimated to be on

average 2 x 10^9 cells/day or equivalent to 5% of the total CD4+ cell population. The life cycle of the virus in the infected individual - from infection of one cell to the production of new progeny virus - is 2.6 days and the half-life of an infected cell is 1.5 days (Perelson *et al.*, 1996). These observations indicate a very high rate of viral replication and CD4+ lymphocyte turnover in HIV infected patients. The level of viral load during HIV infection provides an important prognostic marker of disease progression, with the rate of decline in CD4+ cell counts predicted by the plasma viral RNA level (Saksela *et al.*, 1994, Furtado *et al.*, 1995, O'Brien *et al.*, 1996, Mellors *et al.*, 1997). Although the use of anti-retroviral therapy (e.g. AZT) in primary HIV infection has been shown to reduce viral load and significantly improve the clinical course of the patients (Kinloch-deLoes *et al.*, 1995, Saag *et al.*, 1996) drug-resistant viral strains emerge with monotherapy. This has led to the use of combination therapy or highly active anti-retroviral therapy (HAART) with more than one anti-retroviral drug (e.g. inhibitors of viral protease and RT, (Bartlett & Moore, 1998)) to counter the emergence of drug resistant viral strains and maintain viral load as low as possible (Chun *et al.*, 1997b, Finzi *et al.*, 1997, Perelson *et al.*, 1997, Wong *et al.*, 1997a). However, despite the use of combination therapy (or HAART) in which plasma viraemia has been reduced by *ca.*99% during the first two weeks of treatment, there remains a subset of latently infected cells which may produce low levels of virus, with a half-life of 1-4 weeks. The latter population of cells were thought to contribute to a second phase of decay of plasma viraemia in patients undergoing HAART. In lymph node biopsies from asymptomatic patients undergoing therapy, it was observed that *ca.* 0.5% of resting CD4+ T cells were HIV DNA (unintegrated) positive, of which 0.05% carry the provirus and 10% of the latter subset produce HIV virions (Chun *et al.*, 1997a). Although these studies showed that the percentage of latently infected cells may be low, the absolute number of infected cells is sufficiently high to maintain infection with the potential for further viral replication and dissemination (Ho *et al.*, 1995, Wei *et al.*, 1995, Perelson *et al.*, 1996) and it may be appropriate to quote "We must assume that HIV infection is forever until we know to the contrary." - S. Wain-Hobson, 1997 in (Balter, 1997).

1.7.C Immune Responses

The period from transmission of infection to seroconversion usually ranges from four to twelve weeks (Ho *et al.*, 1985). During this period, virus is detectable in the peripheral blood

mononuclear cells (PBMC) and plasma (Gaines *et al.*, 1987, Clark *et al.*, 1991, Daar *et al.*, 1991) or cerebrospinal fluids of patients (Goudsmit *et al.*, 1986, Pang *et al.*, 1990) and circulating p24 antigen can be detected in some but not all patients. Common symptoms manifested during this period are fever, myalgia, rash, lymphadenopathy, pharyngitis, gastroenteritis or neurologic complications (Cooper *et al.*, 1985, Ho *et al.*, 1985). The acute syndromeis self-limited, after which the patient is usually asymptomatic. The most common diagnostic test is detection of antibodies to *env* products and p24 in patients' sera (Cooper *et al.*, 1987). These antibodies appear with the decline in p24 antigen levels. At the end of the primary infection period, the clinically asymptomatic phase may last 5-12 years prior to the development of AIDS (Levy, 1993, Schrager *et al.*, 1994) although some infected patients progress to this syndrome within 2-4 months (Isaksson *et al.*, 1988, McLean *et al.*, 1990). The reappearance of p24 antigenemia is associated with increased viral replication and the subsequent development of AIDS (Allain *et al.*, 1987). Antibodies to the virus are present throughout the course of infection (Robert-Guroff *et al.*, 1985, Weiss *et al.*, 1985, Ho *et al.*, 1987); however such antibodies are unable to prevent progression of disease (Groopman *et al.*, 1987, Ho *et al.*, 1987).

Cell-mediated immune responses are mounted to HIV infection; in particular by specific CD8+ cytotoxic T-lymphoctyes (CTL) directed against Env, Pol and Gag proteins (Walker *et al.*, 1987, Walker *et al.*, 1988, McMichael, 1998). The CTL responses are detected prior to the appearance of neutralizing antibodies (McMichael, 1998). Major histocompatibility complex (MHC) - restricted, virus-specific CD8+ CTLs are thought to be important in the suppression of HIV replication (Tsubota *et al.*, 1989, Blackbourn *et al.*, 1996, Musey *et al.*, 1997) as the increased viral load observed during the late stage of disease corresponds with a significant loss of these cell types (Borrow *et al.*, 1994, Oldstone, 1997). Recent *in vivo* studies showed that CD8+ cytotoxic T cells can confer some protective immunity (Greenough *et al.*, 1997). As HIV antibodies can remain relatively constant despite increase in viral load and that binding of antibodies to the viral surface glycoproteins is not efficient nor protective, these observations may add to the above reports that CTLs have a crucial role in the control of acute HIV infection (Burton & Moore, 1998).

1.7.D Immune Dysfunction

One crucial manifestation of HIV infection is the progressive depletion of CD4+ lymphocytes; with an attendant destruction of the lymphoid tissues and other components of the immune system, leading to the symptoms of acquired immune deficiency syndrome (Fauci, 1988). The activated/memory T cells are the main targets of HIV infection (Schnittman *et al.*, 1989, Schnittman *et al.*, 1990). CD4+ cells are necessary for many immunologic functions viz. the regulation of antigen-specific chronic inflammatory reactions through activation of macrophages, induction of cytotoxic (CD8) or suppressor T cells and secretion of differentiation factors for lymphoid and haematopoietic cells. In addition, CD4 cells provide the T-helper function to co-operate with B cells via cell surface receptors (immunoglobulins) or in the recognition of foreign antigen in association with the MHC class II molecule on the antigen presenting cell. To effect these immune responses, CD4 molecules interact with the T-cell receptor and CD3 complex to activate the T-helper cells (Meloche *et al.*, 1992, Hasenkrug & Chesebro, 1997, Roitt, 1997).

The precise mechanism of cell killing by HIV infection has not been resolved but may include a number of processes. The high rate of CD4+ cell turnover (see section 1.7.C) may contribute to the decline in this cell-type population by exhausting the regenerative capacity of immune tissues. The change in co-receptor use by predominating viral strains to CXCR4 during the late stage of disease may also contribute to this rapid decline (see sections 1.6.A and 1.7.C). In addition, isolates of the viruses from patients have been observed *in vitro* to change from that of slow/low replication to that of rapid/high characteristics during the change from primary infection to the onset of AIDS (Fenyo *et al.*, 1989). Another possible mechanism is apoptosis as HIV-infected macrophages release tumor necrosis factor-α which is known to induce apoptosis (Poli & Fauci, 1995). Other processes may include the accumulation of viral DNA within the infected cell, the direct cytolytic effects of infection by the virus and cytotoxic T-cell mediated pathology (Zinkernagel & Hengartner, 1994).

The failure of the immune response to eliminate HIV from the infected individual, especially during the clinically latent period, remains a paradox (Pantaleo & Fauci, 1996). The large pool

of latently infected cells in lymphoid tissues may not be recognized by CTLs. The trapping of viral particles in the follicular dendritic cell network may offer a continuous source of viruses for *de novo* infection of cells that migrate through the lymphoid tissues. The virus phenotype and genotype may alter, thus avoiding immune surveillance.

1.8 Aim and scope of this thesis

The aim of this thesis is to study the early events in the synthesis of HIV RNA and integration of viral DNA using a cell-to-cell transmission of infection model. The infection model was developed in our laboratory using H3B cells as viral donors and Hut78 cells as recipients. When mixed in the ratio of one H3B cell to four Hut78 cells this infection model yields a one-step, synchronized viral infection cycle (Li & Burrell, 1992). This model was used in order to study the early kinetics of HIV replication without interference by multiple or unsynchronized rounds of infection. As a virus can infect the susceptible cell by either the cell-to-cell infection or cell-free route, the latter route was also used in these studies for comparison.

1.8.A One step viral infection

The one step viral replication cycle was initially described in the quantitative study of productive phage-bacterium interaction (Ellis & Delbruck, 1939). This classical experiment was based on the initial studies of bacteriophage growth (d'Herelle, 1926) and the one-step or single-burst modification (Burnet, 1929). The central requirement of this one-step growth experiment involved the mixing of sensitive bacteria with a highly virulent strain of bacteriophage. (It is appropriate to quote "...that it is necessary to use a phage of absolutely maximal activity and a highly sensitive bacterial species." (Burnet, 1929)) After a short period of adsorption - less than the minimum latent period between adsorption and bacterial lysis - the mixture was diluted to minimize further phage-bacterium adsorption. Quantitation of progeny bacteriophage production was then determined at intervals by plating for plaque counts on a bacterial lawn. The initial minimal to zero plaque count represents the latent period and the rapid, logarithmic increase ('burst') in plaque counts corresponds to lysis of the infected bacteria. After a certain period, the logarithmic increase levels out suddenly and the plaque counts remain constant. These observations represent a single cycle of phage growth. This one-step growth experiment was later adapted for use in the classical studies of influenza virus multiplication kinetics with

the more technically demanding inoculation procedure in allantoic sacs of chick embryos (Burnet, 1960). In practical terms, a one step viral infection experiment is initiated where all the cells in a particular reaction vessel (e.g. flask or well) are infected simultaneously and the culture is analysed within the time frame of one round of viral infection.

1.8.B Cell-to-cell transmission of HIV infection

Cell-to-cell transmission of infection route has been described earlier with other enveloped viruses viz. herpes simplex virus (Black & Melnick, 1955), varicella-zoster virus (Weller, 1953) and paramyxoviruses (Merz *et al.*, 1980). Multi-nucleated, swollen cells were observed despite the absence of infectious material in the fluid phase of the virus-infected roller tube cell cultures. Focal lesions were observed to increase in size with time, supporting the idea that viruses can spread from an infected cell to adjacent uninfected cells without the release of free virions in the culture media. Cell-to-cell transmission of paramyxovirus was shown not to be inhibited by monospecific antibodies to the HN glycoprotein which mediates viral adsorption. However it was inhibited by antibodies to the fusion protein which mediates viral penetration (Merz *et al.*, 1980). Cell-to-cell spread can occur either by contact between uninfected and infected cells or from parent to progeny cells during cell division. HIV-infected cells can fuse with uninfected, susceptible cells in which the virus can be transmitted directly in the presence of specific neutralizing antibodies (Gupta *et al.*, 1989). In contrast, the cell-to-cell spread of rabies virus infection was inhibited by >75% with antirabies sera, although non-neutralizing antinucleocapsid monoclonal antibodies did not inhibit this viral spread (Lodmell & Ewalt, 1987).

In the cell-to-cell transmission infection model developed in our laboratory, H9 cells persistently infected with HTLV-IIIB (H3B) cells and uninfected target CD4+ lymphocytes were mixed in a ratio of 1:4 to achieve a one-step infection cycle. In this system *de novo* HIV DNA synthesis was observed within 4h post infection (p.i.) (Li & Burrell, 1992), with linear viral DNA accumulating prior to circular forms. Progeny virus was not released until 24h p.i. as judged by supernatant reverse transcriptase activity. This lag phase is equivalent to the observations with the one-step growth experiment using phages described in section 1.8.A, and suggested that the second and subsequent round of infection or superinfection was unlikely within the 24h

p.i. period (Robinson & Zinkus, 1990). The early production of large amounts of HIV DNA within 4h p.i. suggest a highly efficient initiation of infection with this cell-to-cell transmission route. In addition to the early production of viral DNA, giant multi-nucleated cells (syncytia) were observed within 4h of coculture. This was followed by the release of progeny viral particles within 24 - 48 h (Li & Burrell, 1992, Sato *et al.*, 1992, Barbosa *et al.*, 1994). Further kinetic studies have confirmed that cell-to-cell transmission infection with HIV is highly efficient (Dimitrov *et al.*, 1993). The following chapters will discuss the kinetics and pattern of HIV RNA synthesis, in particular the early events and viral DNA integration using the one-step cell-to-cell transmission infection model described above.

Chapter 2

I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me. Sir Isaac Newton (1642-1727)

Materials and Methods

2.1 Cell cultures

2.1.A H3B cells (HIV donors in cell-to-cell transmission infection)

H3B cells are a laboratory clone of H9 cells persistently infected with human T cell-lymphotropic virus type IIIB (HTLV-IIIB) and were originally obtained from the National Institutes of Health, AIDS Research and Reference Reagent Program. Each H3B cell contains two copies of integrated proviral HIV DNA and secretes into the culture supernatant approximately 0.01 $TCID_{50}$ virus/hr (Li & Burrell, 1992). The cells were passaged twice weekly at a concentration of 1-2 x 10^5/ml and were routinely tested for the absence of contaminating mycoplasmas by the polymerase chain reaction (Boehringer Mannheim). (H9 cells are a neoplastic aneuploid T-cell line derived from an adult with lymphoid leukaemia.)

2.1.B Hut78 cells (recipients/targets in cell-to-cell transmission infection)

Hut78 cells, an uninfected CD4+ lymphoblastoid cell line, were obtained from the National Institutes of Health, AIDS Research and Reference Reagent Program. The cells were passaged twice weekly at a concentration of 1-2 x 10^5/ml and were routinely tested for the absence of contaminating mycoplasmas by the polymerase chain reaction (Boehringer Mannheim).

2.2.A Cell-to-cell transmission infection

The cell-to-cell transmission infection model used was a co-culture mix of H3B cells as viral donors and Hut78 cells as recipients/targets (Li & Burrell, 1992). The H3B and Hut78 cells were passaged at a concentration of 1 x 10^6/ml, 18-22hr prior to the infection mix. A synchronous one-step infection mix was obtained in which 4 x 10^6 washed Hut78 cells were infected with 1 x 10^6 washed H3B cells in a volume of 10 ml of RPMI 1640 (Gibco) culture medium supplemented with 10% foetal bovine serum. The infection mix was inoculated into six-well culture plates. The coculture mix was used for viral nucleic acid analyses only when

multi-nucleated giant cells were observed in >10% of the cells by 4h p.i., with 200X magnification in an inverted phase-contrast microscope (Fig. 2.2.Ai).

2.2.B Actinomycin D (AmD) treatment

The virus donor cells (H3B) were incubated in medium containing AmD at 50 μg/ml for 2h at 37°C and then washed three times with culture medium before mixing with Hut78 cells in drug-free medium; or mixed with Hut78 cells and cultured in the presence of the drug throughout the experiment. At the concentration used, >99% of total cellular RNA transcription was irreversibly inhibited (see section 4.1).

2.2.C ^{3}H-uridine incorporation with actinomycin D (AmD) treatment

Aliquots of H3B cells (2 x 10^6) were treated with various concentrations of AmD for 2h, washed three times with drug-free culture medium and incubated in drug-free medium for 0.3h, 2h, 4h or 24h before pulse labelling with [5,6-^{3}H] uridine at 2 μCi/ml for 2h. The control cells were not treated with AmD. After pulse labelling, the cells were lysed with 10% TCA and filtered onto glass fibre filter discs. The filter discs were washed 3 times with 10% TCA followed by 3 washes with 5% TCA and rinsed once with 70% ethanol. The acid-insoluble counts (average from four replicate cultures of H3B cells) were measured in a scintillation analyzer (Packard).

2.2.D Aphidicolin (Aph) treatment

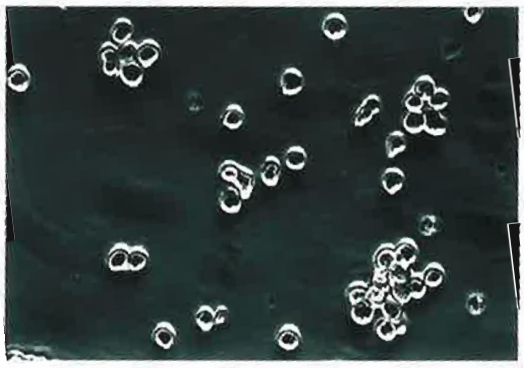
H3B and Hut78 cells were treated with 20 μM (6.8 μg/ml) of Aph 18h prior to cell mixing and the drug was maintained throughout the period of the infection. Under these conditions, cells were arrested in the late G_1 phase of the cell cycle. The cells remained viable and could re-enter the cell cycle upon removal of the drug (Li & Burrell, 1992)

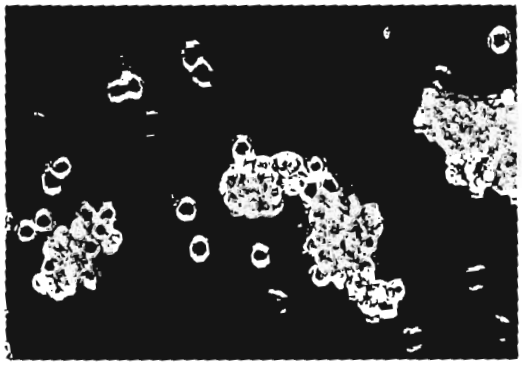
2.3.A Preparation of cell-free HIV stock

A cell-free HIV inoculum was harvested from the clarified culture supernatant fluids of H3B cells at a concentration of 5 x 10^7/ml. (The cells were passaged at 1 x 10^6 cells/ml, 18-22h prior to use.) At one-hourly intervals the H3B cells were spun at 200g/3 min./4°C. The supernatant fluids from each successive centrifugation were pooled and stored at 4°C till the end of the viral

Fig. 2.2.Ai

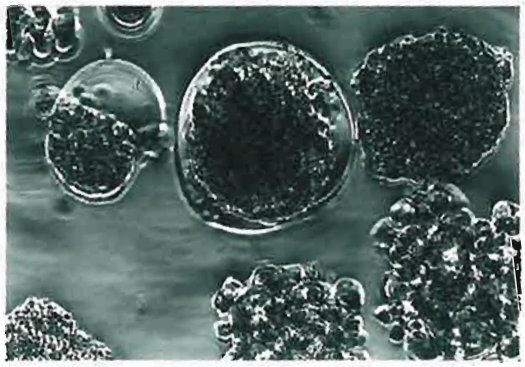
Formation of giant, multinucleated cells produced by the co-culture of HIV donor H3B cells and Hut78 recipient cells in the ratio of 1:4 in the cell-to-cell transmission of infection model.





H3B

Hut78



Giant (ballooning) cells

harvest. The pelleted cells were resuspended in pre-warmed (37°C) RPMI culture medium at the initial concentration of 5 x 10^7/ml. After the last harvest, the H3B cells were vigorously shakened in a vortex mixer for 5 min and then clarified at 2700g/10 min/4°C. The supernatant fluid was then combined with the previous harvests, clarified further by centrifugation at 2700g/10 min/4°C and 5 ml aliquots of the virus culture were stored at -70°C until futher use. An aliquot was used the following day to determine the $TCID_{50}$.

2.3.B Determination of Tissue Culture Infective Dose$_{50}$ ($TCID_{50}$)

Hut78 cells at a concentration of 1.5 x 10^5/ml were treated with 0.001% DEAE-dextran/10 min/37°C. (The cells were passaged at 1 x 10^6/ml, 18-22hr prior to day of use.) The cells were then pelleted at 200g/3 min/4°C, resuspended in fresh culture medium and aliquots of 1.5 x 10^5 cells/Eppendorf tube were prepared. The culture supernatant fluid was discarded and each cell pellet was resuspended in 0.6 ml of each virus dilution prepared in serum-free RPMI medium. This mixture was incubated at 37°C/2hr. The HIV-infected Hut78 cells were then washed twice with serum-free RPMI culture medium and the cell pellet in each tube was resuspended to 1.25 x 10^5/ml in RPMI medium supplemented with 10% foetal bovine serum. Aliquots of 2.5 x 10^4 cells/200µl/well (replicates of six wells/virus dilution) were inoculated into 48-well microplates. The cultures were replenished with fresh medium with half volume changes as required and giant/ballooning cells were observed for the $TCID_{50}$/ml determinations.

2.3.C Cell-free HIV infection of Hut78 cells

Hut78 cells (2 x 10^6) were infected with the cell-free HIV stock at a nominal multiplicity of 0.5 $TCID_{50}$ virus/cell using a centrifugation enhancement method (Pietroboni *et al.*, 1989). The Hut78 cells, passaged at 1 x 10^6/ml 18-22hr prior to use, were pre-treated with 0.001% DEAE-dextran/30 min/37°C. After washing once with RPMI medium (supplemented with 10% foetal bovine serum), the cells were resuspended in the appropriate volume of virus stock at room temperature for 30 min. The cell and virus mix was then clarified at 1000g/25 min/20°C. The supernatant fluid was discarded and fresh virus stock was used to resuspend the Hut78 cells and further incubated at room temperature for 30 min with occasional mixing. After the second infection, the cells were washed three times at 400g/3 min at room temperature before seeding into 6-well culture plates at a concentration of 5 x 10^6 cells/10 ml/well.

2.4 Extraction of HIV DNA by Hirt procedure

HIV DNA was extracted by the Hirt procedure (Hirt, 1967). Infected cells were harvested by low speed centrifugation to minimize cell lysis (200g/3min/room temperature). The cell pellet was washed once with PBS and resuspended with 180 µl of Buffer I (5 mM Tris pH 8.0, 10 mM EDTA and10 µg/µl of proteinase K (Boehringer Mannheim)). The cell pellet was resuspended by gentle inversion of the tube. To the cell suspension, 200 µl of Buffer II (5 mM Tris pH 8.0, 10 mM EDTA and 1.2% SDS) was added. The tube was then gently inverted ten times (to minimize chromosomal DNA damage) and incubated at 37°C/15 min. The chromosomal DNA was then precipitated with 100 µl of 5M NaCl, gently inverted ten times and incubated overnight at 4°C. The precipitated chromosomal DNA was separated from the viral DNA by centrifugation at 17000g/45 min/2°C. The supernatant fluid was then extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with one-tenth volume of 3M sodium acetate (pH 5.5) and two volumes of cold ethanol for >2h at -20°C. The precipitated viral DNA was then pelleted at 15000g/15 min/4°C, washed with one-half volume of cold (-20°C) 70% ethanol and then spun at 15000g/10 min. After removal of the 70% ethanol, residual fluid was allowed to air-dry at room temperature under a table lamp. The DNA pellet was then resuspended in an appropriate volume of TE buffer, treated with RNase for 30 min., extracted once with phenol:chloroform:isoamyl alcohol and re-precipitated as described earlier. The viral DNA was then resuspended in an appropriate volume of TE buffer.

2.5.A Purification of chromosomal DNA with Hirt washing and gel electrophoresis

The Hirt pellet was washed and precipitated three times with the Hirt buffers I & II (see section 2.4). The chromosomal DNA in the Hirt pellet was then extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated in ethanol at -20°C for > 4h. The ethanol was removed by centrifugation (15000g/15 min/4°C) followed by washing with 70% ethanol (15000g/10 min/4°C). The chromosomal DNA was air-dried briefly and resuspended in 100 µl of TE. The suspension was then mixed with an equal volume of 1.6 % low gelling temperature agarose (LGT) and allowed to cool in an Eppendorf tube with the cut bottom supported by clear cellophane tape. After the agarose had cooled sufficiently to form a firm gel, the cellophane tape was removed and the Eppendorf tube (containing the chromosomal DNA

embedded in the agarose) was placed in an electrophoresis tank with 1X TAE buffer. The Eppendorf tube was then prevented from floating by weighing down with a perspex board. Electrophoresis was then applied to the chromosomal DNA - in the agarose gel plug - overnight at 1 V per cm (measured from the cathode to the anode) to remove contaminating unintegrated viral DNA. This was then followed by reversing the polarity of the electrodes and electrophoresis continued for 8 h.

2.5.B Digestion of agarose gel plug (LGT)

Following electophoresis, the agarose gel plug containing the purified chromosomal DNA was heated at 70°C for 5 min. and then cooled to 42°C in a water bath. The agarose was then digested overnight with 1 unit of agarase (Calbiochem/Boehringer Mannheim). The digested agarose was extracted once with phenol (pH 8.0), followed by extraction once with chloroform and then precipitated with 100% ethanol in 0.3 M sodium acetate at -20°C for > 4h. The ethanol was removed by centrifugation (15000g/15 min/4°C) and the purified chromosomal DNA was washed once with 70% ethanol (15000g/10 min/4°C). The chromosomal DNA was allowed to air dry at room temperature and resuspended in the appropriate restriction enzyme digestion or TE buffer.

2.6.A Electrophoretic separation of DNA in Southern gel

DNA samples, mixed with loading dye buffer, were loaded into each lane of 1% agarose prepared in TAE buffer. Electrophoresis was then applied at a voltage of 3V/cm, with the samples moving from the cathode to the anode. When optimal separation of the DNA samples were obtained, as judged by observation of the first dye front migrating to the end of the agarose gel, electrophoresis was stopped. The gel was then treated with 0.2 N HCl for 10 min. followed by further treatment with fresh HCl solution. It was then rinsed briefly in water followed by immersion in denaturing solution, with gentle agitation, for 15 min at RT. The denaturing solution was replaced and the gel further treated for 15 min. The gel was then agitated gently in neutralization solution for 15 min, replenished with fresh solution and neutralized for a further 15-20 min.

2.6.B Southern blotting of DNA

The Southern gel was then inverted and placed face down onto a transfer apparatus containing a wick (Whatman 3MM paper) which was in contact with 20X SSC. An appropriate size of Hybond N+ nylon membrane (Amersham) - prewetted in water and then in 20X SSC - was then placed on top of the Southern gel (the underside). A piece of blotting paper, pre-wetted in 20X SSC, was then placed on the nylon membrane and air bubbles were removed by gentle rolling with a 10-ml pipette. A stack of paper towels was then placed on top of this assembly and weighted with about 400g- weight (usually 4 x 100 ml plastic bottles of distilled water). After overnight transfer, the nylon membrane was placed on top of a piece of blotting paper pre-soaked in 0.4 N NaOH. The DNA samples in the nylon membrane ~~was~~ were denatured for 20 min. followed by neutralization in 5X SSC for 1 min. with constant agitation. Excess fluid was blotted from the nylon membrane and then stored at RT until further use or placed in pre-hybridization solution.

2.7.A RNA preparation (cytoplasmic)

At various times after infection cells were harvested, washed once in ice-cold PBS and then lysed with 200 µl of pre-chilled (4°C) cytoplasmic lysis buffer by mixing vigorously in a vortex mixer for 30 seconds. The cell nuclei were removed by centrifugation at 2500g for 1 min. in a MicroCentaur benchtop centrifuge (MSE). The cytoplasmic RNA (viral and cellular) was mixed vigorously with an equal volume (200 µl) of denaturing solution and 400 µl of phenol/chloroform/isoamyl alcohol (50:50:1)(Gough, 1988). (The latter two reagent mixtures were prepared and stored at 4°C prior to use.) This cytoplasmic RNA, denaturing and phenol/chloroform/isoamyl alcohol mix was then stored at -70°C until further use. After thawing at 4°C, the aqueous phase of the denaturing and phenol reaction mix was separated by centrifugation at 15000g/4°C/5 min. Particular care was taken not to include the white interphase precipitate - which contains mainly proteinaceous materials and DNA - when obtaining the aqueous phase. After precipitation of the aqueous phase overnight at -20°C with absolute ethanol, the RNA samples were obtained by centrifugation at 15000g/15 min/ 4°C, washed once in 70% ethanol (pre-chilled at -20°C) and then dried under a table lamp. The RNA samples were then treated with 0.1 unit/ml DNase (Boehringer Mannheim) in a volume of 100 µl for 5 min at 37°C and then extracted with an equal volume of phenol/chloroform/isoamyl

alcohol (50:50:1), precipitated in two volumes of absolute ethanol and 0.3M sodium acetate (pH 5.5). The RNA was then obtained by similar centrifugation conditions as described above and dissolved in 0.1 mM EDTA solution or RNA loading buffer (Sambrook *et al.*, 1989).

2.7.B Electrophoretic separation of RNA in formaldehyde agarose gel

The RNA samples were heated at 65°C/10 min, chilled in ice and pulse-spinned for 10 seconds prior to loading in Northern gels containing 0.7M formaldehyde. Prior to loading into the gel, running dye (final 1X) was added to the heated RNA samples after chilling in ice. RNA samples were loaded into each lane of 1% Northern agarose gel containing 0.7M formaldehyde and were separated by electrophoresis in 1X MOPS buffer (pH7.0) (Sambrook *et a/.*, 1989). All Northern blot results were from RNA extracted separately from duplicate culture tubes, pooled and loaded in amounts equivalent to 2 x 10^6 cells/lane

2.7.C Northern blotting of RNA

After electrophoresis at 3 volts per cm (distance measured between the anode and cathode electrodes in the tank) RNA was transferred from the gel by capillary blotting onto Hybond-N+ nylon membrane (Amersham) in 20X SSC. The latter membrane, cut to appropriate size, was pre-wetted in sterile distilled water and then in 20X SSC prior to capillary blotting. After overnight transfer the RNA samples on the nylon membrane were fixed with 50 mM NaOH for exactly 5 min, then immediately rinsed for 1 min. in 5X SSC, blotted to remove excess fluid and stored at room temperature until further use or placed in pre-hybridization buffer.

2.8.A Hybridization probe labelling with Megaprime DNA system (Amersham)

For Northern and Southern blot hybridizations, the Sstl fragment of plasmid pBH10 (100 ng) (Hahn *et al.*, 1984) was labelled with 5 µl of α-^{32}P-deoxyadenosine 5'-triphosphate (50 µCi) and 5 µl of α-^{32}P-deoxycytidine 5'-triphosphate (50 µCi) in a total reaction volume of 50 µl at 37°C/40 min. using the Amersham Megaprime DNA labelling kit, according to the manufacturer's instructions. Unreacted label was removed by spun column prepared either in-house with Sephadex G50 (Sambrook *et al.*, 1989) or obtained commercially (Boehringer Mannheim). The columns were spun at 1000g/4 min/4°C and the eluate collected in an

Eppendorf tube. The labelled probe was boiled for 5 min. prior to use in the hybridization mix. The typical specific activity of the labelled probe was 2-5 x 10^8 cpm/μg.

2.8.B ^{32}P labelling of DNA markers (λ -phage DNA/Hind III or SPP-1 phage DNA/EcoRI) or deoxyribonucleotides

1 μg of Hind III digested λ -phage DNA or EcoRI digested SPP-1 phage DNA marker (BRESATEC, South Australia) or deoxyribonucleotide (200 ng) was labelled with 5 μl γ ^{32}P-adenosine 5'-triphosphate (5o μCi) with 2 μl of T4 polynucleotide kinase (5 units/μl) (Pharmacia) in the reaction buffer supplied with the enzyme. The reaction mix, in a volume of 50 μl, was incubated for 1 h/37°C and unincorporated ^{32}P was removed through Sephadex G-25 column (Boehringer Mannheim) for deoxyribonucleotides or precipitation with 0.3M sodium acetate in 100% ethanol at -20°C for > 4 h if labelling DNA markers, followed by washing with 70% ethanol (15000g/10 min/4°C). The labelled DNA marker or deoxyribonucleotide was resuspended in 100 μl of STE buffer. The amount of ^{32}P labelled DNA marker loaded in a Southern gel was *ca* 100,000 cpm. The γ ^{32}P-ATP-labelled deoxyribonucleotide was used without futher treatment as hybridization probe, after removal of unincorporated label.

2.8.C Hybridization of Northern and Southern blots

The Northern or Southern blots were pre-hybridized with 10 ml of hybridization reaction mix at 65°C for >4 hr prior to the addition of the ^{32}P-labelled DNA probe. The membranes were hybridized overnight at 65°C. Unreacted probes were removed by washing with pre-warmed (65°C) buffers which consisted of 2X SSC + 0.5% SDS for 5 min. and then with 2X SSC + 0.1% SDS for 15 min. The membranes were further washed under stringent hybridization conditions with pre-warmed 0.1X SSC + 0.1% SDS for a period of 2 hr with three changes of fresh washing buffer. All washings were at 65°C in a hybridization incubator equipped with a roller mechanism. The membrane was then briefly rinsed in 0.1X SSC and placed onto blotting paper to remove excess fluid but not allowed to dry. The membrane was wrapped in GladWrap and exposed to X-ray films at -70°C/overnight in a cassette with intensifying screens. The films were developed in an automated X-ray film processor (Ilford Ilfospeed 2240).

2.9.A Estimations of the amounts of viral DNA and RNA in Southern and Northern blot hybridizations using phosphorimager analysis

The HIV gag DNA was prepared by reverse transcription PCR of RNA obtained from a co-culture of H3B and Hut78 cells (ratio of 1:4). The primers used corresponded to nucleotide positions 307-322 and 2044-2030 (HIVHXB2R sequence (Myers *et al.*, 1996)). The gag DNA (1738 bp) was gel purified and subsequently TA-cloned into plasmid pBS-dT. The gag RNA standards were then prepared from *in vitro* transcription of the cloned plasmid gag DNA (Davis *et al.*, 1997). The DNA and RNA standards were gifts from A J Davis. Two fold falling dilutions of the *in vitro* transcribed gag RNA standards (range 2000 - 62.5 pg) were loaded into the same Northern gel as the HIV RNA samples obtained from the co-culture of Hut78 and H3B cells in a fresh experiment performed as described above. The Northern hybridization was performed using ^{32}P-Megaprimer (Amersham) - labelled gag DNA (1738 bp) as probe and signals were measured by phosphorimager (Molecular Dynamics, ImageQuant). The amounts of RNA were calculated from a graph of the gag RNA standards used. The graph of the gag RNA standards yielded a linear correlation ($y = 2616x - 166204$, $r = 0.997$; CA-CricketGraph III, Computer Associates). The amounts of HIV 9 kb RNA produced in the co-culture mix were then calculated after adjusting for uniform loading by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA levels on the same tracks. The GAPDH RNAs were detected, on the same nylon membrane, with a ^{32}P-labelled specific probe derived from a plasmid clone (Tso *et al.*, 1985).

Two fold falling dilutions of gag DNA standards (range 500 - 31.25 pg) were loaded into the same Southern gel as HIV DNA samples obtained from the above co-culture of Hut78 and H3B cells. The gag DNA standards were diluted in uninfected Hut78 Hirt supernatant DNA - equivalent to $1x10^6$ cells (see section 2.4). The Southern hybridization was performed using ^{32}P-Megaprimer labelled gag DNA (1738 bp) as probe and signals were measured by phosphorimaging as described above. The amounts of HIV 9 kb DNA produced in the co-culture mix were then calculated from the graph of the gag DNA standards used, which yielded a linear correlation ($y = 14838x - 47679$, $r = 0.999$) (CA-CricketGraph III, Computer Associates).

2.9.B Calculation of copy number of nucleic acid

The copy number of nucleic acids detected in Southern or Northern blot hybridizations was calculated with the formula below:

Copy number = [amount of NA x Avogadro's constant]/[size of NA x 330[@] or 660[@]]

amount of nucleic acid (NA) in grams

Avogadro's number = 6×10^{23}

size of nucleic acid (NA) in number of bases

[@] 330 as average mass of single stranded ribonucleic acid and 660 for double stranded deoxyribonucleic acid.

2.10 HIV reverse transcriptase assay with ^{3}H-thymidine triphosphate

The method used is a modification of that reported by (Hoffman *et al.*, 1985). Clarified HIV-infected cell cultures (1 ml) was precipitated with 30% polyethylene glycol (0.5 ml), mixed well and then incubated on ice for 30 min. The mixture was then spun at 10,000g/10 min./room temperature in a MicroCentaur benchtop centrifuge (MSE). The supernatant fluid was discarded and the pellet was resuspended thoroughly with 100 µl of pre-chilled (4°C) VSB (virus solubilization buffer). The mixture was then stored at -70°C until further use.

To 180 µl of RT reaction mix was added 20 µl of solubilized virus, mixed well and incubated at 37°C/2 hr. The proteins and reactants were precipitated with 50 µl of 50% trichloroacetic acid (TCA) and incubated at 4°C/overnight. The reaction was then mixed well and filtered through Whatman 542 cellulose paper discs. The reaction tubes and discs were then rinsed with three washes of 10% TCA followed by three washes of 5% TCA. The discs were rinsed once with 70% ethanol (analytical grade) and were allowed to dry at room temperature. They were then placed in 5 ml of scintillation fluid (OptiPhase HiSafe, LKB) and counted in a Packard β counter.

2.11 Plasmid clone of HIVHVB2 (Kleen clone)

A plasmid clone (pHXB2 - Kleen) (Australian National Centre in HIV Virology Research, Research Reagents Catalogue 1996) of the proviral DNA was obtained (gift from H. Peng). The pHXB2-Kleen is a subclone of λ HXB2 (NIAID, AIDS Research & Reference Reagent Program) into pBluescript II KS (-) XbaI (5' end of HIV DNA) and SacII (3' end of HIV DNA) restriction sites. The flanking cellular sequences of λ HXB2 were previously removed and only the full length HIV DNA sequence was subcloned. The gel purified HIV DNA fragment from pHXB2-Kleen was used as the reference.

2.12 RNase treatment

The buffer used to digest contaminating RNA in DNA samples consisted of 15mM NaCl and 10 mM Tris pH 8.0. The RNase (Boehringer Mannheim) used was previously boiled for 10 min, cooled to room temperature and stored at -20°C at a stock concentration of 10 mg/ml. DNA samples were treated with 1 µg/ml (equivalent 0.05 U/ml) of RNase/37°C/30 min. in a reaction volume of 100 µl. The treated DNA sample was then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (50:50:1), precipitated in two volumes of absolute ethanol and 0.3M sodium acetate (pH 5.5) at -20°C for >1h. The precipitated DNA was then pelleted at 15000g/15 min/4°C, washed with one-half volume of cold (-20°C) 70% ethanol and then spun at 15000g/10 min. After removal of the 70% ethanol, residual fluid was allowed to air-dry at room temperature under a table lamp. The DNA pellet was then resuspended in an appropriate volume of TE buffer.

2.13 DNase treatment

Contaminating DNA in RNA samples were treated with 0.1 unit of DNase in a reaction volume of 100 µl containing 80 units of ribonuclease inhibitor (RNasin, Promega). The DNase buffer was prepared fresh on the day of use from 10X DNase buffer (100 mM Tris pH8.0, 100 mM $MgCl_2$ and 20 mM DTT). The treated RNA sample was then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (50:50:1), precipitated in two volumes of absolute ethanol and 0.3M sodium acetate (pH 5.5) at -20°C for >1h. The precipitated RNA was then pelleted at 15000g/15 min/4°C, washed with one-half volume of cold (-20°C) 70% ethanol and then spun at 15000g/10 min. After removal of the 70% ethanol, residual fluid was allowed to air-dry at

room temperature under a table lamp. The RNA pellet was then resuspended in an appropriate volume of 1mM EDTA.

2.14 HIV p24 assay

The levels of this viral protein were estimated by the Abbott HIV-1 p24 antigen quantitation assay, Abbott Laboratories, North Chicago.

2.15 Buffers and Reagents

Cytoplasmic lysis buffer

The composition and reaction concentrations of the cytoplasmic lysis buffer for RNA preparation were Tris pH 7.5 (10 mM), $MgCl_2$ (1.5 mM), NaCl (150 mM) and NP-40 (0.65%) (Gough, 1988).

Denaturing solution for Southern blotting of DNA

1.5 M NaCl and 0.5 N NaOH

Denhardt's reagent

This was prepared as 100X stock solution and aliquots stored at -20°C. The composition consisted of 10 g Ficoll (Type 400, Pharmacia), 10 g polyvinylpyrrolidone, 10 g bovine serum albumin (Fraction V, Sigma) and the volume made up to 500 ml with sterile water.

DNA markers

The λ DNA/Hind III markers were obtained from Bresatec, Thebarton, S. Australia 5031. The fragments were 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6 and 0.1 kb. When stained with ethidium bromide or after ^{32}P-labelling and Southern transfer the fragments observed were usually from 23 - 2 kb. The markers were stored at -20°C and thawed in ice prior to use.

Hybridization reaction mix

The final concentration of each reagent in this reaction mix consisted of 6X SSC, 5X Denhardt's, 0.5% SDS and salmon sperm DNA 100 μg/ml. The latter was boiled for 5 min and

chilled in ice for 5 min prior to addition in the reaction mix. The hybridization reaction mix was prepared in 10 ml aliquots and and stored at -20°C until further use.

Loading dye (10X stock)

0.4% bromophenol blue, 0.4% xylene cyanol FF and 25% Ficoll in water (Type 400 Pharmacia)

MOPS buffer 5X stock (pH 7.0).

The composition and reaction concentrations of the 5X MOPS (3-[N-morpholino] propanesulfonic acid) buffer (pH 7.0) were MOPS (0.1 M), sodium acetate (40 mM), NaOH (40 mM) and EDTA pH 8.0 (5 mM) (Sambrook *et al.*, 1989).

Neutralizing buffer for Southern gel

1 M Tris (pH 7.4) and 1.5 M NaCl

Phosphate buffered saline (PBS)

This was prepared with NaCl (130 mM), KCl (2.6 mM), KH_2PO_4 (1.5 mM) and Na_2HPO_4 (8 mM)

Polyethylene glycol 30% (PEG)

This was prepared by autoclaving 30 g PEG (MW 6000) (Sigma) in 100 ml of 0.5 M NaCl.

RNA agarose gel (100 ml)

The agarose gel used for electrophoretic separation of RNA contained 1% agarose and formaldehyde (0.7 M final concentration) prepared in 1X MOPS buffer (Sambrook *et al.*, 1989).

RNA loading buffer

The RNA loading buffer and reaction concentrations were prepared with 40 µl of 5X MOPS buffer, 24 µl of stock 12.3 M formaldehyde (final concentration 0.7 M), 200 µl of formamide (Analar Reagent grade, BDH), 2 µl of ethidium bromide (stock 10 mg/ml) and 134 µl of sterile

water. The total volume was 400 µl. RNA samples were dissolved in 20 µl of this loading buffer, incubated at 65°C/10 min, chilled in ice and spun briefly prior to loading in the agarose gel (Sambrook *et al.*, 1989).

RNA markers

RNA molecular weight markers were obtained from Promega Corporation, Annandale, NSW 2038. These markers were stored at -70°C and thawed in ice prior to use. Two ranges of these markers were obtained from Promega - G3151 or G3191. The size fragments of the earlier markers were 9.5, 6.2, 3.9, 2.8, 1.9, 0.9, 0.6 and 0.4 kb. The G3191 size fragments were 6.6, 5.0, 3.6, 2.6, 1.9, 1.4, 0.9, 0.6 and 0.3 kb.

Reverse transcription reaction mix

The composition and reaction concentration of the RT reaction mix were Tris pH 7.8 (50 mM), $MgCl_2$ (10 mM), dithiothreithol (5 mM), 30 µl of [polyadenylic acid x pentadecathymidylic acid] (Boehringer Mannheim) in a volume of 1 L, 2'-deoxy-adenosine-5'-triphosphate (disodium salt, Boehringer Mannheim) (80 ng/ml) and ^{3}H-thymidine-5'-triphosphate (tetrasodium salt, DuPont NEN) (50 nCi/ml).

STE

10 mM Tris (pH 7.5), 0.1M NaCl and 10 mM EDTA (pH 8.0)

SSC (20X stock)

3 M NaCl and 0.3 M tri-sodium citrate dihydrate (adjusted to pH 7.0-7.2 with a few drops of 10 N NaOH)

TAE buffer (50X stock)

2M Tris base, 50 mM EDTA (pH 8.0) and 1M glacial acetic acid (working solution 1X: 40mM Tris-acetate and 1 mM EDTA)

TE buffer (pH 8.0)

10 mM Tris (pH 8.0) and 1 mM EDTA (pH 8.0)

Urea-SDS solution

The composition and reaction concentrations of the Urea-SDS solution were urea (7 M), SDS (1 %), NaCl (0.35 M), EDTA pH 8.0 (10 mM) and Tris pH 7.5 (10 mM) (Gough, 1988).

Virus solubilization buffer

The composition and reaction concentrations of the virus solubilization buffer were triton X-100 (0.5%), NaCl (0.8 M), glycerol (20%) and Tris pH 7.8 (50 mM).

Chapter 3

There does not exist a category of science to which one can give the name applied science. There are science and the applications of science, bound together as the fruit of the tree which bears it. Louis Pasteur (1822-1895)

Kinetics and pattern of HIV RNA expression in cell-to-cell transmission of infection

3.1 Introduction

Following infection of a cell with human immunodeficiency virus (HIV) or other retroviruses, the positive-sense viral RNA genome is converted to double-stranded DNA by the virus-coded enzyme reverse transcriptase (Baltimore, 1970, Temin & Mizutani, 1970). The newly synthesized DNA then integrates into the cellular DNA and subsequently acts as template for viral RNA transcription. One of the earlier studies with HIV showed that infection of H9 T-cells with cell-free virus yielded full-length linear viral DNA at 4h post-infection (p.i.) which increased in amount to 12 h p.i. (Kim *et al.*, 1989b). Circular DNA forms were detected later, in smaller amounts and in the cell nucleus only.

Infection of a cell with HIV may be initiated by cell-free viral particles or by the interaction of an infected cell with a susceptible cell (Li & Burrell, 1992, Sato *et al.*, 1992, Barbosa *et al.*, 1994). Both processes are assumed to occur in the infected host; however cell-to-cell transmission may be particularly significant once neutralizing antibody has developed. Experimentally, HIV has been transmitted by fusion of infected lymphocytes (obtained from patients with AIDS) with phytohaemagglutin (PHA)-stimulated PBMC cells despite the presence of neutralizing antibodies (Gupta *et al.*, 1989). Transmission of virus from HIV-infected lymphocytes to intestinal epithelial cells was also shown in the presence of anti-gp 120 or antiserum from HIV-antibody positive patients (Phillips & Bourinbaiar, 1992).

In view of the above and to study early replicative events, Li and colleagues (Li & Burrell, 1992, Li *et al.*, 1992) developed a synchronous cell-to-cell transmission model using H3B cells as viral donor cells and CD4+ Hut78 T cells as recipients. H3B cells are a clone of H9 cells persistently infected with human T cell-lymphotropic virus type IIIB (HTLV-IIIB). Each H3B

cell contains two copies of integrated proviral DNA and episomal HIV DNA corresponding to one or two genome equivalents/10^3 cells. Following cell-to-cell transmission, newly-made episomal HIV DNA appears at 4 h p.i., and both linear and circular forms then accumulate with kinetics essentially similar to those seen in cell-free viral infections (Kim *et al.*, 1989b, Li & Burrell, 1992). In a later report Dimitrov *et al.* (Dimitrov *et al.*, 1993) showed that the infectivity of HIV-1 during cell-to-cell transmission is 100 - 1000 times greater than with cell-free virus stocks. This was based on mathematical models relating experimental observations with quantitation of infectious virons per cell. Using the cell-free viral infection route Kim and colleagues (Kim *et al.*, 1989b) showed that HIV RNA was first detected 16 h p.i.and increased progressively to 48h p.i; in contrast to the earlier appearance of HIV DNA at 4 h p.i. The first RNA species found were the spliced 2 kb and 4.3 kb forms encoding regulatory and envelope proteins, and significant amounts of unspliced 9.2 kb RNA capable of providing both new genomic RNA and gag-pol mRNA were not observed until 24 h p.i. These findings have been interpreted as demonstrating a programmed temporal shift from spliced to unspliced RNA transcripts as the infection process develops.

To more fully understand the cell-to-cell transmission infection , the kinetics of HIV RNA expression in this model were examined and correlated with findings for infection with cell-free virus (Kim *et al.*, 1989b).

3.1.A Cell-to-cell transmission infection

The optimal ratio for a synchronous, one-step cell-to-cell transmission of infection HIV donor H3B and target Hut78 lymphoid cells was found to be 1:4 respectively (Li & Burrell, 1992). Each cell line was cultured at a concentration of 1 x 10^6 cells/ml 18h - 22h prior to cell mixing in order to produce cells that were at a similar growth phase and under consistent cell culture conditions in different experiments. On the day of co-culture, the H3B and Hut78 cells were washed once in serum-free, base medium brought to room temperature prior to use and viable cell counts were made by trypan blue exclusion. Cells which yielded less than 95% viability were not used in the experiments. The washed cells were resuspended in growth medium and mixed in the ratio of one H3B cell to four Hut78 cells in a tissue culture flask. Aliquots of the mixed cell suspension were inoculated into six-well tissue culture plates and incubated at 37°C

in 5% CO_2. The co-culture mix of cells, as described above, normally yielded >10% giant cell formation (with cell syncytium and multi-nucleated cells) by 4h p.i. (see Fig. 2.2.Ai). Very occassionally, the cells did not yield optimal giant cell formation and this was corrected by recovering fresh cell stocks from liquid nitrogen storage. At various times post infection the co-culture was harvested, centrifuged at 200g/3 min and viral nucleic acids were extracted from the cells or supernatant RT activity or HIV antigen levels measured. When viral DNA was extracted from this co-culture mix of cells by the Hirt method (Hirt, 1967) (see section 2.4), separated by gel electrophoresis and analysed by Southern blotting a typical profile of the kinetics of HIV DNA production during the 24 h period of cell-to-cell transmission infection is shown in Fig. 3.1.Ai. The Southern blot was hybridized with ^{32}P-labelled-SstI fragment of HIV plasmid pBH10 (Hahn *et al.*, 1984). This DNA profile is similar to the initial report of Li and Burrell (Li & Burrell, 1992). The first appearance of unintegrated, linear HIV DNA was observed at 4h p.i. and the levels continued to increase with appearance of circular forms (supercoiled and relaxed) of viral DNA at *ca.* 8h p.i.. By 24 h.p.i. there is a significant increase in the levels of all three forms of HIV DNA which represents a new round of viral replication.

3.2 HIV RNA expression following cell-to-cell transmission of infection

H3B viral donors and Hut78 recipient cells were co-cultured as described in 3.1.A and at various periods post infection the cells were harvested and cytoplasmic RNA extracted in the presence of 7M urea and 1% SDS according to Gough ((Gough, 1988), section 2.7.A). After treatment with DNase at 0.1 unit/ml for 5 min/37^{o}C (see section 2.13 and below), the extracted cytoplasmic RNA from different time points post infection was separated in 1% agarose gel with 0.7M formaldehyde in 1X MOPS buffer (see section 2.7.B). Without further treatment the Northern gel was capillary blotted onto nylon membrane (Hybond N+, Amersham) in 20X SSC for >16h. After fixation in 50 mM NaOH/5 min the membrane was pre-hybridized for >4h before hybridizing with ^{32}P-labelled-SstI fragment of HIV plasmid pBH10 (Hahn *et al.*, 1984).

Fig. 3.2i shows the changes in cytoplasmic HIV RNA during the period between 0 and 72 h, after coculturing H3B cells and Hut78 cells. Four hours after mixing there was a significant increase in genomic-length 9 kb RNA as compared to the 0 h sample (just after mixing) or a sample containing a five-fold greater number of H3B cells (2 x 10^6 cells; Fig.3.2i, lane H).

Fig. 3.1.Ai

Kinetics of HIV DNA production during the period 0 and 24 h after mixing of Hut78 recipient cells with H3B viral donor cells. The figures at the top of each lane refer to the time (h) at which viral DNA was extracted. Each lane contains an amount of viral DNA equivalent to that from 1 x 10^6 cells representing the average DNA extracted from duplicate cell cultures. RC: relaxed circular, LN: linear and SC: supercoiled forms of HIV DNA.

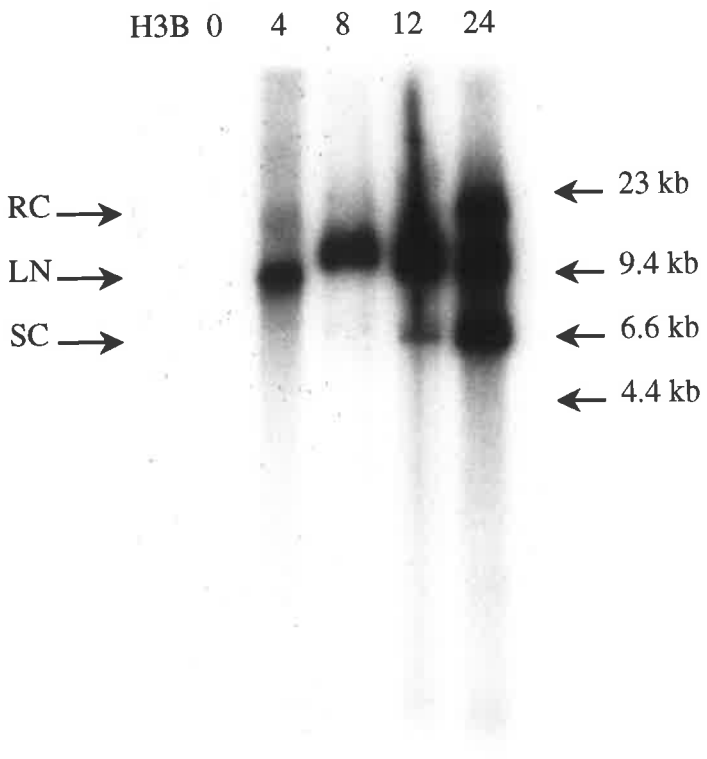
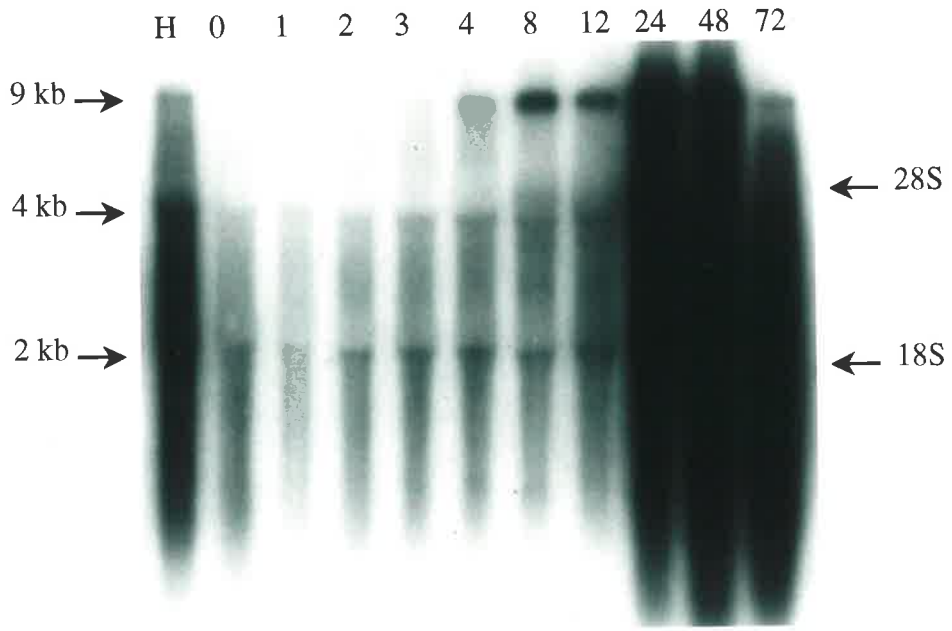

H3B 0 4 8 12 24
RC
LN
SC
23 kb
9.4 kb
6.6 kb
4.4 kb

Fig. 3.2i

Kinetics of HIV RNA production during the period 0 and 72h after mixing of Hut78 recipient cells with H3B viral donor cells. The figures at the top of each lane refer to the time (h) at which RNA was extracted. Lane H, RNA extracted from 2 x 10^6 H3B cells. Each lane contains an amount of RNA equivalent to that from 2 x 10^6 cells representing the average RNA extracted from duplicate cell cultures.


H 0 1 2 3 4 8 12 24 48 72
9 kb
4 kb
2 kb
28S
18S

This increased level of 9kb RNA, and near constant levels of 4 kb and 2 kb spliced RNAs, were maintained until 24 h when a second large increase in all three RNA species was seen. During the period 4-24h p.i. there was a corresponding increase in the number of giant cells followed by the appearance of ghost membranes. Finally, by 72 h p.i. large amounts of 4 kb and 2 kb RNA were present but 9 kb RNA was barely detectable. At 72h p.i. the co-culture mix showed a large number (>50%) of degenerate cells, and giant cells (<30% of total cells) as well as ghost membranes surrounding the latter were also observed.

The early appearance (4h p.i.) of genomic length 9 kb viral RNA in the cell-to-cell transmission infection model (Kok *et al.*, 1993) contrasts with the later appearance of this viral RNA species at 24h p.i. observed with cell-free viral infection (Kim *et al.*, 1989b). The latter study by Kim and colleagues showed that the first species of viral RNA observed were the 2 and 4.3 kb RNAs at 12-16h p.i. In the cell-to-cell transmission of infection model, the steady state in H3B cells contained predominantly 4.3 and 2 kb HIV RNA species in the cytoplasmic fraction, with a barely detectable shadow at 9.2 kb (Fig. 3.2i, lane H). In view of these differences we wished to ensure that the RNA signal detected was not due to contaminating free viral DNA (see section 3.3) and to further investigate the transcription templates responsible for the early appearance of 9kb RNA (see sections 3.4 & 3.5).

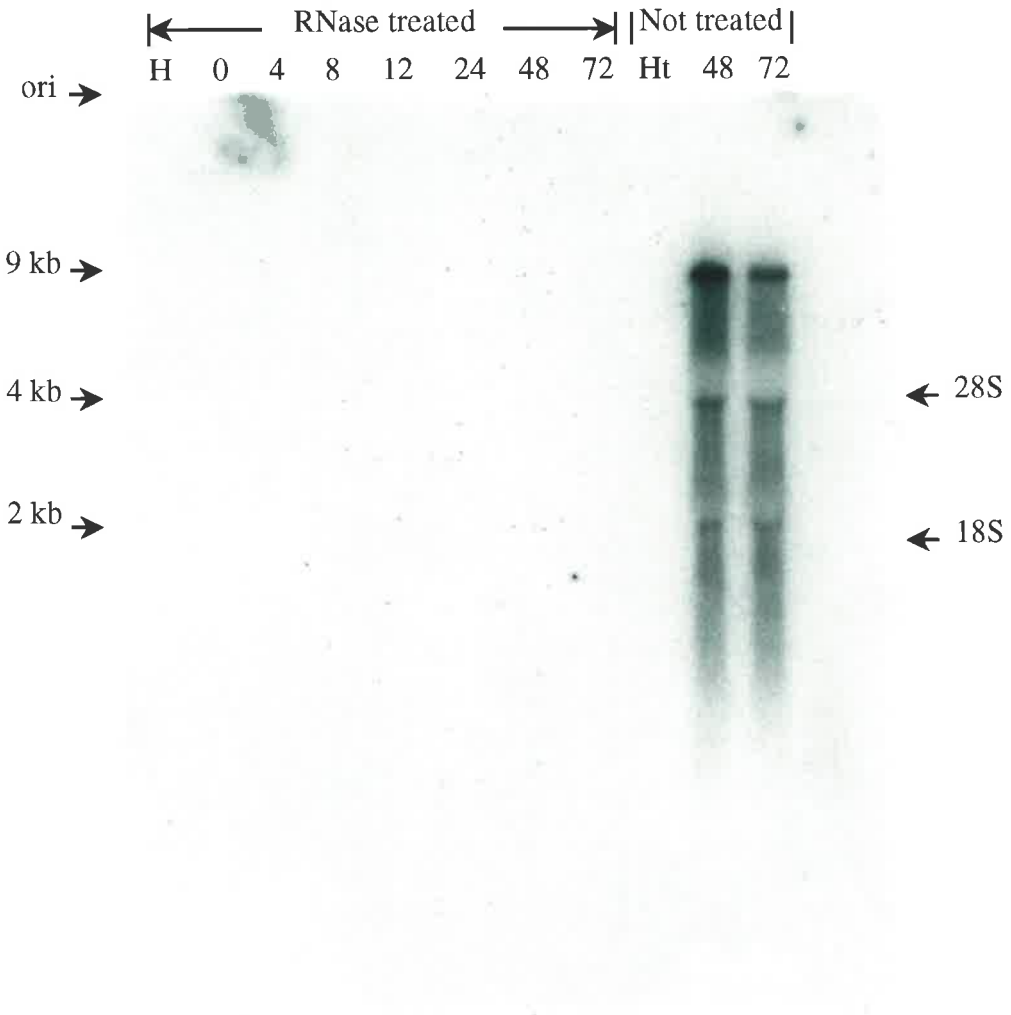
3.3 Specificity of RNA detection

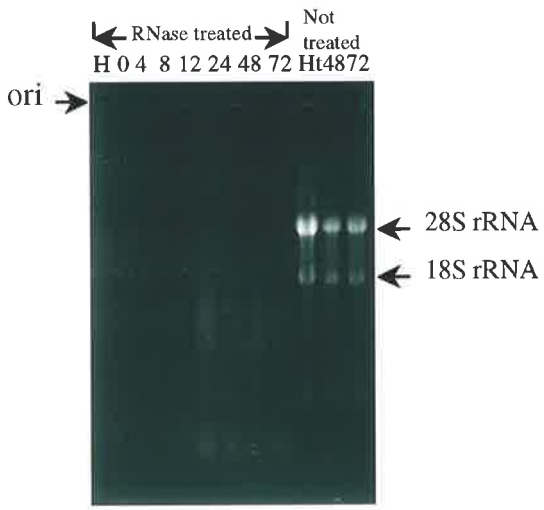
As co-culture of H3B and Hut78 cells yielded HIV DNA ((Li & Burrell, 1992), see also Fig. 3.1.Ai) as well as RNA, it was necessary to be certain that the hybridization signals obtained in the Northern blot hybridizations were specific for HIV RNA and not due to contaminating HIV DNA. This was shown by three approaches viz. (i) abolition of Northern signal by treatment of RNA samples with RNase, (ii) demonstration that the concentration of DNase used to treat RNA was sufficient to completely degrade viral DNA produced during cell-to-cell HIV infection and (iii) absence of Northern blot hybridization signals with DNase-treated HIV DNA. Firstly, treatment with 100 µg/ml RNase abolished all Northern blot hybridization signals as well as 28S and 18S ribosomal RNA detected by ethidium bromide staining (Fig. 3.3i). This RNase treatment has previously been shown not to affect viral DNA detection in Southern blots (Li & Burrell, 1992).

Fig. 3.3i

Northern blot hybridization analysis of RNase-treated HIV RNA samples (duplicate samples obtained from experiment described in section 3.2). The figures at the top of each lane refer to the period p.i. (h) at which RNA was extracted. Lane H, RNA extracted from H3B cells. Lane Ht, RNA extracted from Hut78 cells. Each lane contains an amount of RNA equivalent to that from 1 x 10^6 cells representing the average RNA extracted from duplicate cell cultures.

The lower photograph shows the ethidium bromide-stained agarose gel prior to Northern blotting and hybridization. (N.B. The absence of rRNA in the RNase-treated samples in ethidium bromide stained gel as well as the corresponding absence of Northern signals in the upper photograph.)


RNase treated
Not treated
H 0 4 8 12 24 48 72 Ht 48 72
ori
9 kb
4 kb
2 kb
28S
18S


RNase treated
Not treated
H 0 4 8 12 24 48 72 Ht4872
ori
28S rRNA
18S rRNA

Secondly, the optimal concentration of DNase chosen for treatment of RNA samples, to remove contaminating viral DNA, was obtained by incubating five-fold falling dilutions of DNase (10 units/ml to 0.0032 unit/ml) with HIV DNA obtained in a Hirt supernatant (see section 2.4) from a co-culture of H3B and Hut78 cells at 12 h p.i. The viral DNA was treated in a volume of 100 µl at 37°C/5 min. and then extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The DNase-treated and untreated HIV DNA samples were Southern blotted (see section 2.6A & B) and hybridized with a near full-length HIV plasmid (pBH10) probe (Hahn *et al.*, 1984) (see section 2.8.A). Fig. 3.3.ii shows the Southern blot hybridization results of this titration. At a concentration of $\geq$ 0.016 unit/ml of DNase/37°C/5 min. there was complete degradation of all three forms of HIV DNA. This is the second line of evidence that the Northern blot hybridization signals detected were due to viral RNA. The limit of detection for HIV gag DNA in this Southern blot hybridization was <6 pg. The DNase concentration chosen for subsequent treatment of HIV RNA samples was 0.01 unit in a volume of 100 µl (equivalent to 0.1 unit/ml) at 37°C/5 min. This concentration of DNase chosen is ten-fold greater than the minimum amount required to completely degrade an excess amount of contaminating viral DNA.

Thirdly, HIV DNA was treated with DNase and then examined on a Northern gel with RNA buffers. An excess amount of viral DNA obtained by Hirt extraction of cells from a co-culture of H3B and Hut78 at 12h p.i., was treated with RNase at 1 µg/ml/37°C/30 min. and then phenol-chloroform extracted followed by ethanol precipitation. The HIV DNA were then treated with two-fold falling dilutions of DNase (0.4 unit/100 µl - 0.003125 unit/100µl) at 37°C/5 min. The treated DNA samples were phenol-chloroform extracted, ethanol precipitated and then resuspended in RNA loading buffer and analysed by Northern blot hybridization. Prior to loading into a Northern gel (DNA from the equivalent of 10^6 cells/track), the treated DNA samples were heated at 65°C/10 min. Fig. 3.3iii shows the nucleic acids were degraded to < 0.6 kb fragments as observed in the ethidium bromide stained gel of these treated DNA samples. There were no detectable Northern signals with DNase-treated DNA samples when hybridized with the pBH10 HIV plasmid (data not shown). Even with the untreated DNA there was minimal Northern hybridization signal intensity obtained. Taken together the above

Fig. 3.3ii

Titrations of DNase (from 0 unit/100 µl to 1 unit/100 µl) treatment with HIV DNA, from Hirt supernatant fraction obtained by extraction of a co-culture of H3B and Hut78 cells (12 h p.i.) in the cell-to-cell transmission infection model. The lane numbers at the top of the Southern blot (upper photograph) show the concentration of DNase (unit/ 100 µl) used to treat the HIV DNA (equivalent to that extracted from 1 x 10^6 cells) viz. lane 1: 1 unit/100 µl, lane 2: 0.2, lane 3: 0.04, lane 4: 0.008, lane 5: 0.0016, lane 6: 0.00032 and lane 7: not treated. The lower photograph shows the range (6 - 200 pg) of HIV plasmid DNA (pBH10) (Hahn *et al.,* 1984) detected in the same Southern blot hybridization. RC: relaxed circular, LN: linear and SC: supercoiled forms of HIV DNA. ori: origin of gel

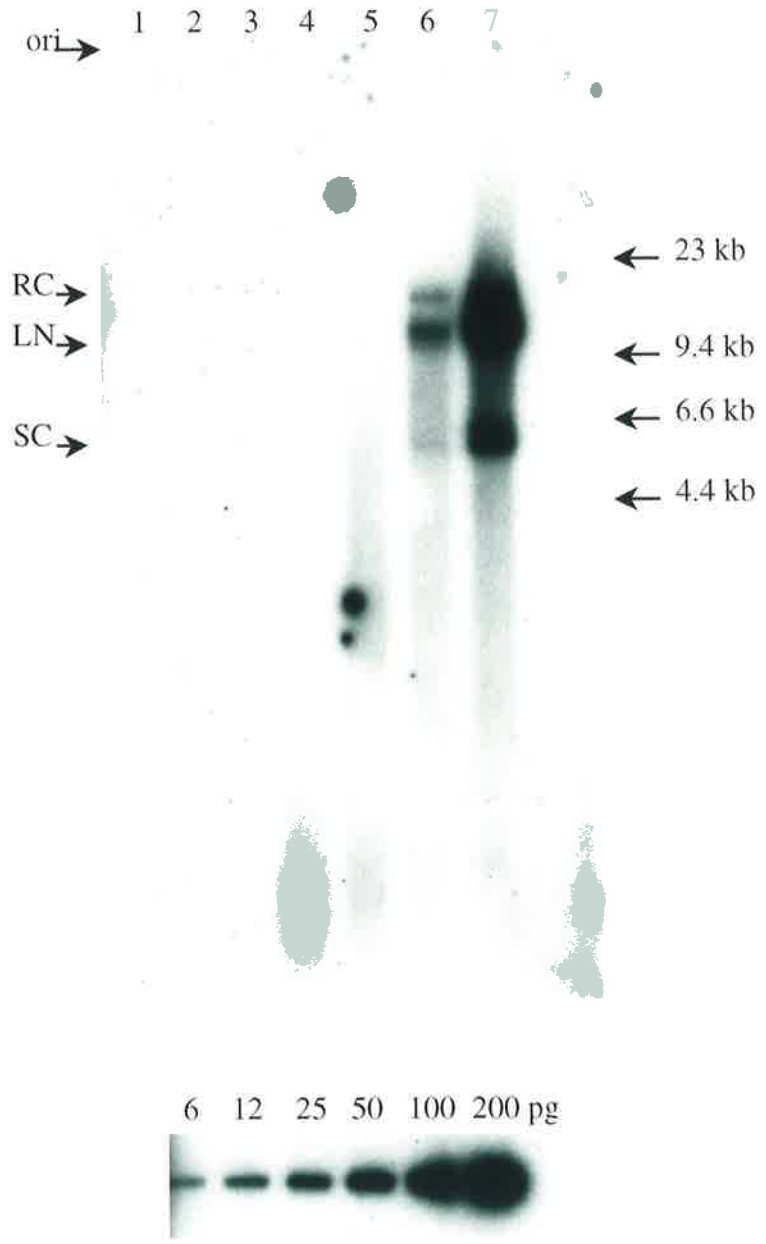

ori
1
2
3
4
5
6
7
RC
LN
SC
23 kb
9.4 kb
6.6 kb
4.4 kb
6
12
25
50
100
200 pg

Fig. 3.3iii

Ethidium bromide stained Northern gel of DNase treated HIV DNA (obtained from the equivalent of 10^6 cells in the co-culture of H3B and Hut78 cells). The DNase treated-DNA samples were then heated at 65°C/10 min prior to loading in the Northern gel. ori: origin of gel The lane numbers at the top of the gel relate to the concentration of DNase (unit/ 100 µl) used to treat the HIV DNA (equivalent to that extracted from 1 x 10^6 cells) viz:

lane 1: 0.4 unit/100 µl

lane 2: 0.2 unit/100 µl

lane 3: 0.1unit/100 µl

lane 4: 0.05 unit/100 µl

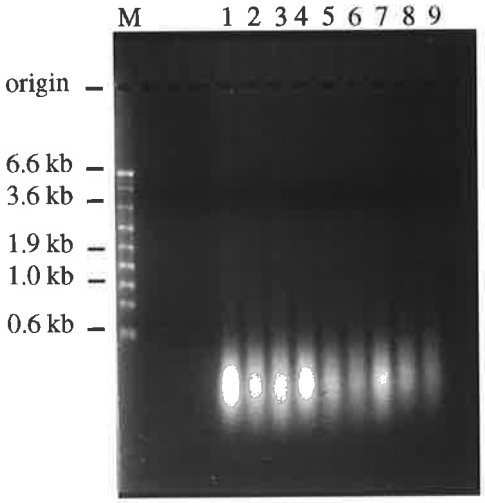
lane 5: 0.025 unit/100 µl

lane 6: 0.0125 unit/100 µl

lane 7: 0.00625 unit/100 µl

lane 8: 0.003125 unit/100 µl

lane 9: no DNase, buffer only.


M
1 2 3 4 5 6 7 8 9
origin
6.6 kb
3.6 kb
1.9 kb
1.0 kb
0.6 kb

evidence indicates that the Northern blot hybridization signals were specific for viral RNA (Fig. 3.2i).

3.4 Use of reverse transcription inhibitors to examine the template for early HIV RNA transcription during cell-to-cell transmission of infection

In the cell-to-cell transmission system described above, three potential templates for early HIV RNA transcription may be considered:

[a] proviral DNA present in the viral donor cells,

[b] newly synthesized unintegrated HIV DNA and

[c] newly synthesized and integrated proviral DNA.

One possible approach to determine which of these is used is to inhibit the production of newly synthesized viral DNA by reverse transcription inhibitors. AZT is a nucleoside inhibitor of HIV reverse transcriptase. It is a thymidine analog which is phosphorylated to the active 5'-triphosphate form by cellular kinases and inhibits reverse transcription by terminating viral DNA chain elongation (Mitsuya *et al.*, 1985, Fischl *et al.*, 1987). Fig.3.4i shows the kinetics of cytoplasmic RNA expression in a similar experiment to that in Fig.3.2i except that both H3B and Hut78 cells were treated with 20 μM AZT 18 h prior to infection. The drug was renewed at the time of infection and maintained throughout the 72 h incubation period. The conditions of infection and RNA extraction were identical to those in Fig.3.2i and the Northern blots were hybridized with the same ^{32}P-labelled probe mix. AZT treatment of H3B cells alone did not reduce viral RNA levels and in fact in some experiments led to a slight increase in 4 kb and 2 kb HIV RNA. However, the early phase (4 - 12 h p.i.) of 9 kb RNA synthesis (Fig. 3.2i) was not observed here (Fig. 3.4i). By 24 to 48h p.i. induction of all three HIV RNA species was obtained, with a similar profile but at lower levels than without AZT. In a similar co-culture of AZT-treated H3B and Hut78 cells, no viral DNA was observed by Southern hybridization during the period 0 -12 h p.i. (Fig. 3.4ii), and at 24 h p.i. the viral DNA level in AZT-treated cells was significantly lower than in untreated cells (Fig. 3.1.Aii).

Two other reverse transcription inhibitors were also used in similar experiments. DDI (2',3'-dideoyinosine) is a nucleoside inhibitor of RT that is converted to the active dideoxyadenosine triphosphate form by cellular enzymes (Mitsuya & Broder, 1986, Nave *et al.*, 1994). DDI has

Fig. 3.4i

Northern blot hybridization analysis of HIV RNA production in the presence of 20 μM AZT commencing 18h before and during the period 0 and 72h mixing of Hut78 recipient cells with H3B viral donor cells. The figures at the top of each lane refer to the period (h p.i.) at which viral RNA was extracted. Lane H, RNA extracted from 2 x 10^6 H3B cells (treated with 20 μM AZT). Each lane contains an amount of RNA equivalent to that from 1 x $1O^6$ cells representing the average RNA extracted from duplicate cell cultures. ori: origin of gel

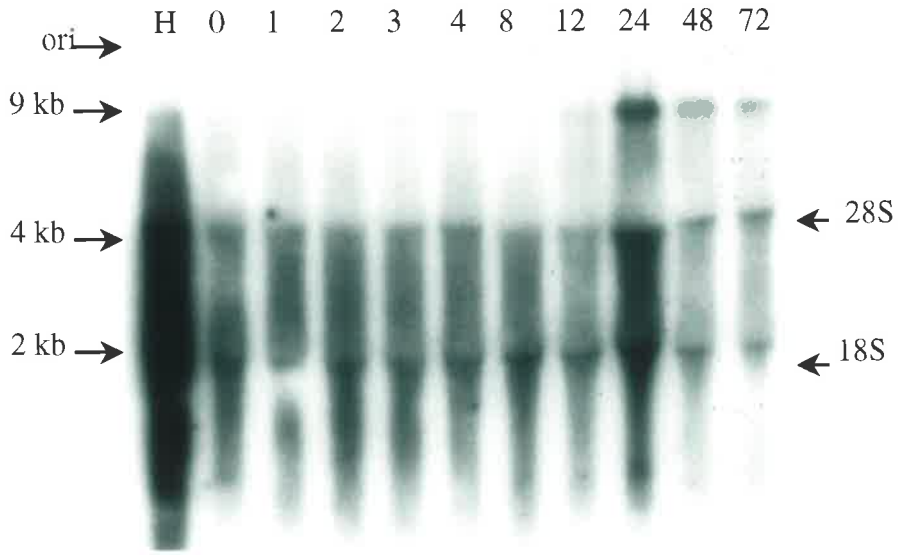
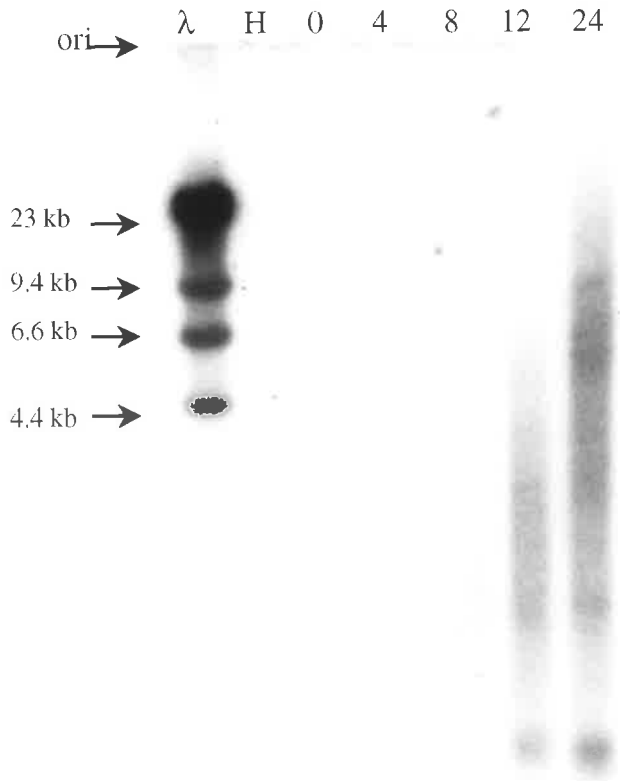

H
0
1
2
3
4
8
12
24
48
72
ori
9 kb
4 kb
2 kb
28S
18S

Fig.3.4ii

Southern blot hybridization analysis of HIV DNA production in the presence of 20 μM AZT commencing 18h before and during the period 0 and 24h mixing of Hut78 recipient cells with H3B viral donor cells. The figures at the top of each lane refer to the period (h p.i.) at which the viral DNA was extracted. Lane H, DNA extracted from 1 x 10^6 H3B cells (treated with 20 μM AZT). Each lane contains an amount of DNA equivalent to that from 1 x 10^6 cells representing the average DNA extracted from duplicate cell cultures. Lane λ, ^{32}P-labelled λ/DNA markers. ori: origin of gel.


λ H 0 4 8 12 24
ori
23 kb
9.4 kb
6.6 kb
4.4 kb

similar activity to AZT and inhibits reverse transcription by terminating viral DNA chain elongation. H3B and Hut78 cells were treated with 20 μM of DDI 18h prior to cell-to-cell co-culture. The drug was renewed at the time of infection and maintained throughout the 72 h infection period. In Northern blot hybridization analysis, the early phase (4 - 12 h p.i.) of viral RNA synthesis was not observed as shown by absence of genomic length 9 kb HIV RNA (Fig. 3.4iii). As with AZT treatment (Fig. 3.4i) the second phase of 9 kb viral RNA synthesis was observed in significant amount by 24 h. p.i. and subsequently. There was no significant increase in the levels of 4kb and 2 kb viral RNA during the early phase of RNA production.

PFA (Phosphonoformic acid or Foscarnet) is believed to inhibit reverse transcription by blocking the pyrophosphate binding site of HIV RT (Oberg, 1983, Wondrak *et al.*, 1988). In a similar cell-to-cell transmission infection experiment, H3B and Hut78 cells were treated with 100 μg/ml of PFA 18h prior to co-culture. The drug was replaced at the time of cell mix and maintained throughout the incubation period. RNA was extracted from the co-culture mix of cells at the indicated periods p.i. and subsequent Northern blot hybridization analysed as described above. As with treatment of the cells with AZT or DDI, genomic length 9 kb HIV RNA was not observed during the early phase (0 - 12h p.i.) of HIV RNA production in the PFA-treated cells, while 4 kb and 2 kb viral RNA levels remained unchanged during this period. The second phase ($\geq$ 24 h p.i.) of viral RNA production was marked by a significant induction of 9 kb HIV RNA as well as the 4kb and 2kb RNA classes (Fig.3.4iv).

The corresponding Southern blot hybridization analysis with DDI or PFA-treated cells in a similar co-culture mix showed minimal or no detectable HIV DNA levels during the period 0 - 12h p.i. (Fig.3.4v & vi). The viral DNA level at 24 h p.i. with DDI-treated cells was significantly lower than PFA-treated (Fig. 3.4v) or untreated cells (see Fig. 3.1.Aii). These experiments showed that in cells pre-treated and maintained with RT inhibitors, the first phase (0 - 12 h p.i.) of HIV RNA production was abolished corresponding to the absence of HIV DNA. Since these inhibitors have not been reported to directly suppress RNA transcription and did not reduce HIV RNA levels in H3B cells, the results suggest that the likely transcription template for the first phase of HIV RNA is the newly made (free and/or integrated) viral DNA.

Fig.3.4iii

Kinetics of HIV RNA production in the presence of 20 μM DDI commencing 18h before and during the period 0 and 24h mixing of Hut78 recipient cells with H3B viral donor cells. The figures at the top of each lane refer to the period (h p.i.) at which the RNA was extracted. Lane H, RNA extracted from 2 x 10^6 H3B cells (treated with 20 μM DDI) Each lane contains an amount of RNA equivalent to that from 2 x lO^6 cells representing the average RNA extracted from duplicate cell cultures. ori: origin of gel.

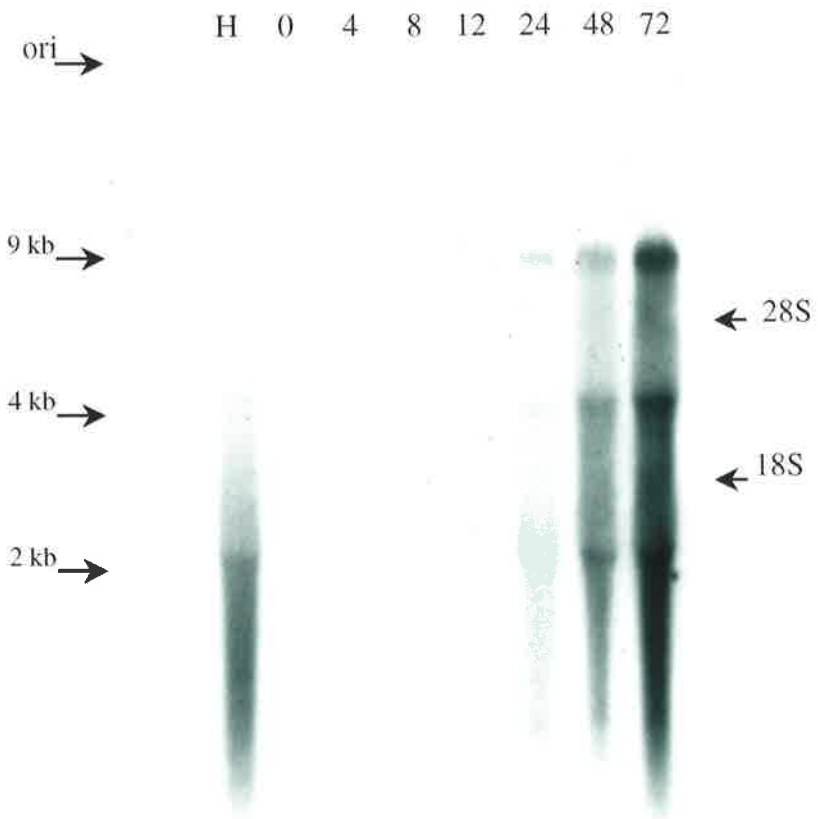

H
0
4
8
12
24
48
72
ori
9 kb
4 kb
2 kb
28S
18S

Fig.3.4iv

Kinetics of HIV RNA production in the presence of 100 µg/ml of PFA commencing 18h before and during the period 0 and 24h mixing of Hut78 recipient cells with H3B viral donor cells. The figures at the top of each lane refer to the period (h p.i.) at which the RNA was extracted. Lane H, RNA extracted from 2 x 10^6 H3B cells (treated with 100 µg/ml of PFA). Each lane contains an amount of RNA equivalent to that from 2 x $1O^6$ cells representing the average RNA extracted from duplicate cell cultures. ori: origin of gel.

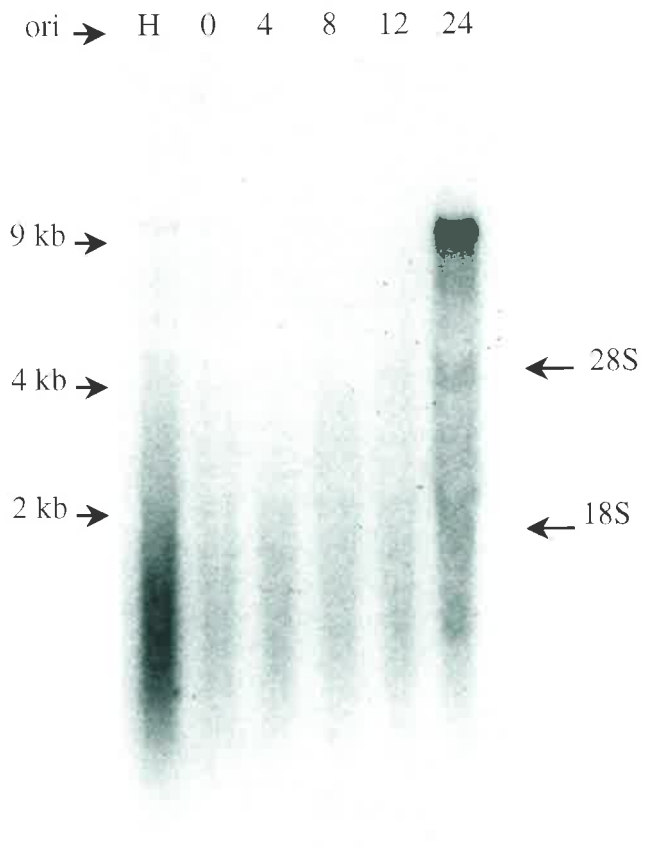

ori
H
0
4
8
12
24
9 kb
4 kb
2 kb
28S
18S

Fig.3.4v

Southern blot hybridization analysis of HIV DNA production in the presence of 20 μM DDI commencing 18h before and during the period 0 and 24h mixing of Hut78 recipient cells with H3B viral donor cells. The figures at the top of each lane refer to the period (h p.i.) at which the viral DNA was extracted. Lane H, DNA extracted from 1 x 10^6 H3B cells (treated with DDI). Each lane contains an amount of DNA equivalent to that from 1 x $1O^6$ cells representing the average DNA extracted from duplicate cell cultures. Lane λ, ^{32}P-labelled λ/DNA markers (section 2.8.B). ori: origin of gel.

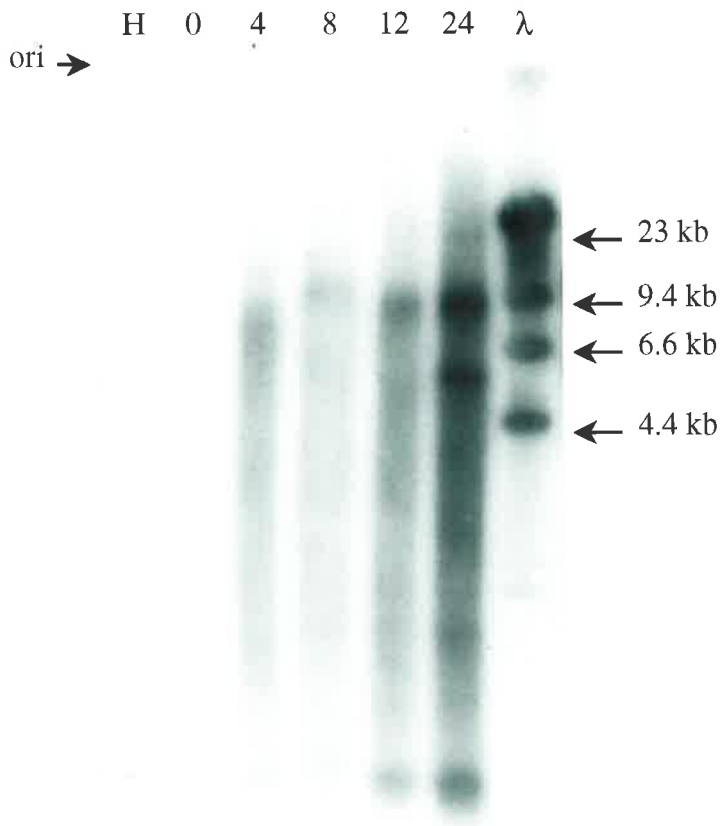
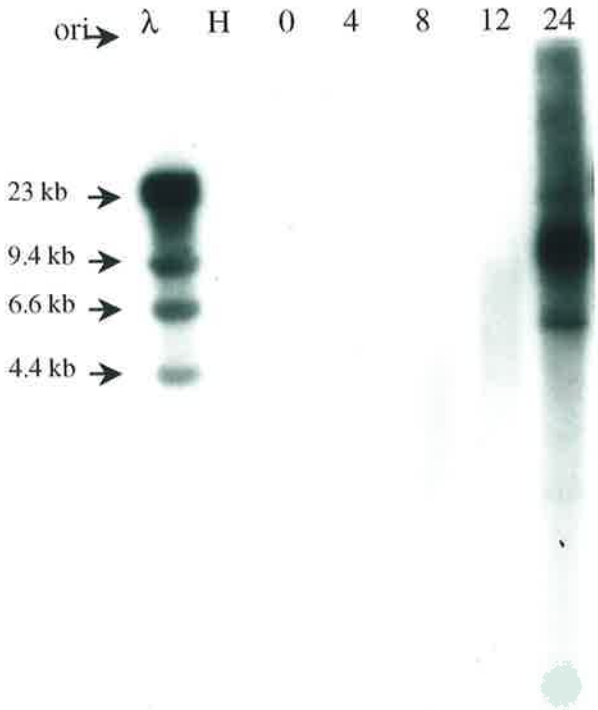

H
0
4
8
12
24
λ
ori
23 kb
9.4 kb
6.6 kb
4.4 kb

Fig.3.4vi

Southern blot hybridization analysis of HIV DNA production in the presence of 100 μg/ml PFA commencing 18h before and during the period 0 and 24h mixing of Hut78 recipient cells with H3B viral donor cells. The figures at the top of each lane refer to the period (h p.i.) at which the viral DNA was extracted. Lane H, DNA extracted from 1 x 10^6 H3B cells (treated with PFA). Each lane contains an amount of DNA equivalent to that from 1 x $1O^6$ cells representing the average DNA extracted from duplicate cell cultures. Lane λ, ^{32}P-labelled λ/DNA markers (section 2.8.B). ori: origin of gel.


ori
λ
H
0
4
8
12
24
23 kb
9.4 kb
6.6 kb
4.4 kb

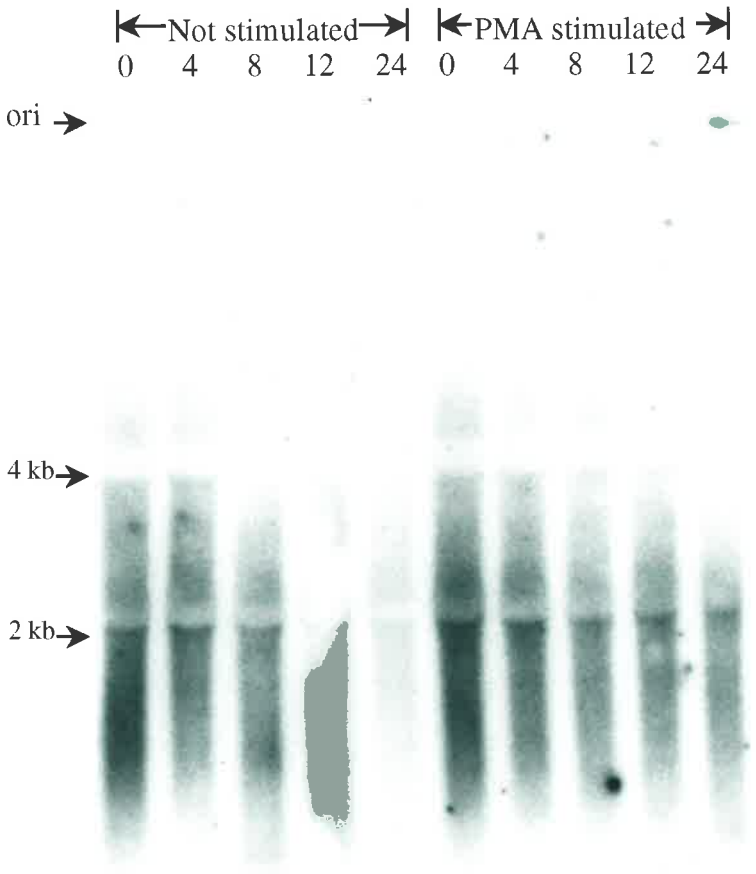
3.5 Studies to determine non-specific induction of genomic HIV RNA expression in H3B cells

Two chronically-infected, but different cell lines with HIV proviral DNA were shown to be induced by cytokines or phorbol esters to produce larger quantities of virus (Folks *et al.*, 1987, Folks *et al.*, 1989). Two separate studies reported that viral RNA expression from proviral DNA could be up-regulated in HIV persistently-infected cells by treatment with some mitogens viz. lipopolysaccharide (LPS), phytohemagglutinin (PHA) or phorbol 12-myristate 13-acetate (PMA) (Pomerantz *et al.*, 1990, Michael *et al.*, 1991). The cell lines used in these studies were U1 - a monocyte/macrophage-like cell line and ACH-2 which is a T-cell line. Both of these cell lines contain substantial amounts of spliced HIV RNAs but significantly less (*ca.* 5%) unspliced, genomic RNA (similar to H3B cells). Induction of U1 or ACH-2 cells with PMA yielded a shift in splicing favoring unspliced genomic RNA (Michael *et al.*, 1991). To determine whether the HIV RNA expression pattern obtained with the cell-to-cell transmission infection experiments described above can be stimulated from the viral donor cells with phorbol ester, H3B cells were cultured with 20nM of PMA and at various periods after stimulation, cytoplasmic RNA was extracted and analysed by Northern blot hybridization (Fig. 3.5i). During the period 0 - 24h of stimulation with PMA there was no increase in any HIV RNA species in the H3B cells compared with unstimulated control cells. The viability of the PMA-treated cells, as judged by trypan blue exclusion, was 100% during the period 0h-12h post treatment and 85% at 24h. Similarly, there was no increase in the levels of the 4 kb and 2 kb RNA species with the PMA stimulated H3B cells. This indicates, firstly, that transcription from proviral DNA in H3B cells is not amenable to upregulation with PMA as it is in ACH-2 or U-1 cells which were used by Pomerantz *et al.* and Michael *et al.*, and secondly that the induction of the genomic length 9 kb HIV RNA early (4 - 12 h p.i.) after co-culture of H3B and Hut78 cells reported is not directly comparable to phorbol ester stimulation of viral RNA expression

It may be suggested that normal cell manipulation may upregulate the expression of 9 kb viral RNA in the H3B cells. To examine this 0.4 x 10^6 H3B cells alone were washed, incubated and cytoplasmic RNA extracted from these cells at regular periods post incubation. There was no induction of 9 kb HIV RNA and the levels of 4 kb and 2 kb RNA remained essentially the same throughout a 24 h incubation period. During this period the number of H3B cells increased two

Fig. 3.5i

Northern analysis of H3B cells (2×10^6) grown in the presence of 20 nM PMA. Cytoplasmic RNA were extracted at different times (h) post stimulation, as indicated by the figures above each lane. ori: origin of gel. Each lane contains 15 μg of total nucleic acids as measured by $OD_{260/280}$.


Not stimulated
PMA stimulated
0 4 8 12 24 0 4 8 12 24
ori
4 kb
2 kb

to three-fold (Fig. 3.5ii). These results suggest that the two phases of HIV RNA induction were unlikely due to manipulation and culture of H3B cells.

3.6 HIV RNA transcription following infection with cell-free virus

As the early phase of HIV RNA production in the cell-to-cell transmission model described above shows a different pattern from that reported in an earlier cell-free virus infection in which unspliced HIV RNA appeared at 20 h p.i. and later (Kim *et al.*, 1989b), we wished to examine the kinetics of HIV RNA production in a similar cell-free viral infection of Hut78 cells. Cell-free HIV was prepared by harvesting the supernatant fluid from a culture of H3B cells at one hourly periods (see section 2.3.A, B & C). After clarification to remove cellular materials, the infectivity titre of the virus was determined ($TCID_{50}$) and then used to infect Hut78 cells by centrifugation enhancement. At regular periods post infection, the cells were harvested and cytoplasmic RNA was extracted as described in section 2.7.A

Fig. 3.6i shows the production of HIV RNA from 0 to 72 h p.i., after infection of Hut78 cells with a cell-free virus inoculum at a nominal multiplicity of 0.5 $TCID_{50}$ virus/cell. The use of centrifugally-enhanced inoculation would have resulted in an effective multiplicity of 5 $TCID_{50}$ virus/cell (Pietroboni *et al.*, 1989). The 9 kb RNA species appeared from 8 h p.i. onwards, whereas significant 4 kb and 2 kb RNA species were observed from 24h p.i. onwards. The 9 kb RNA signals observed were not from input virions, as 2 - 4 h p.i. samples did not produce visible bands corresponding to genomic RNA. These results again are different from the findings of Kim et al. (Kim *et al.*, 1989b). At 24h p.i. a large increase in all three RNA species was seen and by 72 h p.i. the level of 9 kb RNA was lower than that at 8 h p.i. whereas the spliced RNA species remained at high levels. When 20 μM-AZT was added to a duplicate experiment 18h prior to infection and maintained throughout, the HIV RNA signals in Northern blot hybridization were minimal or not detected at any time (data not shown). The cellular RNA levels (28S and 18S ribosomal RNA) were not affected by this treatment throughout the incubation period as judged by ethidium bromide staining (Fig. 3.6ii).

Although the early induction of the 9 kb HIV RNA was slightly later in the cell-free viral infection route (8h p.i.) (Fig. 3.6i) than with the cell-to-cell transmission infection model

Fig. 3.5ii

Effect of manupulation of H3B cells on HIV RNA synthesis. Cells were washed, incubated and sampled at the times shown. Each lane contains the average amount of RNA extracted from duplicate cell cultures of 0 4 x 10^6 H3B cells. The figures at the top of each lane refer to the period (h post incubation) at which the RNA was extracted.

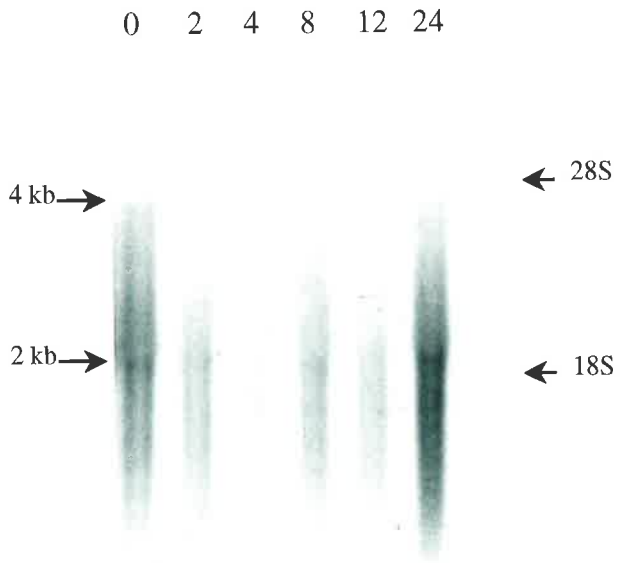

0 2 4 8 12 24
4 kb
2 kb
28S
18S

Fig. 3.6i

Kinetics of HIV RNA production between 0 and 72 h after infeciton of Hut78 cells with cell-free HIV at a nominal multiplicity of 0 5 $TCID_{50}$. The figures at the top of each lane refer to the period (h) at which the RNA was extracted. Each lane contains an amount of RNA equivalent to that from 2 x 10^6 infected Hut78 cells.

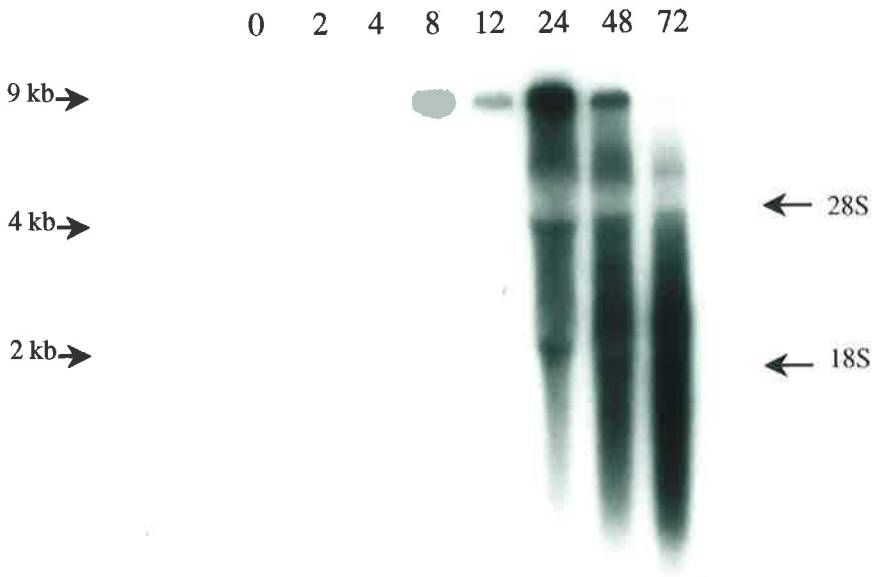
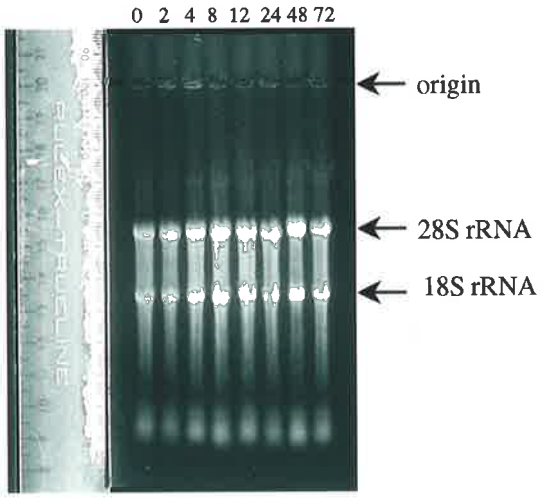

0
2
4
8
12
24
48
72
9 kb
4 kb
2 kb
28S
18S

Fig. 3.6ii

Ethidium bromide-stained Northern gel of cytoplasmic RNA extracted from AZT-treated Hut78 cells infected with cell-free HIV in the presence of the drug (20 μM). The figures at the top of each lane refer to the period (h post incubation) at which the RNA was extracted. ori: origin of gel. Northern blot hybridization analysis did not yield detectable signals with extended period of autoradiograph exposure (data not shown).


0 2 4 8 12 24 48 72
origin
28S rRNA
18S rRNA

described above (4 h p.i.) (Fig. 3.2i), the levels of supernatant reverse transcriptase (Fig. 3.6iii) and HIV p24 antigen (Fig. 3.6iv) as a measure of virion production were similar with both infection routes. The levels of RT and p24 antigen remained relatively uniform during the first 24h p.i. and were then followed by a dramatic and significant increase from 24h to 72h p.i. The latter increase in RT and p24 antigen levels reflect the second round of viral replication.

3.7 Comparison of viral RNA in nuclear and cytoplasmic fractions

The HIV RNA examined above was extracted from cytoplasmic fractions. We then wished to compare these results with findings for nuclear fractions, as altered distribution of viral RNA between the two cellular compartments may influence interpretation of the experimental results. In a similar cell-free HIV infection of Hut78 cells the kinetics of HIV RNA expression in the cytoplasmic and nuclear fractions were analysed. After cell lysis to obtain the cytoplasmic fraction (see section 2.7.A), the nuclei were washed twice with TSE (2500g/1 min) and then resuspended in 200 µl of TSE. The nuclear RNA was then extracted with urea/SDS and phenol-chloroform. Figs. 3.7i & ii show the Northern blot hybridization analyses of HIV RNA production during the period 0 - 72 h p.i. in cytoplasmic and nuclear fractions respectively. In the cytoplasmic fraction the 9 kb genomic length HIV RNA was first observed at 8 h p.i. At 24 h and later, the second phase of RNA production was marked by a significant increase in all three classes of viral RNA. However, in the nuclear fractions, levels of 9 kb RNA at 8h and 12h were much lower than in cytoplasmic fractions and all viral RNA species were significantly lower than the corresponding cytoplasmic fractions, during the period 24 - 72 h p.i. RNase treatment of either cytoplasmic or nuclear fractions abolished Northern blot hybridization signals (data not shown).

3.8 Discussion

In the cell-to-cell infection model, the level of cytoplasmic, 9 kb genomic length HIV RNA increased significantly 4h after coculturing Hut78 and H3B cells. This period corresponded to the first appearance of episomal HIV DNA in the same model (see Fig. 3.1.Ai) (Li & Burrell, 1992). Similar kinetics were observed with other cell-to-cell transmission infection models (Sato *et al.*, 1992, Barbosa *et al.*, 1994). In contrast, the levels of the 4 kb and 2 kb viral RNA transcripts did not show more than twofold increase during the period from 0 to 12 h p.i.

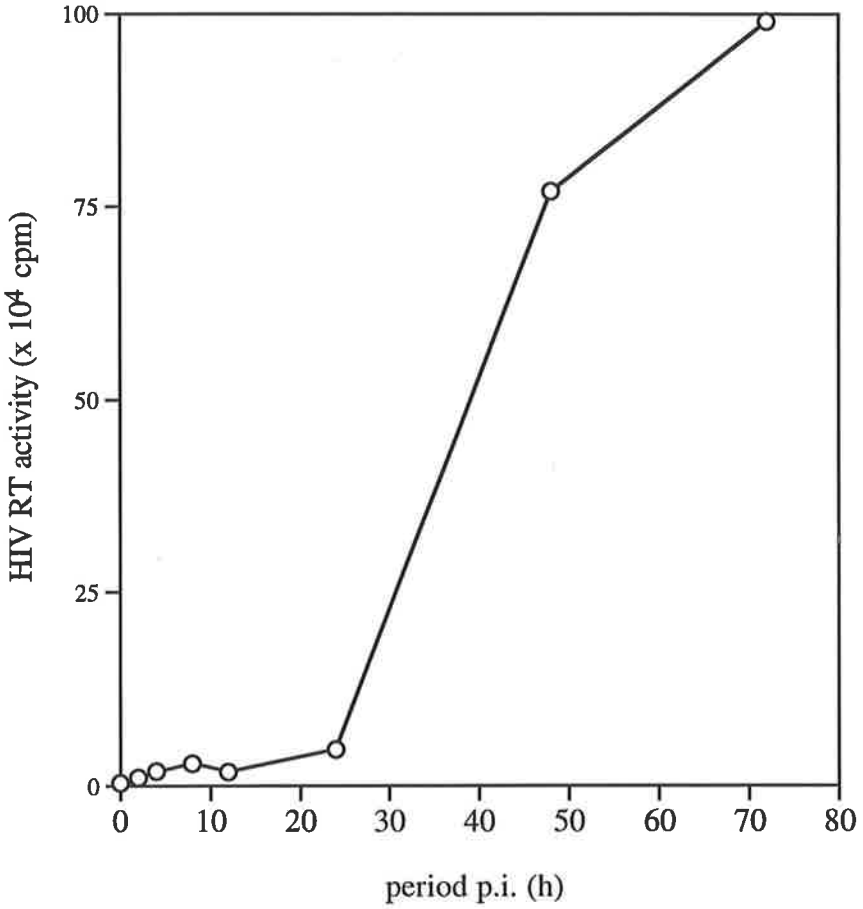


Fig. 3.6iii

Relation between HIV reverse transcriptase activity (cpm/ml) and period (h) p.i. from cell-free HIV infection of Hut78 cells.

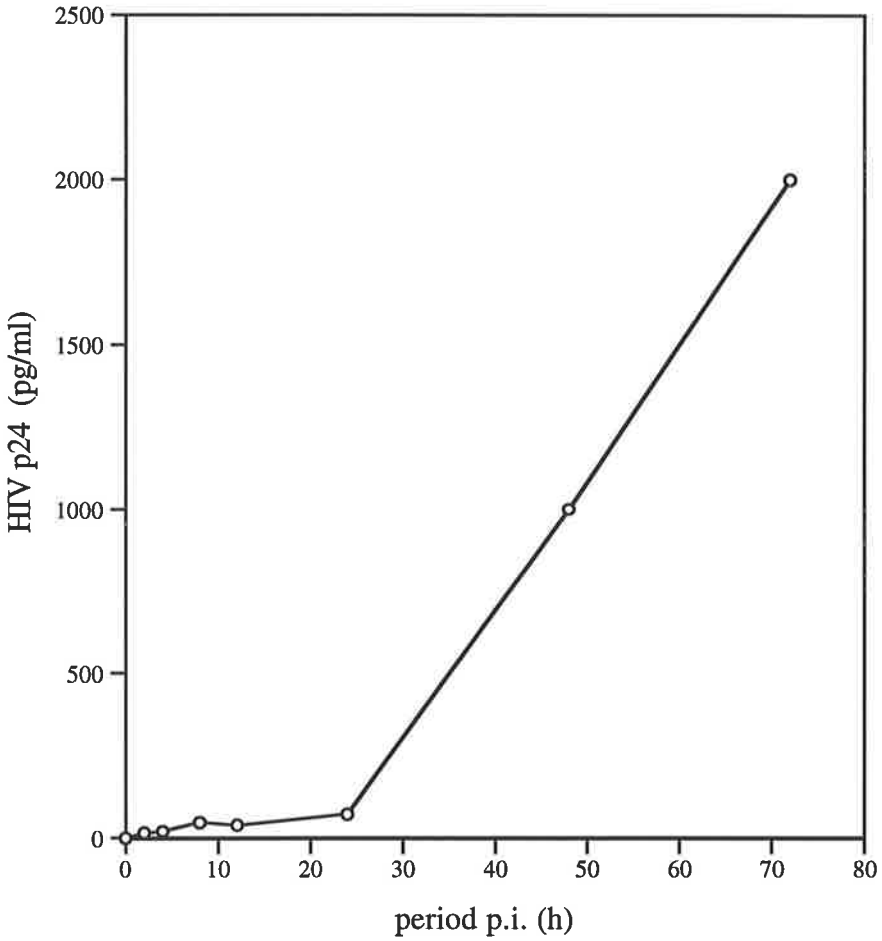


Fig. 3.6iv

Relation between HIV p24 antigen levels (pg/ml) and period (h) p.i. in cell-free viral infection of Hut78 cells.

Fig. 3.7i

Northern blot hybridization analysis of HIV RNA obtained from cytoplasm of Hut78 cells infected with cell-free HIV at a nominal multiplicity of 0 5 $TCID_{50}$ during the period 0 to 72 h p.i. The figures at the top of each lane refer to the period (h p.i.) at which the RNA was extracted. Each lane contains an amount of RNA equivalent to that from 2 x $l0^6$ infected Hut78 cells. ori: origin of gel.

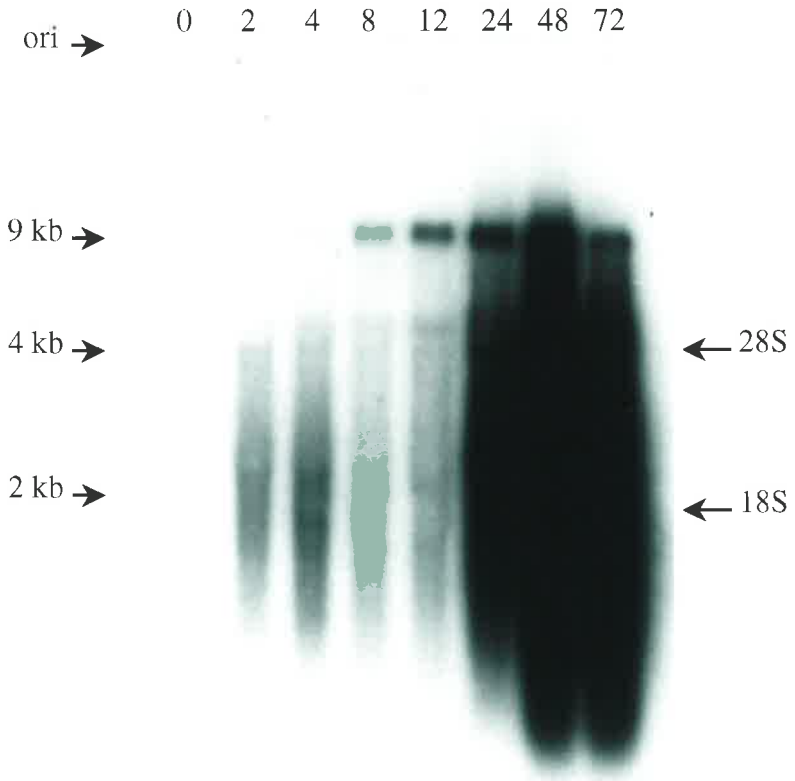
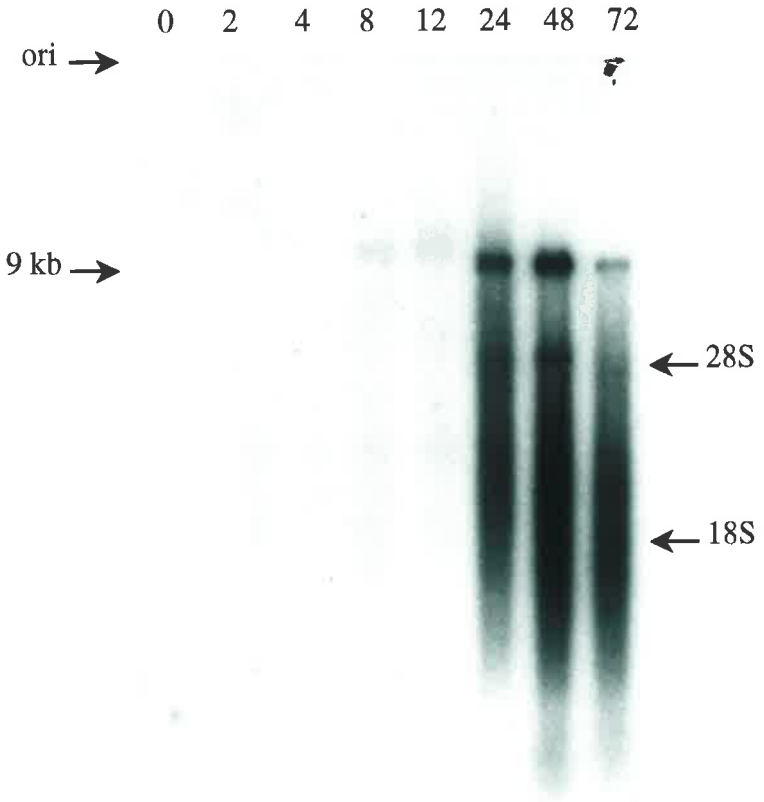

0
2
4
8
12
24
48
72
ori
9 kb
4 kb
2 kb
28S
18S

Fig. 3.7ii

Northern blot hybridization analysis of HIV RNA obtained from nuclei of Hut78 cells infected with cell-free HIV at a nominal multiplicity of 0.5 $TCID_{50}$ during the period 0 to 72 h p.i. The figures at the top of each lane refer to the period (h p.i.) at which the RNA was extracted. Each lane contains an amount of RNA equivalent to that from 2 x 10^6 infected Hut78 cells. ori: origin of gel.


0
2
4
8
12
24
48
72
ori
9 kb
28S
18S

Cytoplasmic RNA levels may be affected by changes in RNA stability (Jacobson & Peltz, 1996), nucleocytoplasmic transport or changes in transcription kinetics. Our results showed that significantly higher levels of HIV RNA were observed in the cytoplasm than in the nucleus. Moreover, cytoplasmic levels of singly and multiply spliced transcripts were also maintained at constant or slightly increased levels, at times when a marked increase in the 9 kb species was observed. Therefore, we believe that the above changes are likely to represent a true increase in HIV RNA transcription, and not merely a change in splicing, RNA transport or other forms of processing. This early induction of the 9 kb transcript was also seen following infection with cell-free virus, although it was somewhat delayed (8 h p.i. instead of 4 h p.i.). This also corresponded to the slightly slower appearance of episomal HIV DNA in a cell-free virus infection, compared to that in the cell-to-cell infection (Li & Burrell, 1992).

The early phase 9kb RNA observed in this study is unlikely to be due to contaminating viral DNA as treatment of the RNA samples with RNase yielded no detectable RNA signals in Northern blot hybridizations, and the viral RNA samples were treated with an optimal concentration of DNase as judged by the absence of viral DNA signals in Southern blot hybridizations. When an equivalent amount of viral DNA, obtained by Hirt fractionation, was treated with the same concentration of DNase used for RNA samples and electrophoresced in a similar Northern gel, no detectable signals were observed.

Both of the above patterns of viral RNA synthesis differ markedly from that seen in the cell-free virus infection model reported by Kim and colleagues (Kim *et al.*, 1989b). These authors reported that the first major RNA species to be induced consisted of the heterogeneous group of 2 kb regulatory transcripts at 16 h p.i. and that significant 9 kb genomic-length RNA did not appear until 24 h p.i. A similar differential RNA transcription pattern in a cell-free virus infection model has previously been reported with visna virus (Vigne *et al.*, 1987). Equine infectious anaemia virus, another member of the lentivirus family, showed a different pattern of viral RNA synthesis, with full-length RNA appearing early after infection (Rasty *et al.*, 1990). However, the kinetics of transcription of visna virus and equine infectious anaemia virus DNA were examined using more infrequent time points. It is not clear at this stage why, in the present study using free virions, the 9 kb viral RNA was detected much earlier than in the

previous report (Kim *et al.*, 1989b). However, Kim et al. suggested that only 10 to 20% of cells in their system were undergoing primary synchronous infection. The virus stocks used in their cell-free inoculum was obtained from transfection of COS cells and subsequent infection of H9 cells followed by viral harvests one to two weeks p.i. The cell-free viral inoculum used in our experiments was obtained from cloned stable viral donor cells (H3B) (also see section 5.3). The observation of Kim *et al.* that genomic-length RNA did not increase until 24 and 36 h p.i. may represent secondary infection within the culture by a cell-to-cell transmission mechanism. Centrifugation enhancement in cell-free viral infection has been reported to increase the resultant multiplicity of infection by ten fold higher than the nominal m.o.i (Pietroboni *et al.*, 1989). The $TCID_{50}$ of our cell-free virus stock was determined without centrifugation enhancement and the inoculum was hence used at ten fold higher titre than the nominal m.o.i. of 0.5. This would theoretically be capable of infecting nearly 100% of cells in the cultures, and may then approach the higher efficiency of cell-to-cell transmission infection (Dimitrov *et al.*, 1993). This suggests that the early phase of HIV 9 kb RNA synthesis (4 - 12 h p.i.) may be seen when high multiplicities of infection are used, either by cell-free virus or cell-to-cell transmission of infection routes. The lack of detection by Kim *et al.* may be either because of sensitivity problems or the events may not occur with a lower m.o.i. per cell.

Li and colleagues have shown previously that 20 μM-AZT completely abolishes *de novo* episomal HIV DNA synthesis for at least 48 h in the cell-to-cell transmission model (Li & Burrell, 1992) (see Fig. 3.4ii). The present study demonstrated that AZT has minimal effect on steady-state 4 kb and 2 kb HIV RNA levels in H3B cells alone, but completely abolishes the first phase of RNA synthesis 4 to 12 h following cell mixing. Theoretically the template for the first phase of HIV RNA induction might be integrated proviral DNA in donor cells, newly made episomal DNA or newly made and integrated proviral DNA (Panganiban & Temin, 1983, Stevenson *et al.*, 1990). Our findings suggest that the transcriptional template for the first phase of HIV RNA expression is likely to be newly synthesized free and/or integrated viral DNA.

The second phase (24 to 72 h p.i.) of RNA induction is more extensive and involves all three RNA species. It is reduced to some extent if *de novo* HIV DNA synthesis is inhibited with

AZT. One conceivable interpretation is that cell fusion might provide stimulatory signals for transcription from the integrated viral sequence of the H3B cells, which is not dependent on *de novo* HIV DNA synthesis. The level of second phase viral RNA synthesis observed in the AZT-treated cells represents only a portion of the viral RNA synthesis observed in the AZT-free culture; by this stage integration of *de novo* synthesized HIV DNA is well established in AZT-free cultures (see section 5.4), and presumably this contributes as transcriptional template when present. In other reports it has been shown that stimulation of the chronically infected ACH-2 and Ul cell lines with phorbol esters leads to an early (2 to 6 h) minor increase in 2 kb RNA followed by a later significant increase in levels of unspliced RNA (Pomerantz *et al.*, 1990, Michael *et al.*, 1991). It was suggested by both groups that the increase in unspliced RNA might be a consequence of increased Rev activity, but these reports differed as to whether there may have been a significant increase in *de novo* RNA synthesis. It is difficult at this stage to make a direct comparison between these experimental systems and the present study; however, since early RNA synthesis in cell-to-cell transmission experiments required *de novo* DNA synthesis, it was clear that this represented a different mechanism from that in PMA-treated cells.

The results differ from those obtained by Davis *et al.* (Davis *et al.*, 1997) who examined the time course of appearance of different mRNA species in the same model system as the current study but used RT-PCR rather than Northern blot hybridization. Davis *et al.* found no increase in any viral RNA species (including gag) above the level at 0 h until 16 h p.i. This discrepancy cannot be easily explained but may be related to the different DNase treatment of RNA. Under less extensive DNase treatment, intermediates in reverse transcription may have had some configurational protection against digestion by DNase or contaminating RNase in the enzyme preparation. In our experience, as well as others ((Goodall *et al.*, 1990) Dr. G. Goodall, *pers. comm.*) different brands and batches of DNase may contain minimal levels of RNase; hence the requirement for titration of optimal enzymic conditions. It may be expected that during titration for optimal conditions of DNase the use of RT-PCR would require a more stringent treatment of the RNA samples than with Southern hybridization. On the other hand, any degraded RNA containing target *gag* sequences is detectable by RT-PCR but not by Northern hybridization which detects the full length 9 kb RNA as a defined species. Notwithstanding the above it was

clear that the 9 kb RNA species described in this chapter was not due to incompletely digested full length DNA, because the signal was readily abolished by RNAse pre-treatment, which an equivalent amount of viral DNA, treated by the DNase protocol in the current study yielded no signals on Northern blot hybridization (see section 3.3). The other difference between this study and that of Davis *et al.* is the different batches of cells used. In our experience the low and variable levels of viral 9 kb RNA were sometimes detectable in different batches of H3B cells. The reason for this variation is not known, but it has been noted to coincide with different batches of media as well as with the particular batch of cells used. (During the extended passage a particular cell line carrying HIV proviral DNA may acquire new proviruses presumably by superinfection (Ott *et al.*, 1995); which may lead to altered levels of viral RNA expression. Although this occurred infrequently, a particular batch of cells was discarded and new stocks of this cell line were recovered from liquid nitrogen storage if detectable 9 kb HIV RNA signals were seen in Northern blot hybridization analysis at the time of cell mix in the cell-to-cell transmission infection experiments. New stocks of the viral donor H3B and Hut78 recipient cells were also recovered from liquid nitrogen storage within six to nine months of routine cell passage.)

Li et al.(1992) have previously demonstrated unequivocally that *de novo* reverse transcription is an essential requirement for HIV replication following cell-to-cell transmission when analysed within the time frame of a single-step synchronized virus growth cycle. However, prolonged incubation with AZT in different cell-free HIV infection and cell-to-cell HIV infection models may be followed by 'escape' from inhibition (a phenomenon different from classical drug resistance) and production of progeny virus (Smith *et al.*, 1987, Gupta *et al.*, 1989, Li & Burrell, 1992). Escape from AZT following cell-free viral infection may be due to incomplete arrest of reverse transcription in the presence of fluctuating AZT triphosphate levels. In the case of cell-to-cell transmission, the second phase of HIV RNA synthesis, defined in the present study, could provide the genomic viral RNA required for progressive virion production in the presence of AZT. In addition, prolonged incubation in a cell-free viral infection is likely to lead to secondary cell-to-cell transmission. Finally, this enhanced genomic-length RNA synthesis observed in our cell-cell infection model may also be reflected in late stage disease when syncytium inducing viral isolates dominate (Schuitemaker *et al.*, 1992), the ratio of genomic

viral RNA and viral DNA in infected cells increases by over 1000-fold when compared to the early stage of disease (Michael *et al.*, 1992). This process may in part explain the long-term failure of AZT or other reverse transcription inhibitors in the treatment of AIDS. The recent studies of HIV patients on triple therapy regimen of AZT, lamivudine (3TC) and indinavir (IDV), or combinations of other anti-retroviral drugs, led by Richman (Wong *et al.*, 1997b) and Siliciano (Finzi *et al.*, 1997) showed that despite the levels of viral RNA falling below the threshold of detection in blood (<50 copies/ml) for 52 to 92 weeks, the virus was recoverable from PBMCs and quiescent CD4+ T cells.

Chapter 4

Discovery is not a result of logical thought, even though the end result is intimately bound to the rules of logic. Albert Einstein (1879-1955), Nobel Laureate for Physics 1922.

Further characterization of HIV DNA and RNA synthesis early after cell-to-cell transmission of infection

4.1 Introduction

All current models of reverse transcription predict that either one or two molecules of input viral RNA are required to produce one molecule of progeny DNA, i.e. there is no capacity for amplification of genome copy number by this process (Coffin, 1996). Despite this, early after retrovirus infection, infected cells frequently contain large numbers of episomal and newly integrated viral DNA, without similar levels of viral RNA being reported very early in infection (Harris *et al.*, 1981, Haase *et al.*, 1982, Li & Burrell, 1992, Sato *et al.*, 1992, Barbosa *et al.*, 1994). Although unintegrated viral DNA accumulates in the cytoplasm, only a small proportion of these molecules are integrated to form proviruses (Fan *et al.*, 1978, Keshet & Temin, 1979, Rice *et al.*, 1989, Robinson & Zinkus, 1990). We wished to further investigate the relation between the newly made genomic length viral RNA and unintegrated DNA during the early phase of viral replication after cell-to-cell transmission of HIV infection. The early (4 - 12 h p.i.) synthesis of the genomic length viral RNA described in chapter 3 is a process distinct from the extensively studied activation of transcription from proviral DNA. When H3B viral donor and Hut78 recipient cells are cocultured in this cell-to-cell transmission infection, there are potentially three transcription templates for HIV RNA synthesis viz. newly made viral DNA, newly made and newly integrated provirus and proviral DNA from H3B cells. Results presented in chapter 3 showed that *de novo* synthesis of HIV DNA was necessary for early RNA synthesis. However, involvement of RNA transcription from proviral DNA or other cellular genes in this process could not be excluded. Therefore actinomycin D was used to inhibit transcription of the integrated proviral DNA in the H3B donors prior to coculture with Hut78 cells. The use of actinomycin D was considered as this drug inhibits transcription irreversibly by intercalating double-stranded DNA (Sobell *et al.*, 1971). Earlier reports have shown that actinomycin D inhibited growth (Temin, 1963) and DNA-dependent transcriptional activities (McDonnell *et al.*, 1970) of Rous sarcoma virus.

4.1.A Parameters of transcriptional inhibition by actinomycin D

Actinomycin D (AmD) was used to inhibit total transcription in H3B viral donor cells, including that from proviral DNA. The optimal concentration of AmD to inhibit total cellular RNA synthesis was determined by measuring the incorporation of tritiated uridine into acid-insoluble material. Donor H3B cells were pretreated with different concentrations of the drug for 2h and then pulsed with 2 μCi/ml of [5,6-^{3}H] uridine (Amersham) for a further 2h in the continued presence of the drug (Fig. 4.1Ai). At a concentration of 4μg/ml the incorporation of ^{3}H-uridine was reduced by >90%, compared to the untreated cells, and at 50 μg/ml the reduction was >99%. The highest concentration (50 μg/ml) of AmD was chosen to treat the H3B donor cells in subsequent experiments (see below). At this concentration viability of the pretreated and washed H3B cells, as judged by trypan blue exclusion, remained at 100% during the first 8h post treatment. At 12h post treatment it was 90% and by 24h, 65%. Inhibition of RNA synthesis in H3B cells treated with 50 μg/ml AmD for 2h was shown to be irreversible, as the incorporation of ^{3}H-uridine cells pretreated with 50 μg/ml of AmD for 2h/37°C followed by washing three times with culture medium and further incubation in drug-free medium remained less than 1% compared to the untreated cells for the next 0.3 - 24h (Table 4.1). This irreversible inhibition of transcription by AmD in pre-treated H3B cells was exploited to inhibit only transcription from H3B provirus, but not transcription from any viral DNA newly made in cocultures involving pretreated H3B as viral donors (see below). When *in vitro* reverse transcriptase activities, as measured by ^{3}H-thymidine triphosphate incorporation on a poly r(A)-oligo (dT) template-primer (see 2.10), were assayed using standard virus stocks (H3B culture supernatant fluids) in the presence of AmD (5 μg/ml or 50 μg/ml) in the RT reaction mix, there was no significant difference from assays without the drug (Fig. 4 .1Aii). This suggests that AmD does not inhibit reverse transcriptase activity from an artificial template *in vitro*.

4.2 Effects of Actinomycin D on HIV reverse transcription in acutely infected cells

AmD inhibits RNA transcription by intercalating adjacent GpC sites and binding to the deoxyguanosine residue on double-stranded DNA (Sobell *et al.*, 1971) In addition, binding of AmD to non-GpC sites on duplex DNA has also been reported (Takusagawa & Berman, 1983, Snyder *et al.*, 1989). We first wished to determine whether AmD pretreatment of donor cells at

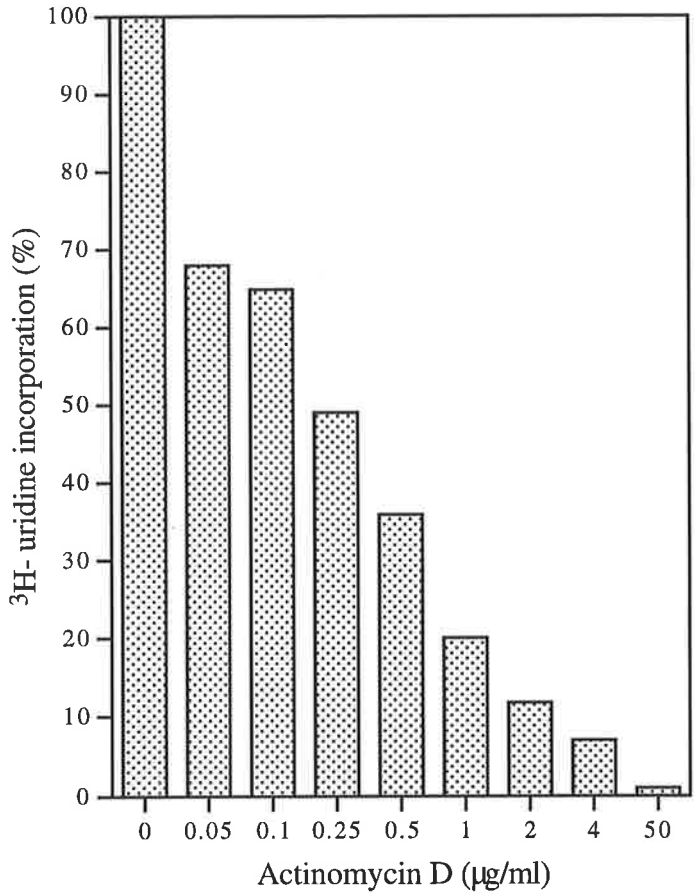


Fig. 4.1Ai. Effect of actinomycin D treatment on ^{3}H-uridine incorporation by H3B cells.

Aliquots of H3B cells (2 x 10^6) were incubated for 2h with AmD at the concentrations shown and then washed (X3) prior to pulse labelling with [5,6-^{3}H]-uridine at 2 µCi/ml for 2h in the continued presence of the drug. The cells were washed once with Tris-saline, lysed with 10% trichloroacetic acid (TCA) and filtered onto glass fibre filter discs (Whatman). The filter discs were washed 3 times with 10% TCA followed by 3 washes with 5% TCA and rinsed once with 70% ethanol. The acid-insoluble counts of AmD-treated cells were expressed as the proportion (%) of total acid-insoluble counts from untreated cells which were incubated similarly.

Control (not treated)	Maintained with AmD	0.3h-post wash	2 h-post wash	4 h -post wash	24 h - post wash
58907 cpm	459 cpm	512 cpm	440 cpm	435 cpm	440 cpm
100%	0.8%	0.9%	0.8%	0.8%	0.8%

Table 4.1. Effect of actinomycin D treatment on ^{3}H-uridine incorporation by H3B cells after removal of drug

Aliquots of H3B cells (2 x 10^{6}) were treated with 50 µg/ml of AmD for 2h, washed three times with drug-free culture medium and incubated in drug-free medium for 0.3h, 2h, 4h or 24h before pulse labelling with [5,6-^{3}H] uridine at 2 µCi/ml for 2h. The control cells were not treated with AmD. After pulse labelling, the cells were lysed with 10% TCA and filtered onto glass fibre filter discs. The filter discs were washed 3 times with 10% TCA followed by 3 washes with 5% TCA and rinsed once with 70% ethanol. The acid-insoluble counts (average from four replicate cultures of H3B cells) were shown.

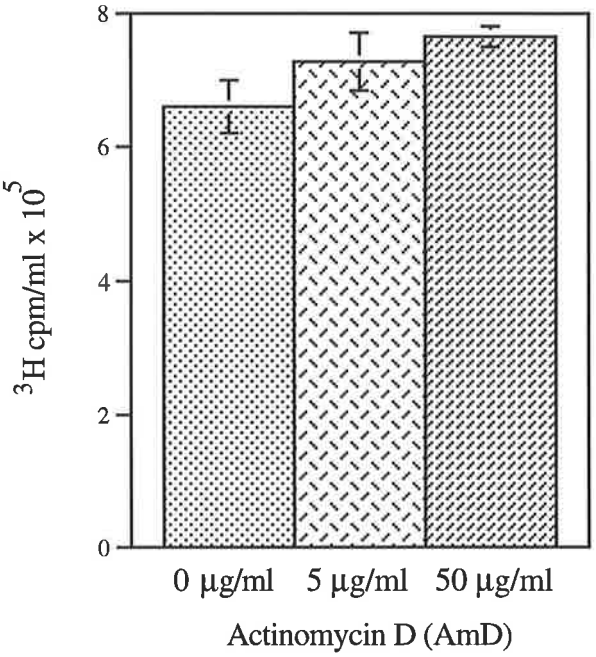


Fig. 4.1ii.

Reverse transcriptase activity of standard HIV-containing supernatant using exogenous template (expressed as ^{3}H cpm/ml of virus supernate), in the absence or presence of AmD (5 mg/ml or 50 mg/ml). The bars at the top of the columns denote the standard error of the mean of four assays for each concentration.

the drug concentration that inhibited total cellular RNA transcription would have any effect on HIV reverse transcription following cell-to-cell transmission of infection. Figure 4.2i (Control) shows the kinetics of viral DNA synthesis in a one step cell-to-cell infection of Hut78 cells with untreated H3B cells. There was an early and progressive increase in linear viral DNA (commencing 4h p.i.) followed by the accumulation of circular viral DNA. When H3B cells that had been pretreated with 50 μg/ml AmD for 2h, washed (x3) to remove unbound drug and then mixed with Hut78 cells in a similar experiment and incubated in the absence of AmD (Fig. 4.2, Pre-treated), the levels of linear viral DNA synthesized during the period 4h to 12h p.i. were similar to levels seen using untreated H3B cells (Fig. 4.2i, Control), although the onset of synthesis was slightly delayed. However viral DNA accumulation was abolished when similarly pre-treated H3B cells were incubated with Hut78 recipient cells in the continued presence of AmD (50 μg/ml) (Fig. 4.2i, Pre-treated & Maintained). These results suggest that when transcription was inhibited in H3B cells (presumably from both proviral DNA and cellular genes as templates), subsequent viral DNA synthesis in a standard coculture took place with similar kinetics to untreated cells. However, in the continued presence of the drug in the coculture mix, *de novo* HIV reverse transcription was not seen. A recent report showed that AmD inhibits minus-strand DNA transfer by preventing the annealing of (-) ssDNA to the viral RNA during reverse transcription of HIV RNA (Guo *et al.*, 1998).

4.3 Effects of Actinomycin D on HIV RNA expression in cell-to-cell transmission infection

The experiments described in section 4.2 showed that AmD pretreatment of H3B cells did not prevent subsequent reverse transcription after mixing with untreated Hut78 recipient cells. Next we wished to study the early phase of HIV RNA expression under the same conditions. In a similar cell-cell infection experiment using untreated H3B cells mixed with untreated Hut78 cells (Fig 4.3i, Control), Northern blot hybridization demonstrated two distinct phases of RNA induction as reported previously (Chapter 3) (Kok *et al.*, 1993). The first phase of viral RNA transcription at 4h - 12h p.i. comprised a significant increase in the genomic-length 9.2 kb RNA compared to that seen at 0 h. The levels of 4.3 kb and 2 kb spliced RNAs remained near constant during this first phase, except for a slightly increased level of 2 kb RNA species at 4h

Fig. 4.2i.

Kinetics of synthesis of unintegrated HIV DNA during cell-to-cell infection using actinomycin D-treated H3B cells. Hut78 recipient cells (2 x 10^6) were co-cultured with virus donor cells using untreated H3B cells (0.5 x 10^6) **(Control);** H3B cells (0.5 x 10^6) which had been pre-treated with 50 μg/ml of AmD for 2h and washed three times **(Pre-treated);** or same as with 'Pre' but with the continued presence of 50 μg/ml of AmD in the culture mix **(Pre-treated & Maintained).** At different times (h) after infection (figures at the top of each lane) extrachromosomal DNA was extracted and viral DNA analysed as described in sectin 2.4. Lanes denoted by 'pg' represent 12 pg, 25 pg, 50 pg and 100 pg of *SstI* fragment of plasmid pBH10 respectively. ori - origin of agarose gel. Each lane contains an average amount of DNA equivalent to that from a total of 1 x 10^6 cells. The Southern blots were hybridized with the same probe mix.

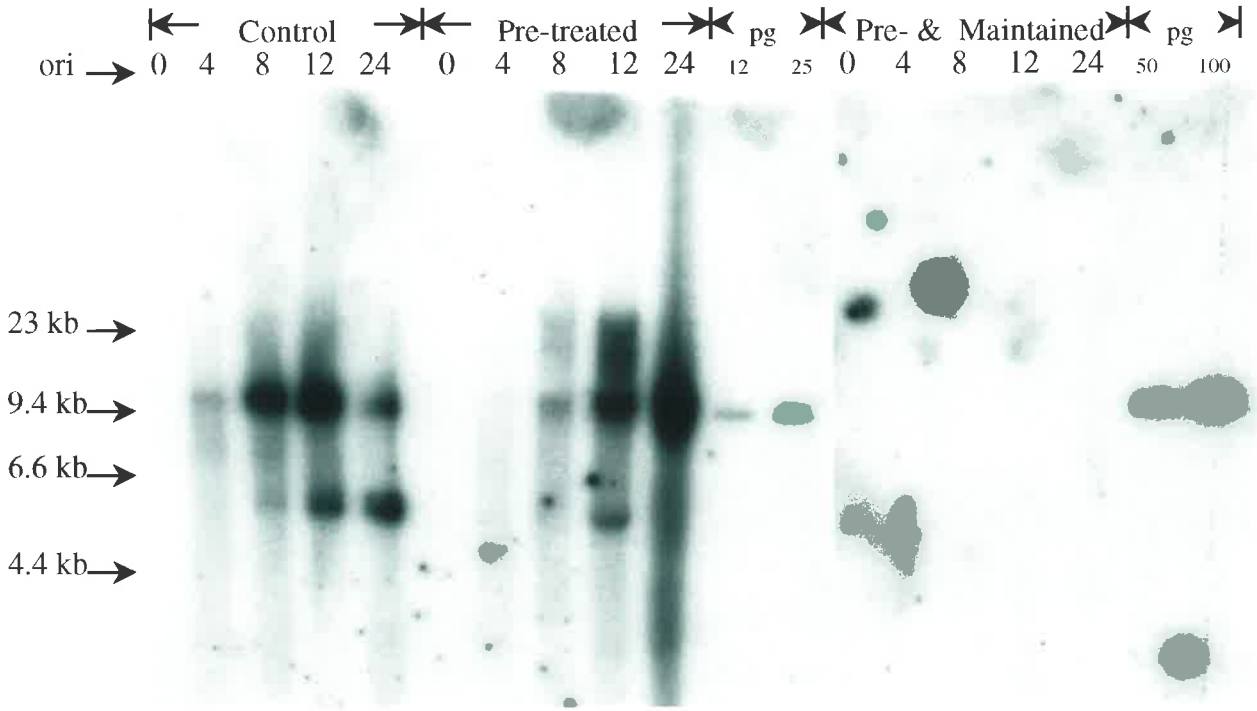
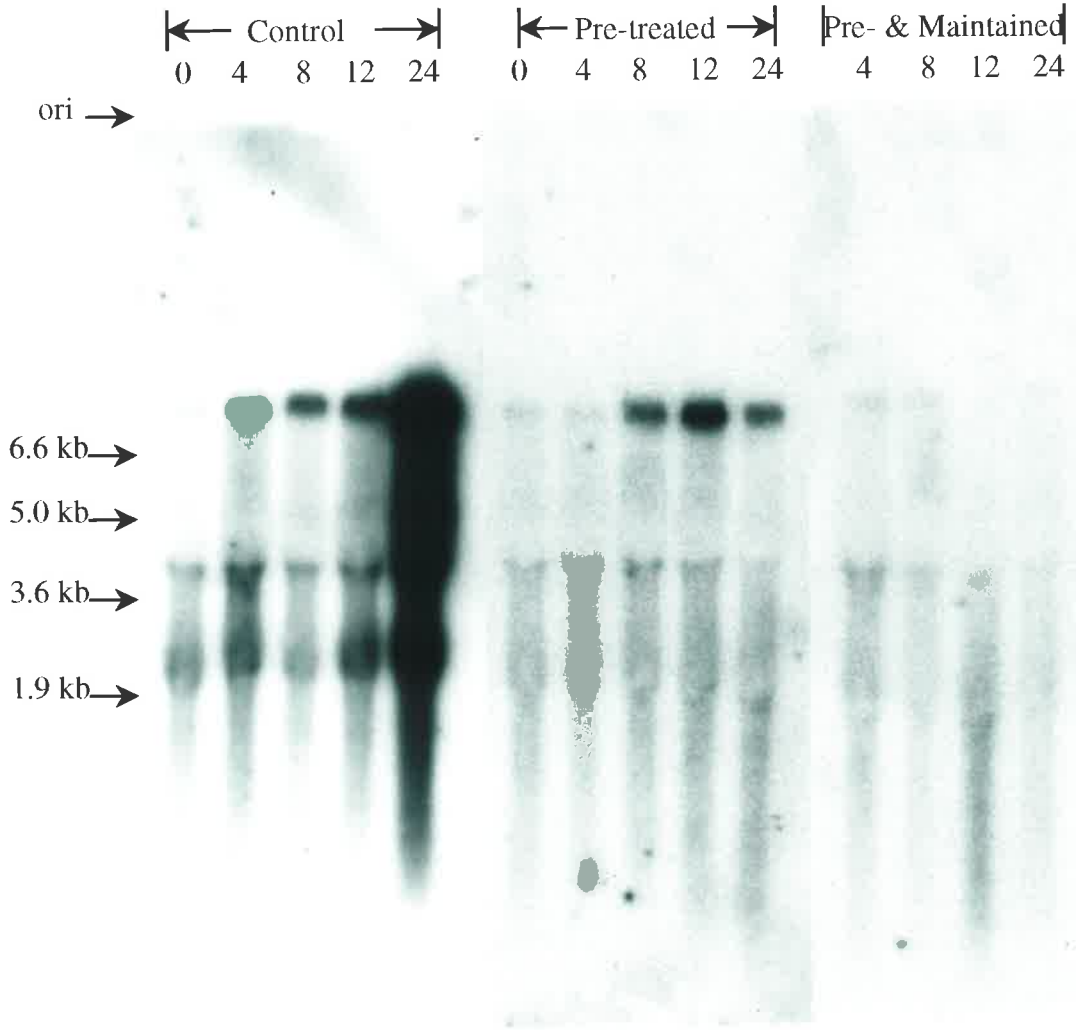

Control
Pre-treated
pg
Pre- & Maintained
pg
ori
0 4 8 12 24 0 4 8 12 24 12 25 0 4 8 12 24 50 100
23 kb
9.4 kb
6.6 kb
4.4 kb

Fig. 4.3i.

Kinetics of HIV RNA synthesis during cell-to-cell infection using actinomycin D-treated H3B cells. Hut78 recipient cells (2 x 10^6) were co-cultured with virus donor cells using untreated H3B cells (0.5 x 10^6) **(Control),** or H3B cells (0.5 x 10^6) which had been pre-treated with 50 μg/ml of AmD for 2h and washed three times **(Pre-treated),** or identically to 'Pre-treated' but with the continued presence of 50 μg/ml of AmD in the culture mix **(Pre-treated & Maintained)**. At different times (h) after infection (figures at the top of each lane) cytoplasmic RNA was extracted and viral RNA analysed as described in section 2.7.A. Each lane contained an average amount of RNA equivalent to that from 2 x 10^6 cells ori - origin of agarose gel. All these Northern blots were hybridized with the same probe mix.


Control
Pre-treated
Pre- & Maintained
0 4 8 12 24
0 4 8 12 24
4 8 12 24
ori
6.6 kb
5.0 kb
3.6 kb
1.9 kb

and again at 12 h p.i. in some experiments. The second phase of viral RNA synthesis at 24 h p.i. consisted of a dramatic increase in the levels of all three viral RNA species.

When H3B cells were pretreated with 50 μg/ml of AmD, washed and then mixed with untreated Hut78 cells, the increase in genomic-length 9.2 kb RNA during the period 0h - 12h p.i. was not significantly affected (Fig. 4.3i, Pre-treated). As the AmD irreversibly inhibited >99% of RNA transcription from pre-existing H3B cellular DNA including proviral HIV DNA (Table 4.1), the induced 9.2 kb HIV RNA must have been synthesized from the newly made HIV DNA. Pre-existing levels of spliced 4.3 kb and 2 kb RNA species at 0h were similar in the untreated or treated H3B cells; however, during the period 4h - 24h p.i. the levels of these spliced RNAs appeared lower in the AmD-pretreated H3B cultures (Fig. 4.3i, Pre) than in the untreated cultures (Fig 4.3i, Control). These spliced RNA species present after cell mixing may represent both pre-existing spliced RNA before cell mixing and processing of RNA synthesized after cell mixing. The fact that levels of spliced RNA did not increase during the first phase of RNA synthesis when using AmD-pretreated donor cells, while levels of 9 kb RNA increased similarly with treated or control donor cells, suggested that H3B proviral DNA may serve as a major template for synthesis of the primary transcripts from which spliced RNAs were made at this phase of infection. The second phase of RNA synthesis (24h p.i.) was markedly reduced in the AmD-pretreated cultures as indicated by lower levels of all RNA species (Fig. 4.3i, Pre-treated), compared to the untreated cells (Fig. 4.3i, Control). When similarly pre-treated H3B cells were mixed with Hut78 recipient cells in the presence of AmD (50 μg/ml) in the culture mix throughout the period 0h - 24h p.i., both the first phase and the second phase of viral RNA synthesis were abolished (Fig. 4.3i, Pre-treated & Maintained). This is an expected result. When 50 μg/ml of AmD was maintained in the co-culture, synthesis of both linear viral DNA and first phase 9 kb viral RNA were inhibited (Pre-treated & Maintained - Figs. 4.2i and 4.3i), despite the fact that this concentration of AmD did not affect minus strand DNA synthesis from an exogenous template in a standard *in vitro* RT reaction. Earlier studies using Rous sarcoma virus (Garapin *et al.*, 1970, McDonnell *et al.*, 1970) and Moloney mouse leukemia virus (Manly *et al.*, 1971) have shown that AmD at concentrations of 5-50 μg/ml did not inhibit the formation of single-stranded DNA from a viral RNA template although formation of double-stranded DNA was reduced. The *in vitro* activity of reverse transcriptase was not affected by

AmD (see section 4.1 and Fig. 4.1ii). This is because the *in vitro* assay using poly r(A)-oligo dT as template-primer reflects only the first component of the retroviral reverse transcription process *in vivo* and presumably does not include an AmD-sensitive step.

4.4 HIV RNA expression in acutely infected cells treated with Aphidicolin to inhibit viral DNA integration

The AmD pretreatment experiments described above effectively eliminated the possibility that the H3B provirus was the template for the early 9 kb viral RNA synthesis. Two remaining potential transcription templates in the cell-to-cell transmission infection system are the newly made episomal and newly-made, integrated viral DNA. Aphidicolin is an inhibitor of eukaryotic DNA polymerase alpha and delta (Huberman, 1981, Spadari *et al.*, 1982, Kornberg & Baker, 1992) and arrests cells at the late G_1 phase of the cell cycle. Its effect is reversible, as treated cells that are washed free of the drug can re-enter the cell cycle (Li & Burrell, 1992).(Li & Burrell, 1992) In an earlier report, aphidicolin (Aph) was shown to inhibit murine leukemia viral DNA integration (Roe *et al.*, 1993). It has been reported that some strains of HIV can infect and integrate in aphidicolin-treated, growth-arrested cells (Li *et al.*, 1993a, Lewis & Emerman, 1994). However, using the cell-to-cell HIV infection model system and the HXB2 strain of HIV, Li and Burrell showed that aphidicolin inhibited the production of unintegrated circular viral DNA and progeny virus production, but not the synthesis of linear unintegrated viral DNA (Li & Burrell, 1992). Furthermore, at a similar concentration of this drug (20 μM, equivalent to 6.8 μg/ml) and in the cell-to-cell transmission infection model described above, HIV DNA integration was inhibited (see section 5.5). The HXB2 strain lacks a functional Vpr due to truncation at amino acid position 81 of the protein (GenBank Accession K03455, HXB2 sequence, see also (Nie *et al.*, 1998, Zhou *et al.*, 1998)). As Vpr carries a nuclear localization sequence (see section 1.5.A.i), truncation of this protein might interfere with viral DNA integration in aphidicolin-arrested cells and lead to a situation similar to that with murine leukemia virus which cannot integrate in growth-arrested cells (Roe *et al.*, 1993, Lewis & Emerman, 1994). To examine whether integration of newly made viral DNA was required for its role as template for each phase of HIV RNA synthesis, viral RNA expression was studied in cells treated with aphidicolin which prevent viral DNA integration and arrests cells at the late G_1 phase of the cell cycle. Treatment of H3B and Hut78 cells with 20 μM aphidicolin 18h prior to

and during the infection period did not inhibit the first phase (4h - 12h p.i.) of viral RNA synthesis as judged by the similar levels of genomic-length 9.2 kb RNA in the untreated or Aph-treated cells (Fig. 4.4i). However, aphidicolin treatment had a dramatic impact on the second phase of viral RNA synthesis. At 24h p.i. the levels of all three RNA species in the aphidicolin-treated cells were reduced significantly compared to those of the untreated cells and by 48h p.i. the levels of all three viral RNAs in the drug-treated cells were minimal or not detectable. These results showed that the 9 kb RNA expression during the early phase (4 - 12 h p.i.) was likely to be transcribed from the unintegrated viral DNA template as the latter has been prevented from integrating into the cellular DNA. In contrast, the major reduction in RNA levels at 24h and beyond suggested that second phase HIV RNA synthesis used integrated viral DNA as template.

4.5 HIV RNA expression in cell-to-cell transmission of infection after aphidicolin treatment

To confirm the above conclusion that early 9 kb RNA synthesis used newly-made, episomal viral DNA as template, similar experiments were carried with AmD and Aph treament combined to block transcription from both H3B proviral DNA and newly-integrated DNA in the same experiment. H3B cells were pre-treated with AmD as well as aphidicolin 18-20h prior to the coculture. Hut78 recipient cells were pre-treated with aphidicolin only. On the day of coculture the pretreated cells were washed to remove the drugs, mixed in the ratio of 1:4 H3B and Hut78 cells respectively, and incubated with the same concentration of aphidicolin but without addition of AmD. At various periods p.i. cytoplasmic RNA was extracted from the infection mix and examined by Northern blot hybridization (Fig. 4.5i). At the time of cell mix (0 h p.i.) there was no detectable 9 kb viral RNA in either treated or untreated cells. The untreated cells showed the typical early phase of 9 kb RNA synthesis (with a slightly later appearance at 8h p.i.) and the late phase as described above. The treated cells also showed the first phase of 9 kb viral RNA synthesis but there was minimal RNA detectable at 24h p.i. or later with the treated cells, in contrast to the large amounts of all three species of viral RNA produced during the same period with untreated cells. These results, taken together with the observations in sections 4.2 to 4.4, further suggest it is the unintegrated viral DNA synthesized *de novo* following cell-to-cell

Fig. 4.4i

Kinetics of HIV RNA production during cell-to-cell infection using aphidicolin (Aph)-treated cells. Untreated Hut78 recipient cells (1.6 x 10^6) were co-cultured with untreated virus donor H3B cells (0.4 x 10^6) left panel. Hut78 cells (1.6 x10^6) and H3B cells (0.4 x 10^6) - each pre-treated with 20 μM Aph for 18h - were co-cultured in the presence of 20μM Aph (right panel). At different times (h) after infection (figures at the top of each lane) cytoplasmic RNA was extracted and viral RNA analysed as described in section 2.7.A. Each lane contained an average amount of RNA equivalent to that from 2 x 10^6 cells. The Northern blots were hybridized with the same probe mix.

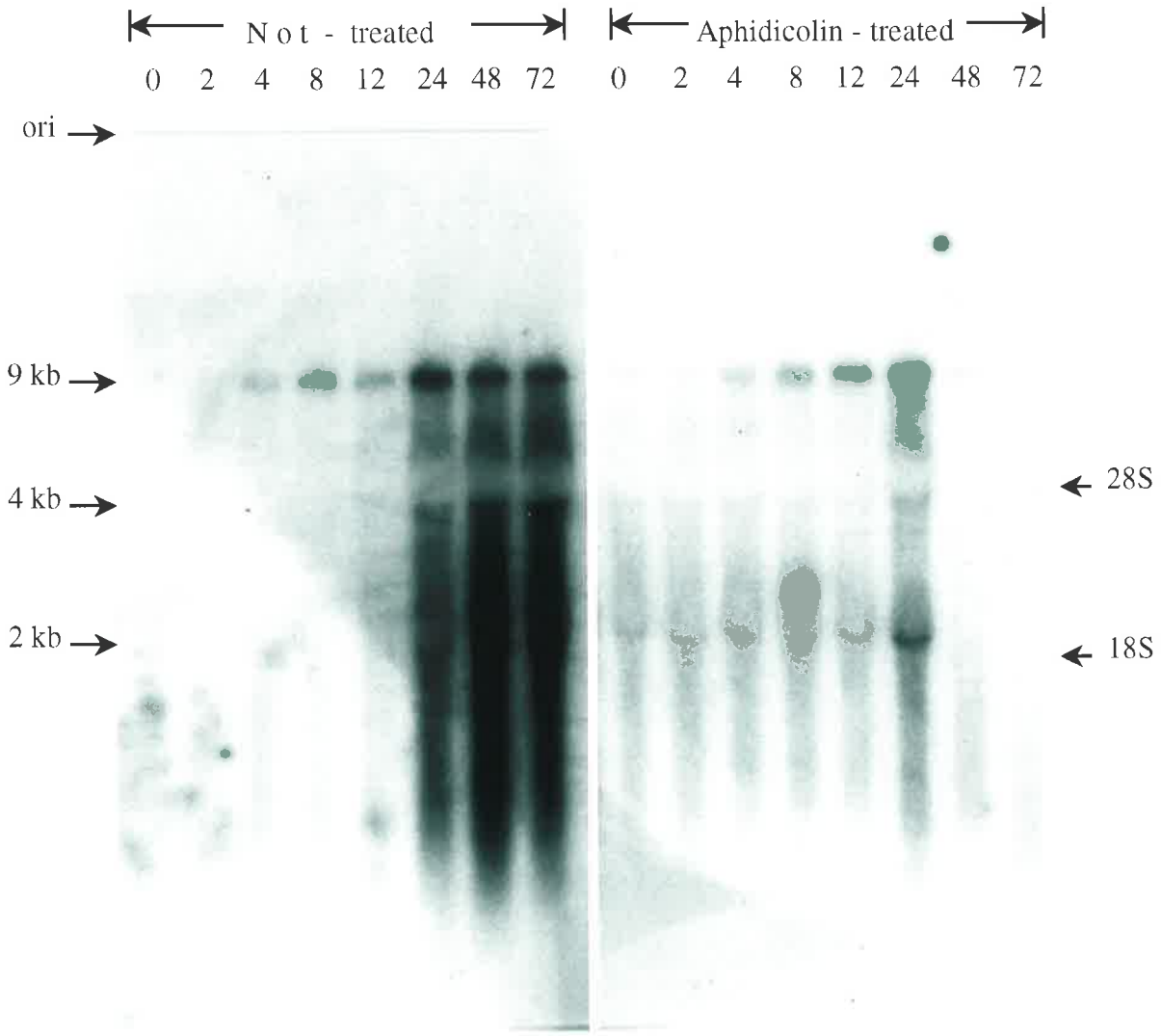
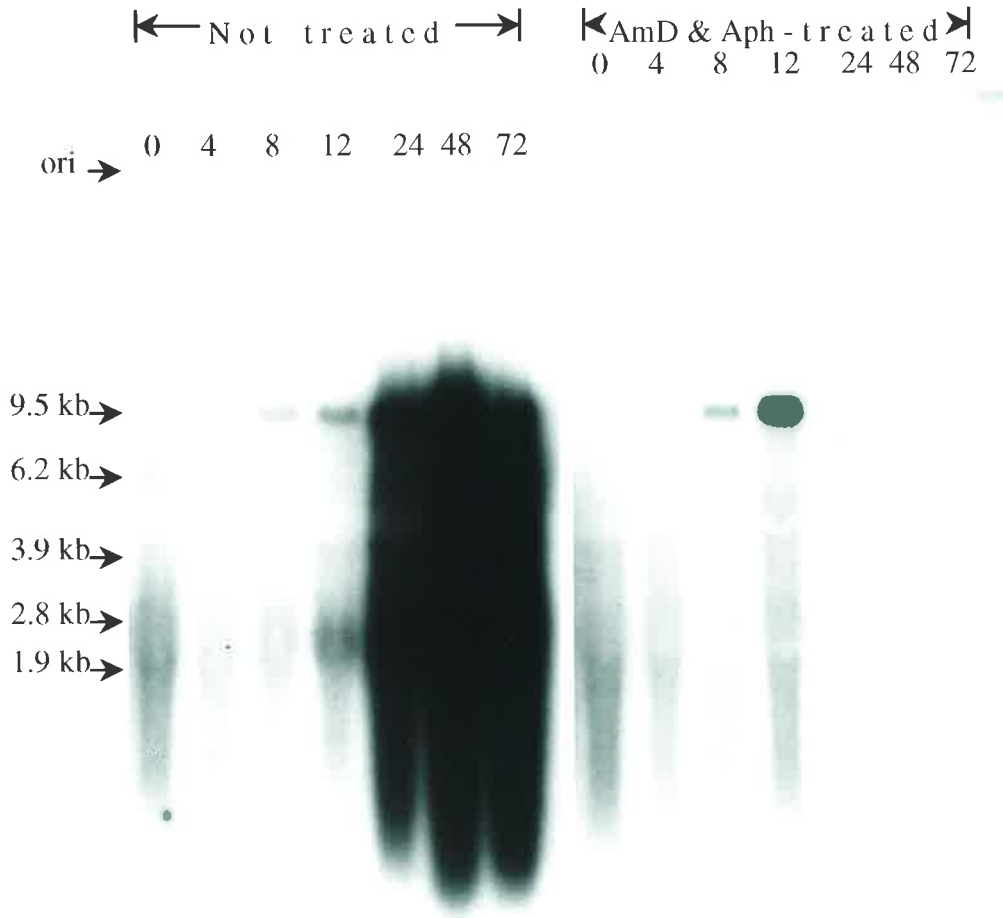

Not - treated
Aphidicolin - treated
0 2 4 8 12 24 48 72
0 2 4 8 12 24 48 72
ori
9 kb
4 kb
2 kb
28S
18S

Fig. 4.5i

Kinetics of HIV RNA production during cell-to-cell infection, using AmD-pretreated H3B donors and aphidicolin-treated donor as well as Hut78 recipient cells, and maintained in the presence of aphidicolin. Untreated Hut78 recipient cells (1.6×10^6) were co-cultured with untreated virus donor H3B cells (0.4×10^6) left panel. Hut78 cells (1.6×10^6) and AmD (50 μg/ml) pretreated H3B cells (0.4×10^6) - each pre-treated with 20 μM Aph for 18h - were co-cultured in the presence of 20μM Aph (right panel). At different times (h) after infection (figures at the top of each lane) cytoplasmic RNA was extracted and viral RNA analysed as described in section 2.7.A. Each lane contains an average amount of RNA equivalent to that from 2×10^6 cells. Both Northern blots were hybridized with the same probe mix. ori - origin of gel


Not treated
AmD & Aph - treated
0 4 8 12 24 48 72
0 4 8 12 24 48 72
ori
9.5 kb
6.2 kb
3.9 kb
2.8 kb
1.9 kb

transmission infection that serves as the major template for the synthesis of 9 kb HIV RNA during the early phase (4 - 12 h) p.i.

4.6 The effect of progressive inhibition of reverse transcription by AZT on the expression of early phase 9 kb viral RNA

The link between early phase viral RNA production and DNA synthesis was further analysed by reducing the amount of unintegrated viral DNA template available for viral RNA production. When the amount of unintegrated viral DNA was reduced, a corresponding reduction of early phase 9 kb viral RNA might be expected if the newly made unintegrated viral DNA served as the template for early phase viral transcription as previously suggested (sections 4.2 to 4.5). The amount of viral DNA produced during the early phase of cell-cell transmission infection was reduced by addition of 20 μM of AZT to the co-culture mix at either 2h or 4h p.i., and the amounts of viral RNA produced under each set of conditions were then examined by Northern blot hybridization. As expected, when AZT was added at 2h p.i. the amount of viral DNA synthesized throughout the culture was less than when AZT was added after 4h p.i. (Fig. 4.6i) Similarly, when AZT was added at 2h p.i., less 9 kb viral RNA was observed than when the drug was added at 4h p.i.(Fig. 4.6ii). The addition of AZT 18h prior to cell mix or at 0h p.i. totally inhibited the production of unintegrated viral DNA as well as 9 kb viral RNA during the early phase (0-12h p.i.) of co-culture but not the second phase (24-72h p.i.) (Kok *et al.*, 1993). This experiment showed that the amount of 9 kb RNA synthesized correlated with the amount of viral DNA synthesized, further supporting the role of newly made viral DNA as template. In the Northern hybridization blots shown in Fig. 4.6i and 4.6ii, the amounts of 9 kb viral RNA produced was significantly less than in an earlier report in which AZT was added 18h prior to cell mix (Kok *et al.*, 1993). This apparent difference may likely be due to the use of different batches of cells.

4.7 Estimation of the levels of viral RNA and DNA produced during cell-cell transmission infection

Newly made viral DNA might be derived wholly from input RNA from the donor H3B cells. However, we wished to explore the possibility that newly made 9 kb RNA could also serve as a template for futher reverse transcription, thus increasing the copy number of viral genomes

Fig. 4.6i

Southern blot hybridization analysis of HIV DNA produced in cell-to-cell transmission infection without addition of AZT (control), with AZT added at 2h and 4h p.i. The probe used was ^{32}P-multiprimer labelled Sst I fragment of plasmid pBH10.

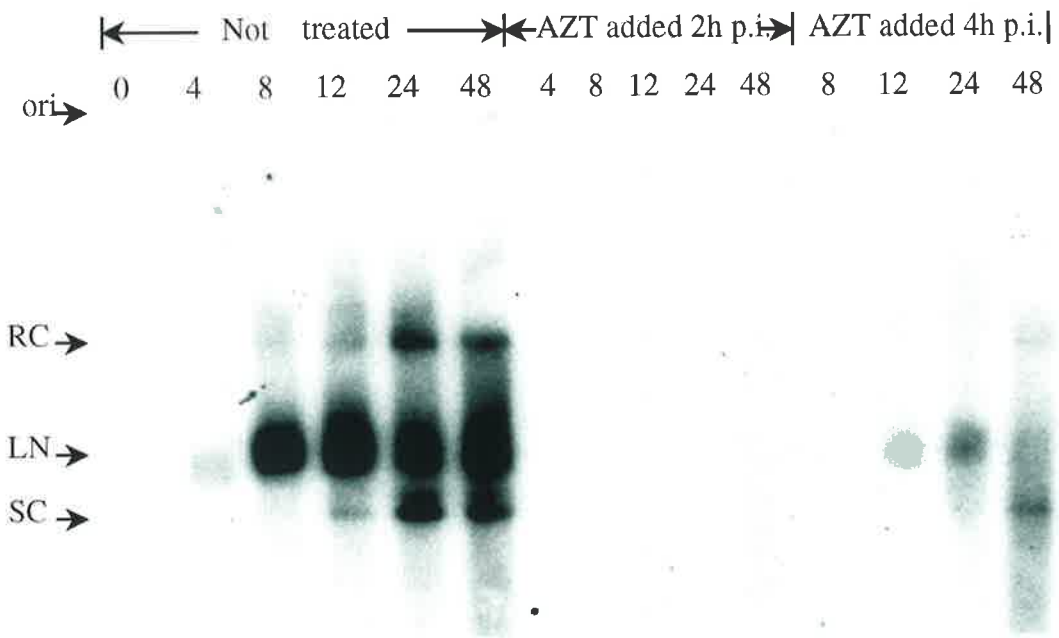
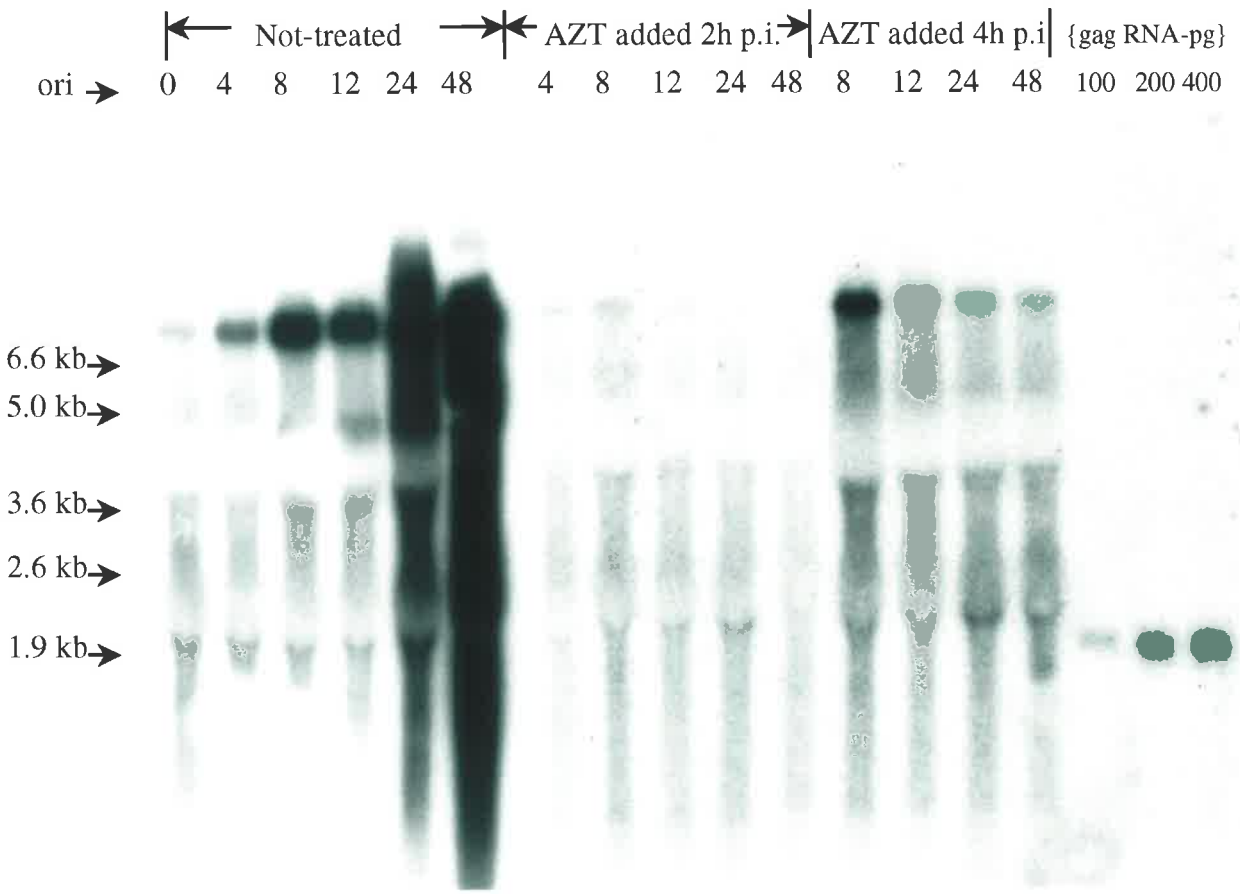

Not treated
AZT added 2h p.i.
AZT added 4h p.i.
0 4 8 12 24 48 4 8 12 24 48 8 12 24 48
ori
RC
LN
SC

Fig. 4.6ii.

Northern blot hybridization analysis of HIV RNA produced in cell-to-cell transmission infection without addition of AZT (Not-treated), or with AZT added at 2h and 4h p.i. The probe used was ^{32}P-multiprimer labelled Sst I fragment of plasmid pBH10.

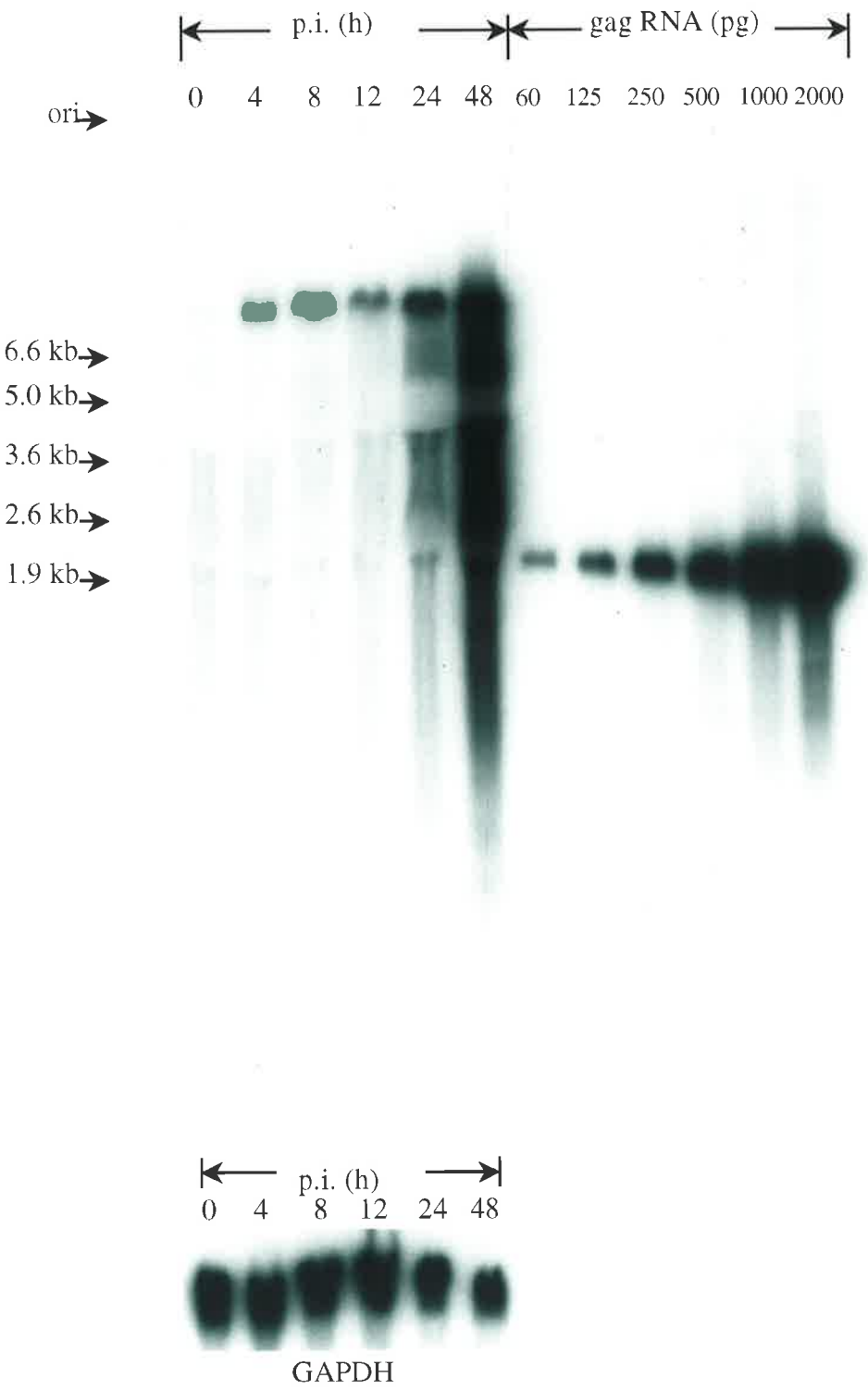

Not-treated
AZT added 2h p.i.
AZT added 4h p.i
{gag RNA-pg}
ori
0 4 8 12 24 48 4 8 12 24 48 8 12 24 48 100 200 400
6.6 kb
5.0 kb
3.6 kb
2.6 kb
1.9 kb

beyond that present at 0 h p.i. Therefore the amounts of viral RNA and DNA produced in the cell-to-cell transmission infection model described above were quantitated with a view to testing this possibility.

The levels of early phase 9 kb HIV RNA and unintegrated viral DNA produced during cell-cell transmission infection were compared with a range of gag RNA and DNA standards in Northern (Fig. 4.7i) and Southern blot hybridizations (Fig. 4.7ii) respectively. The gag RNA and DNA standards were a gift from A J Davis. The gag DNA was prepared by reverse transcription PCR of RNA obtained from a co-culture of H3B and Hut78 cells (ratio of 1:4). The primers used corresponded to nucleotide positions 307-322 and 2044-2030 (HIVHXB2R sequence (Myers *et al.*, 1990)). The gag DNA (1738 bp) was gel purified and subsequently TA-cloned into plasmid pBS-dT. The gag RNA standards were then prepared from *in vitro* transcription of the cloned plasmid gag DNA (Davis *et al.*, 1997). At various periods p.i. viral DNA or RNA were extracted from the cells in a cell-to-cell transmission infection mix. After treatment with DNase (section 2.13) or RNase (section 2.12) respectively, the viral RNA or DNA was separated in Northern or Southern gels together with a range of gag RNA or DNA standards. After capillary blotting onto nylon membranes followed by fixation for RNA or DNA respectively, the membrane filters were hybridized with a ^{32}P-labelled gag DNA probe with the same sequence as the standards used. The Northern and Southern blot hybridization signals were then analysed in a PhosphorImager (Molecular Dynamics) using the "Volume Integration" function of the software provided by the manufacturer. In order to adjust for loading differences in the Northern gel, the membrane filter was hybridized with ^{32}P-labelled GAPDH probe after removal of gag RNA signals by immersing the membrane filter in hot (95°C) 0.5% SDS which was allowed to cool to room temperature (according to manufacturer's instructions, Amersham). The SDS-treated membrane filter was then exposed in an X-ray cassette with intensifying screen for 48 h to ensure absence of gag RNA signals. The specific GAPDH RNA was then detected by hybridization with a cDNA probe derived from a plasmid clone (Tso *et al.*, 1985) labelled with ^{32}P by Megaprimer (Amersham) (see section 2.8.A). The Northern signals were similarly analysed in the PhosphorImager. The amounts of viral gag RNA or DNA expressed in the coculture mix were then semi-quantitated for each period p.i. by extrapolation from the respective gag standards using a computer programme for graph analysis

Fig. 4.7i.

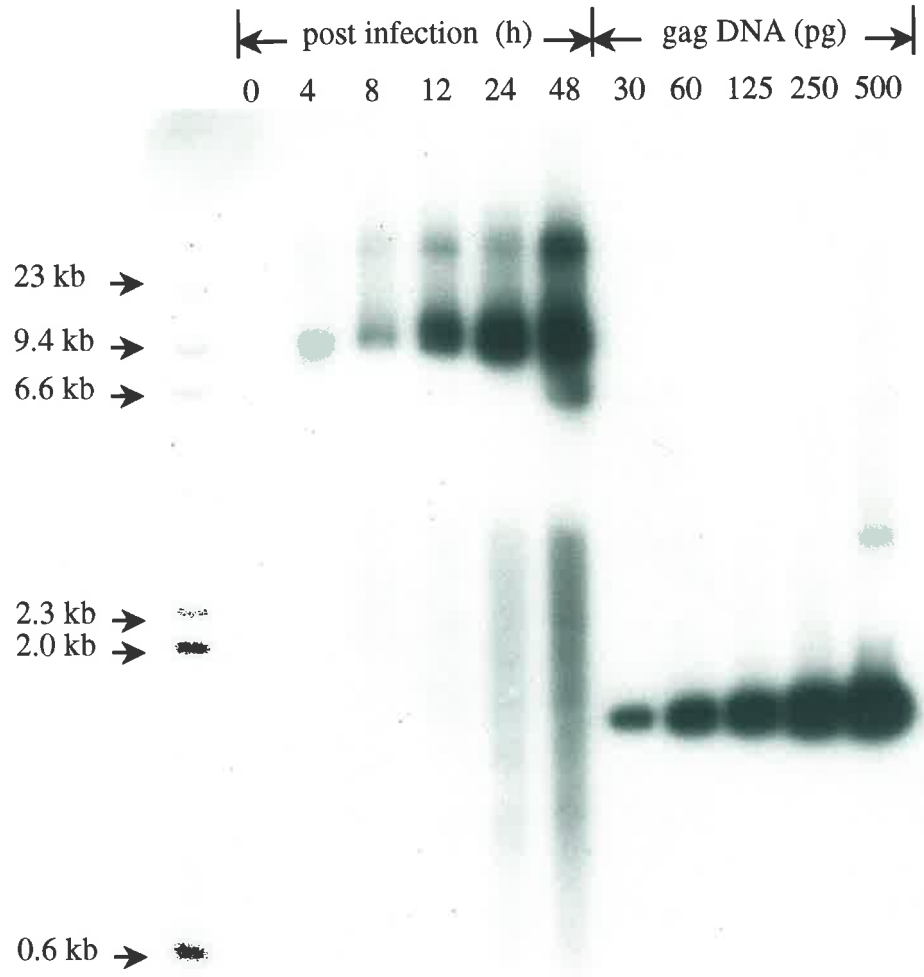
Northern blot hybridization analysis of HIV RNA produced in cell-to-cell transmission infection during the period 0 - 48h p.i. with gag RNA standards (pg). The probe used was ^{32}P-labelled Multiprimer labelled 1.7 kb gag DNA (see section 2.8.A). The *gag* RNA standards were *in-vitro* transcribed from plasmid gag DNA and were a gift from A. J. Davis.


p.i. (h)
gag RNA (pg)
0 4 8 12 24 48 60 125 250 500 1000 2000
ori
6.6 kb
5.0 kb
3.6 kb
2.6 kb
1.9 kb

p.i. (h)
0 4 8 12 24 48
GAPDH

Fig. 4.7ii.

Southern blot hybridization analysis of HIV DNA produced in cell-to-cell transmission infection during the period 0 - 48h p.i. with gag DNA standards (pg). The probe used was ^{32}P-labelled Multiprimer labelled 1.7 kb gag DNA (see section 2.8.A). The *gag* DNA standards were a gift from A. J. Davis.


post infection (h)
gag DNA (pg)
0 4 8 12 24 48 30 60 125 250 500
23 kb
9.4 kb
6.6 kb
2.3 kb
2.0 kb
0.6 kb

(CA-CricketGraph III, Computer Associates). The amounts of viral gag RNA expressed at each period p.i. were calculated after correcting for differences in loading, blotting and transfer by adjusting for the GAPDH signals. At 0 h p.i. the amount of gag RNA was undetectable (<60 pg/10^6 cells, equivalent to <33 copies/cell) (Fig. 4.7i). As the lower amount of gag RNA standard loaded in the gel was 60 pg, the minimal to undetectable signal observed at 0 h p.i. was taken to be less 60 pg. During the early phase of infection (0-12 h p.i) the level of 9 kb RNA produced increased to 60 copies/cell and by 24 h and 48 h p.i. the levels were 254 to 1264 copies/cell respectively (Table 4.7). The corresponding levels of HIV DNA (Fig. 4.7ii) were less than 17 copies/cell at the time of cell mix and at 4h p.i. By 8 -12 h p.i. the levels of HIV DNA had increased to 38 copies/cell and by 24h and 48h p.i. the HIV DNA levels increased to 68 and 104 copies/cell respectively (Table 4.7). These results confirmed that early in infection, HIV DNA and gag RNA appeared at similar levels. Later in infection a further increase in both species occurred, particularly with viral RNA. This may be a reflection of integration and transcription from the more efficient integrated proviral template.

4.8 Discussion

The studies in this and the previous chapter have identified an early phase of viral RNA synthesis in infected cells at 0-12 h after cell-to-cell HIV infection (Figs. 3.2i and 4.3i). The first phase of viral RNA induction following cell-to-cell HIV infection was detected as long as the accumulation of linear unintegrated viral DNA took place, and was seen independently of the concurrent inhibition of (i) cellular DNA polymerase alpha & delta activities and cell cycle progression (Fig. 4.4), (ii) circularization of newly synthesized episomal HIV DNA (Li & Burrell, 1992), (iii) progeny virus production and (iv) RNA transcription from the virus donor cells (Table 4.1, Fig. 4.3i). On the other hand, whenever the accumulation of linear unintegrated viral DNA was inhibited, the first phase of viral RNA synthesis was also abolished. This was seen in cells in the continued presence of AZT (Kok *et al.*, 1993)(see section 3.4) or AmD (Figs. 4.2i and 4.3i). When the levels of viral DNA were partially reduced by AZT there was a corresponding partial reduction in the level of 9 kb RNA produced (Fig. 4.7ii). Furthermore, aphidicolin treatment inhibited integration of newly synthesized viral DNA in the cell-to-cell transmission infection system, based on restriction enzyme digestion and Southern blot hybridization analysis of extensively washed and electrophoresis-purified high

Table 4.7.

Relation between the levels of HIV RNA and DNA production (gag copies/cell) during the period 0 - 48 h p.i. in cell-cell transmission infection

Period p.i. (h)	RNA (gag) copies/cell	DNA (gag) copies/cell
0	<33	<17
4	37	<17
8	60	14
12	60	38
24	254	68
48	1264	104

molecular weight cellular chromosomal DNA (see section 5.5), however 9 kb RNA synthesis was not reduced by this treatment. All these suggest that this early phase of induced viral RNA synthesis requires the newly synthesized episomal viral DNA and only this species, as template. The question of whether the newly synthesized 9 kb RNA might be used as template for further reverse transcription was then considered. All retroviral reverse transcription models to date predict that only one unintegrated viral DNA molecule could be made from one or two genomic length viral RNA (Li *et al.*, 1993b, Jones *et al.*, 1994, Coffin, 1996). Semi-quantitative Northern and Southern blotting studies (Figs.4.7i & ii, Table 4.7) showed that the initial level of genomic length (9 kb) viral RNA at the time of infection was minimal, relative to the levels observed at 4h p.i. and later; similarly no viral DNA was detectable at 0 h p.i. (see Fig. 3.1.Ai and (Li & Burrell, 1992)). However, the levels of viral RNA produced during the period 0 -12 h p.i. were *ca.* two fold higher than the viral DNA levels (Table 4.7). Thus, we found no quantitative evidence for immediate initiation of reverse transcription of the newly synthesized HIV RNA.

These findings may be compared with studies using integration-deficient mutant retroviruses. In one of these studies, integration appeared to be required for production of infectious human immunodeficiency virus; unintegrated HIV-1 DNA was shown to be a possible template for transcription, although the level and size of transcripts generated were not reported (Stevenson *et al.*, 1990). Similarly, using murine leukemia virus with extensive deletions in integrase, Schwartzberg and colleagues (Schwartzberg *et al.*, 1984) showed that these mutants could produce viruses but with reduced infectivity. More recently, HIV-1 DNA molecules were shown to be transcriptionally active (Engelman *et al.*, 1995, Wiskerchen & Muesing, 1995) (also reviewed in (Brown, 1997)). After the newly synthesized viral DNA molecules become integrated, they appear to provide more efficient templates for the second phase of viral RNA transcription. Furthermore, these latter transcripts may be more accessible to the cellular splicing machinery, leading to the later production of singly and doubly-spliced RNAs. However the early phase RNA transcripts are clearly largely unspliced; it is likely that these are produced in the cytoplasm and are presumably inaccessible to the splicing machinery of the cell, although it has not been formally excluded that they may be produced in the nucleus and transported to the cytoplasm under the influence of the pre-existing levels of Rev protein present

in the viral donor cells. If this RNA is produced in the cytoplasm, the enzyme/s responsible and its mechanism of action are at present unknown. Thus, during the early phase of replication (0-12h) following cell-to-cell transmission infection, reverse transcription and HIV RNA synthesis occur concurrently; RNA synthesis is dependent on newly synthesized DNA as template while the role played by newly synthesized RNA in further reverse transcription remains to be clarified.

Chapter 5

Nothing in this world can take the place of persistence.....Persistence and determination alone are omnipotent. Calvin Coolidge (1872-1933), US President.

Kinetics and pattern of HIV DNA integration during cell-to-cell transmission infection

5.1 Introduction

Two major steps in the replication cycle of retroviruses are the synthesis of viral DNA by the virion reverse transcriptase, and the subsequent integration of this newly-made viral DNA into the host chromosome to form the provirus (see sections 1.6.C & D). The integration step is catalyzed by the viral integrase (IN) and this enzyme has been shown by *in vitro* studies to be both necessary and sufficient to process the LTR ends of the linear viral DNA for integration (Katz *et al.*, 1990, Wiskerchen & Muesing, 1995, Leavitt *et al.*, 1996). The provirus is always colinear with the unintegrated linear viral DNA with each LTR end covalently joined to the host DNA while integration sites in the cellular DNA are random (see section 1.6.D). Although there are three unintegrated viral DNA forms in the infected cell viz. relaxed circular, linear and supercoiled (Li & Burrell, 1992), *in vitro* studies of sequences at the junction of viral and cellular DNA suggest that the linear viral DNA is the immediate precursor to the provirus (Brown *et al.*, 1987, Fujiwara & Mizuuchi, 1988, Brown *et al.*, 1989). The endonucleolytic action of IN processes the linear viral DNA in the cytoplasm by removing two bases from the 3' termini to produce the recessed 3' OH group (Brown, 1990). The processed linear viral DNA in a pre-integration complex (Bukrinsky *et al.*, 1992, Karageorgos *et al.*, 1993, Farnet & Bushman, 1997) is translocated to the nucleus where it interacts with the host DNA and is subsequently integrated. *In vitro* studies have also identified a DNA-splicing activity of the viral IN in which the viral DNA integration reaction is reversed, a process initially called disintegration (Chow *et al.*, 1992). The role of disintegration *in vivo* has not been studied. Integration is an important step in the replication of retroviruses, in particular for productive infection (Panganiban & Temin, 1983, Schwartzberg *et al.*, 1984, LaFemina *et al.*, 1992, Sakai *et al.*, 1993, Englund *et al.*, 1995). Transcription from the proviral DNA is regulated by contributions from the host transcription machinery and viral regulatory proteins, although

transfected viral DNA (Hwang & Gilboa, 1984) and unintegrated HIV-1 DNA can also be transcribed [Kok, 1993 #961; Engleman, 1995 #1332; Wiskerchen, 1995 #1333; Cara, 1997 #1626]. Formation of the proviral DNA to become a functional part of the host DNA facilitates the propagation and persistence of the virus in the host.

Although some of the molecular mechanisms of retroviral DNA integration have been characterized *in vitro*, this process has not been comprehensively characterized biologically in infected cells. An earlier report (Harris *et al.*, 1984) that the visna lentiviral DNA may not integrate has been re-investigated recently and it has been shown that visna viral DNA did integrate in infected sheep choroid plexus cells (List & Haase, 1997). These reports and the necessity of distinguishing the large amounts of contaminating unintegrated viral DNA (Keshet & Temin, 1979, Weller *et al.*, 1980, Li & Burrell, 1992, Sato *et al.*, 1992), from proviral DNA produced in the course of retroviral integration indicates difficulties in interpreting integration studies. HIV infection of susceptible cells with the cell-free virus (Kim *et al.*, 1989b) or by cell-to-cell transmission produces large amounts of unintegrated HIV DNA early (4h p.i.) in the infection (Li & Burrell, 1992, Sato *et al.*, 1992, Barbosa *et al.*, 1994). In cell-to-cell and cell-free viral transmission of infection there is also a corresponding early induction of genomic length viral RNA (Kok *et al.*, 1993, Kok *et al.*, 1998) (see chapters 3 & 4). This chapter analyses the kinetics of HIV DNA integration in relation to the earlier studies of unintegrated viral DNA and RNA production, a similar cell-to-cell transmission infection model was used.

In this work, the cell-to-cell transmission model described in the previous two chapters was used. During the course of infection, the large amounts of unintegrated viral DNA, as well as viral RNA, produced need to be separated from chromosomal DNA in order to analyse the integrated sequences. In one approach, the cell cultures were harvested at different time point post infection and chromosomal DNA was prepared by Hirt extraction (Hirt, 1967) (see section 2.4). Chromosomal DNA was then purified by electrophoresis to remove unintegrated viral DNA and digested with restriction enzymes. The kinetics of viral DNA integration were then analysed by Southern blot hybridization with a near full-length HIV DNA probe (pBH10) (Hahn *et al.*, 1984) (see section 2.8.A). Restriction enzyme cleavage of the chromosomal DNA

followed by Southern blot hybridization would identify subgenomic HIV DNA fragments and high molecular weight DNA containing proviral and host flanking sequences. These latter high molecular weight species, appearing as a smear in Southern blots, include a specific 5' or 3' terminal restriction fragment covalently linked to host sequences of different sizes due to random integration into the host DNA.

A second appproach to analysing the integrated DNA is the use of PCR with specific primers that amplify only integrated viral DNA and not unintegrated forms. One method is to use PCR with outward directed primers (inverse PCR) in conserved regions of the HIV LTR and *gag* sequence. Chromosomal DNA containing the proviral DNA is digested with a selected restriction enzyme that cleaves the HIV DNA at one site, and subsequent intramolecular ligation, is allowed to occur and the outward primers are then used in a PCR to amplify the ligated DNA (Chun *et al.*, 1995). Restriction enzyme digestion of unintegrated viral DNA would not yield circular viral DNA due to incompatible ends (blunt end at the 5' LTR and overhang 3' end), and there would be no corresponding PCR amplification product. Further specificity and sensitivity can be increased by using nested PCR amplification of the ligated DNA with primers specific for HIV LTR (Chun *et al.*, 1997b). Alternatively, integrated DNA in macrophages has been analysed by *Alu*-PCR sequences in host DNA as targets for PCR primers (Sonza *et al.*, 1996). Although these PCR-based approaches have been used with some studies, Southern blot hybridization analyses were used for studies of HIV integration kinetics in this thesis to allow more direct comparison with the earlier Southern and Northern blot hybridization analyses of viral DNA and RNA (see Chapters 3 and 4). In order to maximize removal of contaminating unintegrated viral DNA from the chromosomal DNA in Hirt pellets, extensive gel electrophoresis was used. The use of gel electrophoresis to remove contaminating unintegrated retroviral DNA, including HIV DNA, has been reported previously (Humphries *et al.*, 1981, Harris *et al.*, 1984, Stevenson *et al.*, 1990, List & Haase, 1997).

5.2 Purification of chromosomal DNA (Hirt pellet) to remove contaminating unintegrated viral DNA

Extraction of virus-infected cell harvests by the Hirt procedure yields two fractions - the Hirt supernate and pellet (Hirt, 1967) (see section 2.4). The Hirt supernate contains mainly

mitochondrial DNA, unintegrated viral DNA and viral and cellular RNA while the Hirt pellet contains mainly chromosomal DNA which contains the integrated proviral DNA. Reliable identification of integrated viral DNA requires requires purification of the Hirt pellet - free of contaminating unintegrated viral DNA.

To achieve this the chromosomal DNA was first washed with Hirt solution at 56°C/1 h, precipitated in 0.2 M NaCl at 4°C/>4 h or overnight and then centrifuged at 17000g/45 min/4°C (see section 2.5.A & B). This washing and precipitation cycle was repeated once. The washing temperature of 56°C yielded lower background signals in Southern blot hybridization analysis of the chomosomal DNA than washing at 37°C in preliminary experiments. During these washing and precipitation procedures, it was noticed that the chromosomal DNA appeared as a gelatinous, clear, mucoid-like blob. The approximate volume of the blob of chromosomal DNA from the equivalent of 2 x 10^6 cells was 100 µl. Following two phenol/chloroform extractions and ethanol precipitation, contaminating RNA was removed by treatment with RNase (1 µg/ml at 37°C/30 min) followed by phenol/chloroform extraction. The purified Hirt pellet DNA was then resuspended in 1% low gelling temperature agarose (gel plug) and subjected to overnight electrophoresis. (The optimal concentration of RNase and treatment conditions used were determined by prior titrations.) The electrode polarity was reversed on the following day and electrophoresis was continued for a further 8 hours. This extensive electrophoresis procedure was adopted in order to maximize removal of unintegrated viral DNA that was not covalently associated with chromosomal DNA. After electrophoresis, the agarose in the gel plug was digested by agarase (Calbiochem/Boehringer Mannheim) and the released chromosomal DNA was then extracted with Tris-buffer saturated phenol (pH 8.0), followed by chloroform extraction and precipitation in ethanol. The purified chromosomal DNA pellet was then dissolved (with occasional vigorous mixing in a Vortex mixer set at maximal speed) in restriction enzyme digestion buffer at 37°C/6-8 h or overnight at room temperature to ensure maximal dissolution, prior to digestion with the selected enzyme. During preliminary experiments, the purified chromosomal DNA was sheared by passing through 19G and then 23G hypodermic needles instead of vortexing to facilitate dissolution. Although this procedure sheared the chromosomal DNA sufficiently for dissolution, this was not used in subsequent

experiments due to the variable recovery of the sheared DNA and as a potential occupational hazard in an infectious disease laboratory. Although preliminary experiments showed that the minimal period for complete restriction digestion of chromosomal DNA was 6 - 8 h/37°C, this was invariably incubated at 37°C overnight in a water bath to ensure complete reaction. After RE digestion the chromosomal DNA was extracted once with phenol/chloroform and precipitated with ethanol at -20°C/>4h. After RE digestion the characteristic, clear, mucoid-like appearance of the chromosomal DNA described earlier has changed to a more fluid consistency. Due to the long incubation periods used for purifying the chromosomal DNA, extra care was taken to prevent the inadvertent introduction of nucleases and where possible all reagents used were treated as if for RNA preparations. The purified and enzyme restricted chromosomal DNA was then separated by agarose gel electrophoresis, Southern blotted and hybridized with a selected HIV probe labelled with α-^{32}P-deoxyadenosine 5'-triphosphate and α-^{32}P-deoxycytidine 5'-triphosphate.

The restriction enzyme selected for this work was KpnI. Digestion of linear, unintegrated HIV DNA with KpnI yields a convenient pattern of five fragments in Southern blots (Fig. 5.2i) consisting of the 5' terminal 3.8kb, the internal 2.7 and 2.2 kb fragments, the 3' terminal 0.7 kb and an internal 0.3 kb fragment. Cleavage of the 2-LTR circular HIV DNA would yield two fragments viz. 4.5 kb and 5.2 kb in size. Integrated HIV DNA examined in the same way would be expected to yield only the internal 2.7, 2.2 and 0.3 kb HIV DNA fragments and high molecular weight DNA fragments containing the 3.8 kb and 0.7 kb terminal viral fragments. Since HIV integrates at random sites (see section 1.6.D) the KpnI cleavage fragments containing the 5' and 3' viral terminal sequences would yield a smear of larger fragments due to inclusion of random lengths of chromosomal DNA sequences.

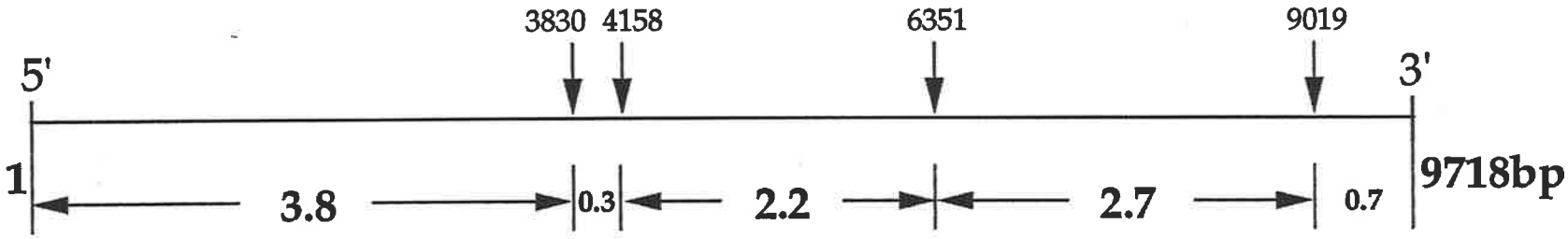
5.3 Reconstruction experiments to confirm removal of unintegrated viral DNA from chromosomal DNA

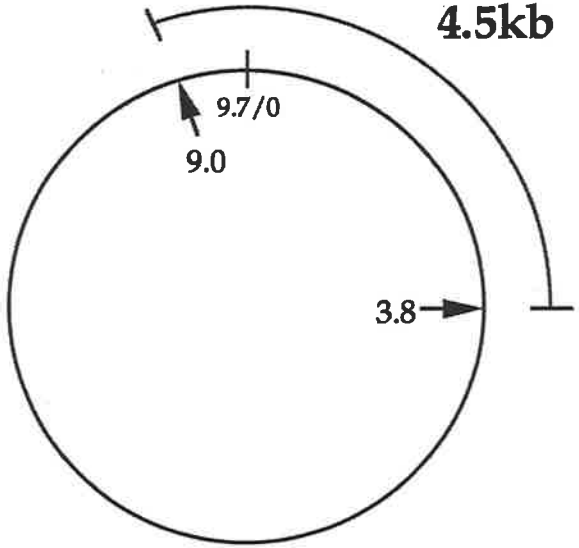
In a reconstruction experiment to confirm removal of the unintegrated viral DNA from the chromosomal DNA (equivalent to 2 x 10^6 cells) by the above procedure, Hut78 or H3B chromosomal DNA was prepared by Hirt extraction (see section 2.4), resuspended in TE buffer

Fig. 5.2i

KpnI restriction endonuclease sites of linear and two-LTR circular HIV DNA.

In order of decreasing size, restriction of HIV DNA with KpnI yields the 5' terminal 3.8kb, the internal 2.7 and 2.2 kb fragments, the 3' terminal 0.7 kb and an internal 0.3 kb fragment. Cleavage of the 2-LTR circular HIV DNA would yield two fragments viz. 4.5 kb and 5.2 kb in size.


3830
4158
6351
9019
5'
3'
1
3.8
0.3
2.2
2.7
0.7
9718bp


4.5kb
9.7/0
9.0
3.8

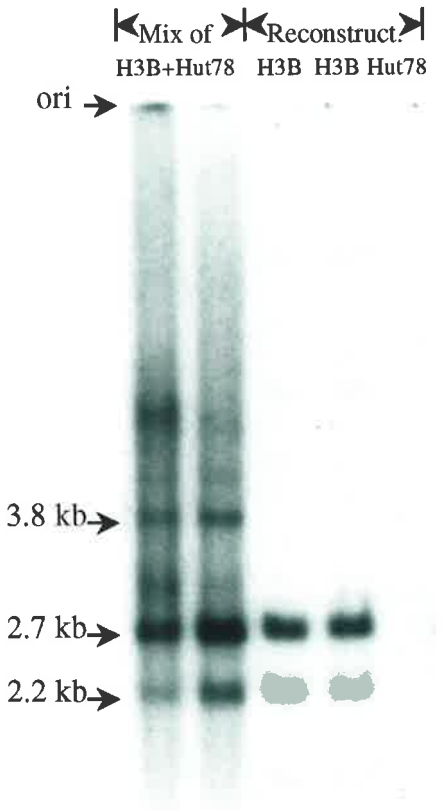
and then incubated (37°C/30 min) with Hirt supernate prepared from a co-culture infection mix of Hut78 and H3B cells harvested at 9h p.i. Previous Southern blot hybridization has shown 50-100 copies/cell of HIV unintegrated DNA is present at this time point p.i. (Li & Burrell, 1992) (see Fig.3.1.Ai). The reconstructed DNA samples containing unintegrated HIV DNA with Hut78 chromosomal DNA or H3B chromosomal DNA were purified as described in section 2.5.A and KpnI restricted chromosomal DNA was then Southern blotted and hybridized with a HIV probe labelled with α-^{32}P-deoxyadenosine 5'-triphosphate and α-^{32}P-deoxycytidine 5'-triphosphate.

The above reconstruction experiment with Hut78 (uninfected) chromosomal DNA did not yield HIV specific Southern blot hybridization signals. Reconstruction with H3B chromosomal DNA which contained two proviral DNA copies/cell showed no 3.8 kb or 0.7 kb HIV specific terminal fragments (Fig. 5.3i), while the 2.7 and 2.2 kb bands observed were the predicted internal KpnI restricted fragments from proviral DNA. These reconstruction experiments showed that extensive purification of the Hirt pellet by the procedures described above eliminated contaminating unintegrated viral DNA detectable by Southern hybridization analysis and that the terminal HIV proviral fragments were not seen as bands when purified chromosomal DNA was being analysed.

In a different experiment in which chromosomal DNA from H3B cells was extracted and similarly purified by agarose gel electrophoresis followed by KpnI restriction, the two internal fragments (2.7 and 2.2 kb) were again observed in Southern blot hybridization but not the terminal viral fragments (Fig. 5.3ii). Two high molecular weight fragments (one > 23 kb and the second < 23 kb) were obtained (indicated by arrowheads). The signal intensities from the latter fragments were not as high compared to the internal fragments. This may be due to lower Southern blot transfer efficiency with high molecular weight DNA. As H3B cells are a clone containing two proviral DNA copies, the presence of two high molecular fragments may represent the two 5' integration sites if the shorter 3' terminal fragments were undetectable in this assay. However, no further confirmation of this was performed.

Fig. 5.3i

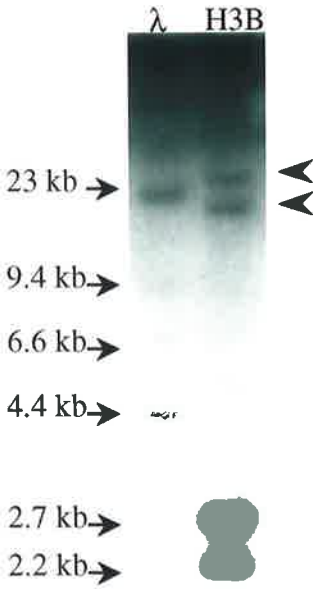
Southern blot hybridization analysis of chromosomal DNA extracted from a reconstructed mix of H3B or Hut78 DNA incubated (1h/37°C)with unintegrated HIV DNA prior to Hirt wash and gel electrophoresis. The two tracks on the left side of the Southern blot shows the hybridization signals obtained from chromosomal DNA extracted from a co-culture mix of H3B and Hut78 cells. The purified chromosomal DNA were cleaved with KpnI restriction enzyme prior to Southern blotting and hybridization with a near full-length HIV DNA probe as described in sections 2.5.A - 2.7.A and 2.8.A.


Mix of
Reconstruct.
H3B+Hut78
H3B
H3B Hut78
ori
3.8 kb
2.7 kb
2.2 kb

5.3ii

Monoclonality of H3B cells

Southern blot hybridization analysis of KpnI restricted purified chromosomal DNA from H3B viral donor cells hybridized with the plasmid pBH10 HIV DNA probe [Hahn, 1984 #793] (section 2.8.A). The presence of the two high molecular weight fragments suggest that the H3B cells are of clonal origin, and are likely to represent KpnI fragments at ends of the provirus to which upstream flanking cellular sequences are covalently joined. The 2.7 and 2.2 kb fragments are from internal KpnI restriction sites of HIV DNA.


λ
H3B
23 kb
9.4 kb
6.6 kb
4.4 kb
2.7 kb
2.2 kb

5.4 Kinetics of HIV DNA integration by cell-to-cell transmission infection

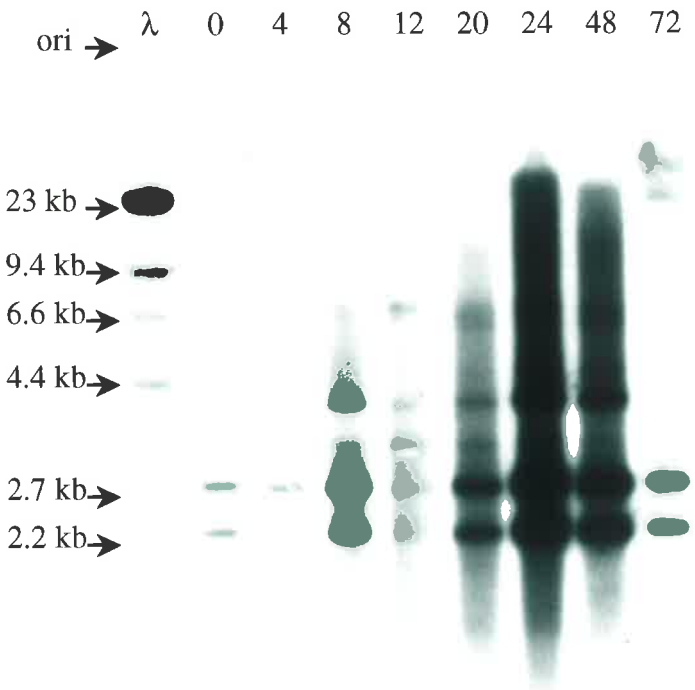
To analyse the biological processes and kinetics of HIV integration in the synchronous cell-to-cell transmission model described above HIV donor H3B cells were cocultured with Hut78 recipient cells in the ratio of 1:4 (see section 2.2.A) and cells from the co-culture mix were harvested at regular periods post infection. The chromosomal DNA fraction (Hirt pellet) was obtained from the harvested cells (2×10^6) and purified to remove unintegrated HIV DNA as described in section 5.3. After purification, the chromosomal DNA was restricted with KpnI enzyme, Southern blotted and hybridized with a near full-length ^{32}P-HIV probe (Hahn *et al.*, 1984) (see section 2.8.A).

Fig. 5.4i shows the time course of HIV DNA integration obtained as above. As the cell mix contains H3B cells, as well as Hut78 recipient cells, KpnI enyzme restriction of the Hirt pellet from the harvested cells yielded the major 2.7 kb and 2.2 kb HIV internal proviral DNA fragments (Fig. 5.4i). The 0.3 kb internal fragment is not clearly evident, except at 24 h and 48 h p.i., in all the tracks possibly due to the lower sensitivity in detecting smaller fragments. Two fragment sizes of *ca.* 6.6 kb and 3.5 kb with relatively weak signal intensities were seen during the period 8h to 48h p.i. but not from 0 - 4 h p.i. and at 72 h p.i. These relatively distinct fragments were not observed in other similar experiments and their significance is not known.

HIV DNA integration was first shown at 8h p.i. by the appearance of a smear of Southern hybridization signals increasing in molecular weight from the 3.8 kb position. Integration reached a maximum by 24h p.i. as indicated by the more intense signals representing the 2.7 and 2.2 kb internal proviral fragments. In addition, the total signals within each time point increased to a maximum by 24h p.i., began to decrease by 48 h p.i. and by 72 h p.i. the pattern was less complex, consisting of the internal 2.7 kb and 2.2 kb KpnI restricted fragments and two prominent high molecular weight fragments (*ca.* 23 kb). The latter presumably are cleavage products (5' or 3' terminal fragments) of proviral DNA with covalently joined KpnI restricted chromosomal DNA (see Fig. 5.5i). These discrete as well as the smear of Southern signals may represent the population of cells with newly-integrated viral DNA and the surviving H3B cells. The Southern blot hybridization of Fig. 5.4i also shows a smear of signals beginning at 0.7 kb position and increasing in intensity at 24 h and 48 h p.i. This is likely due to the KpnI

Fig. 5.4i

HIV DNA integration kinetics showing Southern blot hybridization analysis of purified chromosomal DNA extracted from co-culture mix of H3B and Hut78 cells in cell-to-cell transmission infection during the period 0 - 72 h p.i. Unintegrated viral DNA was removed from the chromosomal DNA as described in the text. The purified chromosomal DNA was digested with KpnI RE, Southern blotted and then hybridized with a near full length HIV DNA probe (pBH10) (see section 2.8.A). The amount of KpnI restricted chromosomal DNA loaded was equivalent to 2 x 10^6 cells/track. The cells used are from the same coculture mix as described in Fig. 5.4ii.


ori
λ
0
4
8
12
20
24
48
72
23 kb
9.4 kb
6.6 kb
4.4 kb
2.7 kb
2.2 kb

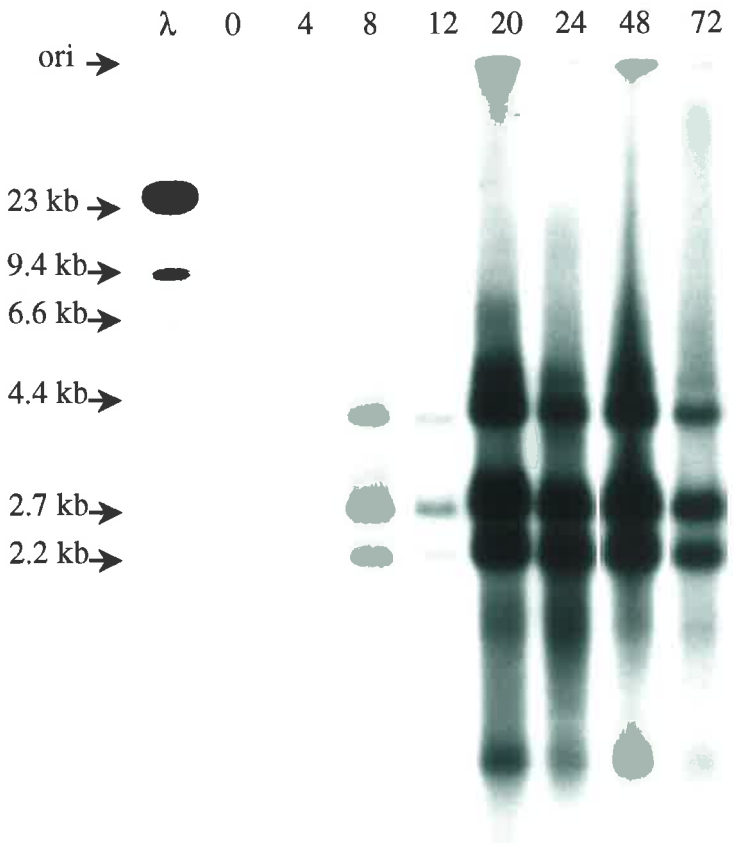
restriction site towards the 3' end of the HIV DNA (see Fig. 5.2i) as well as random cleavage sites along the chromosomal DNA downstream of the integration junction. It is noted that distinct fragments were observed at 3.8 kb from 8 - 48 h and a band around 0.7 kb position was also seen. It might be argued that these two bands (0.7 kb and 3.8 kb) represent contaminating, unintegrated viral DNA. This is unlikely as all Hirt pellets extracted from the co-culture mix of cells were washed twice in Hirt solution and electrophoresis was applied extensively, with reverse polarity, as described earlier; reconstruction experiments showed that these procedures were sufficient to remove contaminating, unintegrated viral DNA. Furthermore, at 72 h p.i. these terminal fragments were not detectable in the Southern blot shown in Fig. 5.4i despite large amounts of unintegrated viral DNA being detectable at this time point (Fig. 5.4ii, see also (Li & Burrell, 1992)). In addition, there are large amounts of circular viral DNA present from 12 h p.i. in this experimental system ((Li & Burrell, 1992), Fig. 5.4ii). KpnI RE digestion of circular viral DNA (2-LTR forms) would be expected to yield 4.5 kb fragments (Fig. 5.2i). However, there were no detectable 4.5 kb fragments in the Southern blot hybridization analysis (Fig. 5.4i). Taken together these observations make it unlikely that contaminating unintegrated viral DNA was being detected in the purified chromosomal DNA. The possible identity of the 3.8 kb KpnI fragment in the extensively purified chromosomal DNA preparations has been studied further and will be discussed in section 5.6.

5.5 Effect of aphidicolin on HIV DNA integration in cell-to-cell transmission of infection

Unlike the oncogenic murine leukemia virus (MLV) (Humphries & Temin, 1974, Roe *et al.*, 1993, Lewis & Emerman, 1994), HIV-1 can infect non-dividing cells such as macrophages productively (Saag *et al.*, 1988, Weinberg *et al.*, 1991, Schmidtmayerova *et al.*, 1997). This may be due to active nuclear import of the HIV pre-integration complex (Bukrinsky *et al.*, 1992, Karageorgos *et al.*, 1993) in contrast to that of MLV which is thought to require nuclear membrane disruption at mitosis (Roe *et al.*, 1993). The Vpr protein of HIV contains a nuclear localization signal at the carboxyl end and is thought to be involved for translocation of the HIV preintegration complex from the cytoplasm to interphase nucleus (see section 1.5.A.i). Analysis of the HIV HXB2 sequence, which is the proviral DNA in the H3B cells, shows that its *vpr* region contains a premature stop codon (at position 81) towards the 3' end which prevent

Fig. 5.4ii

Southern blot hybridization analysis of unintegrated HIV DNA synthesis in cell-to-cell transmission infection during the period 0 - 72 h p.i (see section 2.4). Each track contains unintegrated viral DNA, cleaved with KpnI, obtained from the equivalent of 1 x 10^6 cells. The cells used are from the same coculture mix as described in Fig. 5.4i. The Southern blot was hybridized with the near full length HIV DNA probe (section 2.8.A).


λ
0
4
8
12
20
24
48
72
ori
23 kb
9.4 kb
6.6 kb
4.4 kb
2.7 kb
2.2 kb

the complete expression of this gene. Table 5.5 shows the two termination codons of the amino acid sequence in the HIV HXB2 *vpr* gene. In contrast, pNL4-3 strain of HIV does not have premature stop codons in the *vpr* gene and this viral strain has been shown to integrate in non-dividing cells (Fouchier *et al.*, 1998, Zhou *et al.*, 1998). The arginine residues at the carboxyl end of Vpr are important for nucleocytoplasmic transport. Aphidicolin (Aph) is an inhibitor of eukaryotic DNA polymerase alpha and delta and arrests cells at the late G_1 phase of the cell cycle (Huberman, 1981, Spadari *et al.*, 1982, Kornberg & Baker, 1992). Using a similar cell-to-cell transmission infection of Hut78 cells with viral donor H3B cells, in which both cell lines were pretreated with 20μM aphidicolin and co-cultured in the presence of the drug, the levels of linear viral DNA synthesis were similar to untreated cells. However, circular viral DNA synthesis was significantly reduced and progeny virus production, as judged by supernatant reverse transcriptase activity, was inhibited in the presence of aphidicolin (Li & Burrell, 1992). To relate these observations to viral integration, the extent of integration at each time point was examined in aphidicolin-treated growth-arrested cells using a similar cell-to-cell transmission infection of Hut78 with viral donor H3B cells.

Both target Hut78 and viral donor H3B cells were pre-treated with 20 μM of Aph 18-20 h prior to cell mix, washed three times with drug-free cell culture medium and then co-cultured in the presence of the drug. At various periods p.i. the cells were harvested and chromosomal DNA (Hirt pellet) were prepared as described in section 2.5.A. The unintegrated viral DNA were removed by extensive washing of the Hirt pellet and gel electrophoresis. The purified chromosomal DNA was then digested with KpnI RE (37°C/overnight) and analysed by Southern blot hybridization with a near full-length HIV DNA probe (Hahn *et al.*, 1984) labelled with α-^{32}P-deoxyadenosine 5'-triphosphate and α-^{32}P-deoxycytidine 5'-triphosphate (Amersham) (section 2.8.A) (Fig. 5.5i). There was minimal or no integration of HIV DNA obtained in the growth-arrested cells as judged by absence of any increase of Southern hybridization signals compared to untreated cells. Thus treatment of both cell lines with aphidicolin prevented the integration of newly made viral DNA into the H3B or Hut78 cellular DNA. Two high molecular weight bands (around 23 kb) are seen in the untreated cells at 24 - 72 h p.i. It is not known what these bands represent although they are unlikely to be clonal integration sites. Importantly, the absence of the 3.8 kb KpnI terminal (5' end) fragment in

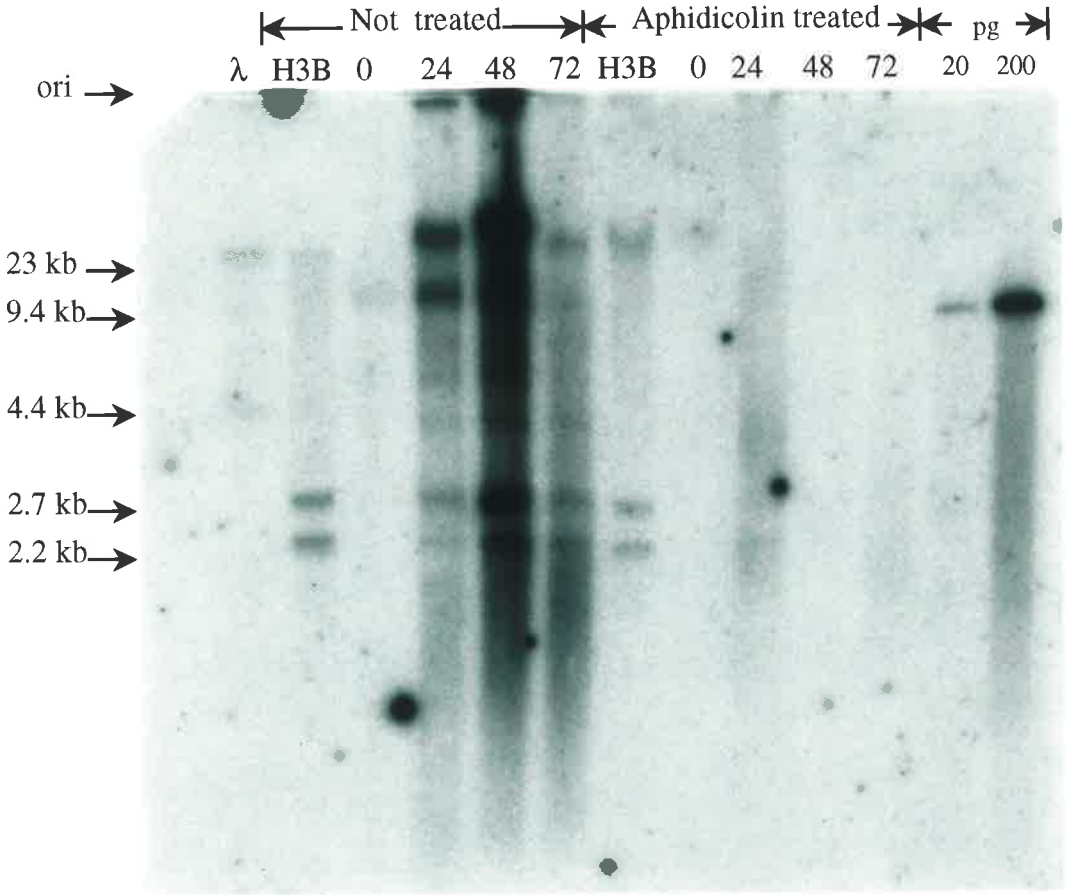
Table 5.5

Amino acid sequences of Vpr (amino to carboxyl ends) from HIV HXB2 (GenBank Accession K03455, nt 5559 - 5795, 25Mar1997) and HIV pNL4-3 (GenBank Accession U26942, nt 4937 - 5227, 15Nov1995) adapted from Clustal W (1.5) multiple sequence alignment program. Bold letters within the alignment indicate different amino acids between the two sequences. Amino acids abbreviations are shown in Appendix I. (* Indicates termination codon and - indicates absence of corresponding amino acid)

HXB2	1	M E Q A P E D Q G P Q R E P **H** N E W T **T**
pNL4-3		M E Q A P E D Q G P Q R E P **Y** N E W T **-**
HXB2	21	L E L L E E L K **N** E A V R H F P R I W L
pNL4-3		L E L L E E L K **S** E A V R H F P R I W L
HXB2	41	H **G L** L G Q H I Y E T Y G D T W A G V E
pNL4-3		H **N -** L G R H I Y E T Y G D T W A G V E
HXB2	61	A I I R I L Q Q L **L** F I H F **Q N W V S T**
pNL4-3		A I I R I L Q Q L **P** F I H F **R - - I G C**
HXB2	81	*
pNL4-3		R H S R I G V T R Q R R A R N G A S R S *

Fig. 5.5i

Southern blot hybridization analysis of chromosomal DNA extracted at 0, 24, 48 and 72 h p.i. from co-culture mix of aphidicolin (20 μM) - treated H3B as well as Hut78 cells in cell-to-cell transmission infection and maintained in the presence of the drug during the incubation period. The chromosomal DNA were purified to remove unintegrated viral DNA as described in section 5.2, KpnI restricted and then Southern blotted. Each track contains chromosomal DNA extracted from the equivalent of 2 x 10^6 cells. The ^{32}P-labelled, near full-length HIV DNA, probe used was purified from pBH10 (see section 2.8.A).


Not treated
Aphidicolin treated
pg
λ H3B 0 24 48 72 H3B 0 24 48 72 20 200
ori
23 kb
9.4 kb
4.4 kb
2.7 kb
2.2 kb

Aph-treated cells provide further support that contaminating, unintegrated viral DNA has been removed from the chromosomal DNA fractions, despite the presence of significant levels of unintegrated viral linear DNA.

5.6 Unresolved Southern hybridization signals observed with viral DNA integration kinetic studies

The presence of the 3.8 kb KpnI restricted fragment in the purified chromosomal DNA (section 5.4) is unlikely to be due to contaminating unintegrated viral DNA for several reasons. (i) The extracted chromosomal DNA (Hirt pellet) was purified with extensive (reverse polarity) agarose gel electrophoresis (see section 5.2). The successful use of electrophoresis to remove unintegrated viral DNA has been reported previously with Rous sarcoma (Humphries *et al.*, 1981), HIV (Stevenson *et al.*, 1990) and visna (List & Haase, 1997) viral integration studies. (ii) The reconstruction experiments showed the absence of unintegrated viral DNA signals after electrophoresis (see section 5.3). (iii) Although large amounts of unintegrated linear viral DNA were present in the aphidicolin-treated co-culture mix of H3B and Hut78 cells (Li & Burrell, 1992), there were no detectable 3.8 kb KpnI restricted fragment in the Southern blot hybridization of chromosomal DNA from the drug-treated cells (see section 5.5).

The possibility the 3.8 kb band might be due to incomplete integration at the 5' end of each viral DNA strand was considered. The integration process may be interpreted schematically in Fig. 5.6i. *In vitro* studies with the murine leukaemia viral DNA showed that the 3' end of each viral DNA strand is integrated first (Fujiwara & Mizuuchi, 1988, Brown *et al.*, 1989). KpnI cleavage of chromosomal DNA containing partly integrated HIV DNA contains five possible DNA cleavage fragments (Fig. 5.6i). Three of these are the internal 2.7 kb, 2.2 kb and 0.3 kb viral DNA fragments (Fig. 5.6i (d)). The remaining two fragments are the possible structures formed by partially integrated viral DNA (3' HIV DNA ends joined to 5' ends of host DNA).

If the 5' end of each viral DNA strand were incomplete or damaged and consequently not able to integrate, this could lead to substantial numbers of partial integration structures. If the single-stranded joining regions in these structures (Fig. 5.6 a, b & c) were labile, for example to trace nuclease activity, the 3.8 kb terminal fragment would result. (Although star activities of some

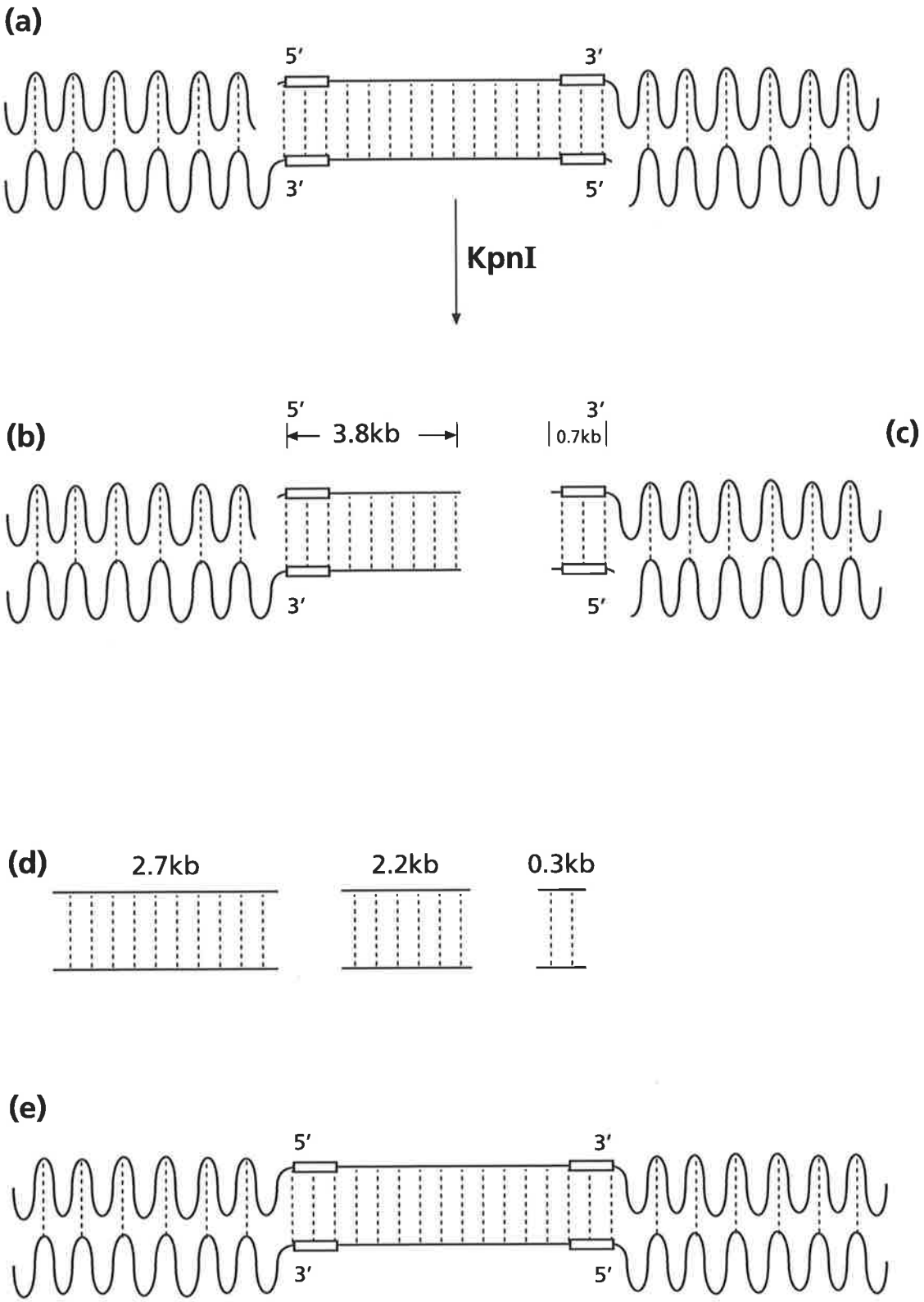
Fig. 5.6i

a Possible partially integrated HIV DNA structure showing the covalently joined 3' viral terminus, but with the 5' end not yet joined to host DNA (wavy lines).

b & c The putative partially integrated HIV DNA structure after KpnI restriction showing the 5' terminal (3.8 kb) and 3' terminal (0.7 kb) HIV DNA fragments (double strand).

d The KpnI restricted internal fragments of HIV DNA.

e The fully integrated form of HIV DNA in relation to chromosomal DNA.


(a)
5′
3′
3′
5′
KpnI
(b)
5′
3.8kb
3′
0.7kb
(c)
3′
5′
(d)
2.7kb
2.2kb
0.3kb
(e)
5′
3′
3′
5′

restriction enzymes may contribute to spurious and unexpected DNA fragments, restriction enzymes, throughout the work in this thesis, were used according to the manufacturer's recommendations and unlikely to yield star activities. Star activities of RE may yield spurious cleavage patterns and are usually caused by high (>5%) glycerol concentrations, incorrect salt or pH concentrations during enzymic reaction.) Alternatively, the formation of partial reverse transcripts (Zack *et al.*, 1992) can result in the inefficient completion of DNA synthesis which could lead to partial integration structures. However, Southern blot analysis with specific oligodeoxyribonucleotide probes which hybridize to the 5' or 3' termini of unintegrated viral DNA - extracted from a co-culture mix of H3B and Hut78 cells at 9 h p.i. - showed no difference in hybridization signals when compared with a reference strain of HIV plasmid DNA (pHXB2 - Kleen, see section 2.11) (data not shown).

Two other restriction enzymes (BamHI and PstI, which cleaves the HIV DNA at positions 8475 and 1419 respectively, see Fig. 5.6ii) used to treat similarly prepared and purified chromosomal DNA from the co-culture mix of H3B and Hut78 cells, yielded Southern blot hybridization results which paralleled those from KpnI restricted patterns. The BamHI and PstI restriction enyzmes each cleave the HIV DNA molecule at one position. Fig. 5.6iii shows the BamHI restricted chromosomal DNA with a discrete fragment at about 8.5 kb position as well as a smear of Southern signals extending to >23 kb in size. In addition, a discrete fragment of 1.2 kb in size is observed and a smear of Southern signals extending above this position. The latter signals may represent integrated HIV DNA (with chromosomal DNA) cleaved by BamHI. Fig. 5.6iv shows a similar experiment using PstI. Two discrete fragments - corresponding to positions *ca* 8.3 kb and 1.4 kb - are observed, together with a smear of Southern signals which likely represent randomly integrated HIV DNA cleaved with PstI enzyme. Thus, the Southern blot hybridization patterns observed with BamHI, KpnI or PstI restriction suggest the possibility of unintegrated 5' viral DNA ends (with the 3' viral DNA covalently joined to the chromosomal DNA), as well as fully integrated forms. This issue is unsolved and requires further investigation.

Fig. 5.6ii

BamHI (position 8475) and PstI (position 1419) restriction sites of HIV-1 DNA

Fig. 5.6iii

HIV DNA integration kinetics showing Southern blot analysis of chromosomal DNA extracted from co-culture mix of H3B and Hut78 cells in cell-to-cell transmission infection during the period 0 - 72 h p.i. Unintegrated viral DNA was removed from the chromosomal DNA as described in the text. The chromosomal DNA were digested with **BamHI** RE, which cleaves HIV DNA once at position 8475, and Southern blotted. This was then hybridized with a near full-length HIV DNA probe (see section 2.8.A). Hence, cleavage of H3B chromosomal DNA with BamHI would be expected to yield two fragments which also suggests the clonal origin of this cell. The amount of BamHI restricted chromosomal DNA loaded was equivalent to 2 x 10^6 cells/track. ori: origin of gel. λ: ^{32}P-labelled λ phage DNA/HindIII restricted (see section 2.8.B).

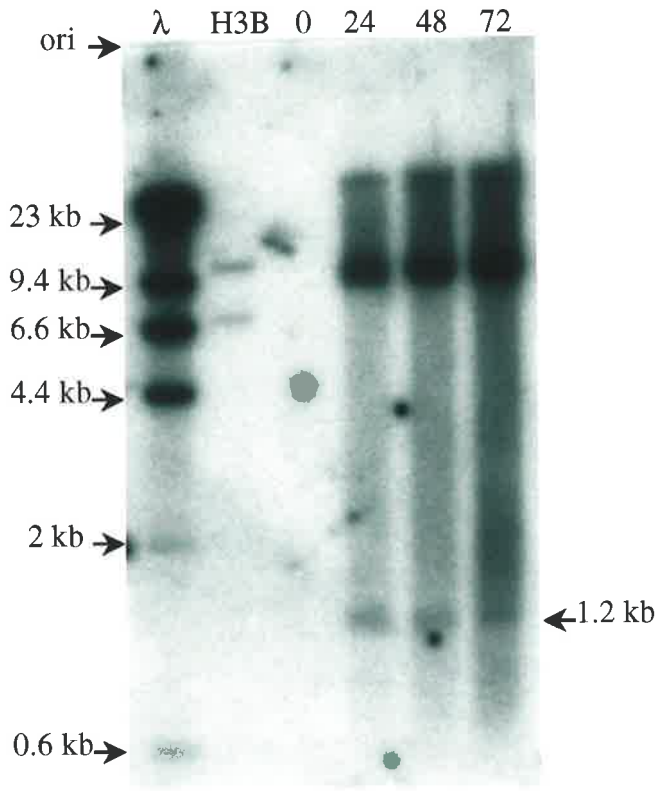
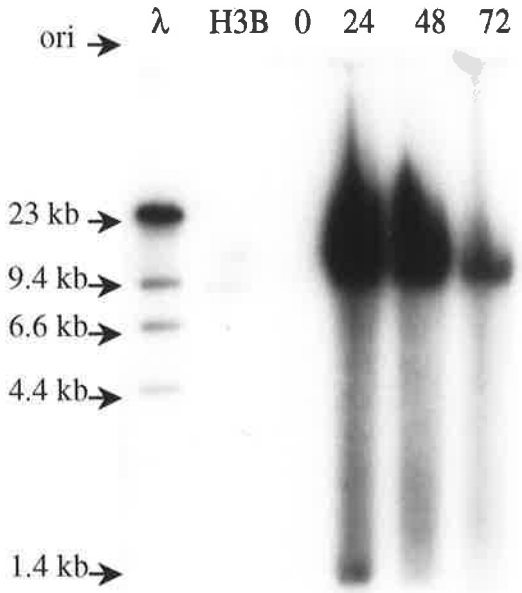

λ
H3B
0
24
48
72
ori
23 kb
9.4 kb
6.6 kb
4.4 kb
2 kb
0.6 kb
1.2 kb

Fig. 5.6iv

HIV DNA integration kinetics showing Southern blot hybridization analysis of chromosomal DNA extracted from co-culture mix of H3B and Hut78 cells in cell-to-cell transmission infection during the period 0 - 72 h p.i. Unintegrated viral DNA was removed from the chromosomal DNA as described in the text. The chromosomal DNA were digested with **PstI** RE, which cleaves HIV DNA once at position 1419, and Southern blotted. This was then hybridized with a near full-length HIV DNA probe (see section 2.8.A). Hence, cleavage of H3B chromosomal DNA with PstI would be expected to yield two fragments which also suggests the clonal origin of this cell. The amount of PstI restricted chromosomal DNA loaded was equivalent to 2 x 10^6 cells/track. ori: origin of gel. λ: ^{32}P-labelled λ phage DNA/HindIII restricted.


λ
H3B
0
24
48
72
ori
23 kb
9.4 kb
6.6 kb
4.4 kb
1.4 kb

5.7 Discussion

Although the patterns and kinetics of unintegrated HIV DNA and RNA have been characterized in both cell-to-cell transmission or cell-free viral infection routes (see sections 3.4 and references therein), the time course of HIV DNA integration within infected cells has been less studied. In contrast, the molecular events of retroviral DNA integration have been studied more extensively using *in vitro* assays (reviewed in (Brown, 1997)). More recently, the integration of another lentivirus - visna virus DNA was re-investigated and found to integrate, using Southern blot hybridization analysis (List & Haase, 1997). This was in contrast to an earlier report, from the same group of researchers, who showed that visna viral DNA did not integrate as judged by absence of Southern blot signals in chromosomal DNA which were subjected to electophoresis to remove unintegrated viral DNA (Harris *et al.*, 1984).

Using the cell-to-cell transmission infection of Hut78 target cells with viral donor H3B cells, we investigated the kinetics of HIV DNA integration by Southern blot hybridization. To reliably identify integrated DNA, it is critical that contaminating unintegrated viral DNA was removed. A number of different lines of evidence supported the successful removal of unintegrated viral DNA in this work (section 5.2, 5.3 & 5.6). In the cell-to-cell transmission infection used in these experiments, HIV DNA integration was first seen at 8 h p.i.(Fig. 5.4i), shortly after the initial production of unintegrated viral DNA at 4 h p.i. (see Fig.3.1.Ai, (Li & Burrell, 1992)). This is similar to the recent report of visna viral DNA integration detected between 4 - 16 h p.i. in a cell-free viral infection (List & Haase, 1997). Using the cell-free viral infection route, Rous sarcoma viral DNA was observed to integrate as early as 3h p.i. (Humphries *et al.*, 1981). During the analysis of HIV DNA integration in the cell-to-cell transmission infection, an incomplete integration structure was suspected from studies with KpnI cleavage of the chromosomal DNA. A similar incomplete integration structure, although not fully defined, was also suggested in the recent report on visna viral DNA integration; the latter was observed as a 1.8 kb BamHI terminal (5' end) fragment by Southern blot analysis (List & Haase, 1997).

Experiments in this thesis to characterize the suspected incomplete integration structure showed that it is unlikely that the 5' end of each viral DNA strand is damaged or incompletely synthesized as judged by Southern hybridization analysis of 5' termini of HIV DNA (obtained

from a co-culture mix of H3B and Hut78 cells at 9 h p.i.) with specific deoxyribonucleotides, compared to a reference strain of HIV plasmid DNA (pHXB2 - Kleen, see section 2.11) (data not shown). When fully integrated, the presence of this incomplete 5' terminal fragment would not be expected as it would be covalently joined to the 3' end of each chromosomal DNA strand. When cleaved with KpnI RE, the 5' terminal fragment of the proviral DNA would be expected to be longer than the corresponding (after similar RE digestion) terminal fragment of unintegrated viral DNA due to random RE sites on the flanking cellular sequence. Our results of the HIV DNA integration analysis showed that the terminal 3.8 kb KpnI terminal fragment was observed both as an intense discrete signal as well as being associated with a smear of higher molecular weight Southern signals. These observations suggest that at least a proportion of the HIV DNA molecules were integrated into the chromosomal DNA (see Fig. 5.4i). However, the intense and discrete Southern signal of the 3.8 kb fragment suggest that there are molecules which are not fully integrated at the 5' end of HIV DNA. The reports that linear retroviral DNA integrates first at the 3' end, prior to the 5' end (Fujiwara & Mizuuchi, 1988, Brown *et al.*, 1989) support the possible existence of the incomplete integration structure in our experiments. Recently, the report that there is a one hour lag period between the distinct steps of 3'-end and 5'-end joining to the host DNA (Roe *et al.*, 1997) provide further support for the possible presence of the incomplete integration structure (see Fig. 5.6i (a)). If the latter structure exists, a mechanism still needs to be identified to account for the discrete 3.8 kb KpnI viral DNA fragment in the extensively purified chromosomal DNA preparations.

Chapter 6

... much of the great future in biology lies in gaining a detailed understanding of the inner workings of the cell's many marvelous protein machines.
Bruce Alberts (1998), Cell 92: 291-294

Overview and General Discussion

6.1 Transmission of viral infection

The studies in this thesis are generally directed towards understanding the early events of HIV replication. The spread of virus, which initiates a new round of replication, can be mediated by either cell-free viral infection or by direct cell-to-cell transmission infection. The latter is a general mechanism used by many enveloped viruses viz. herpesviruses, paramyxoviruses, rhabdoviruses and retroviruses (see section 1.8.B). Retroviruses, in particular, seem to have exploited cell-to-cell transmission infection in many ways. For example, Human T-cell leukaemia virus type 1 (HTLV-1) is spread almost exclusively through direct cell-to-cell transmission from virus-producing cells rather than cell-free virus. This is demonstrated by extensive epidemiology studies showing that cells and blood, but not cell-free blood products (e.g. factor VIII) from HTLV-1 carriers are capable of spreading viral infection; and human contacts which involve the transfer of cells e.g. infant breast-feeding and sexual intercourse are the major routes of HTLV-1 transmission (Cann & Chen, 1996, Uchiyama, 1997). This indicates that HTLV-1 has evolved to successfully use direct cell-to-cell transmission infection for its survival. Porcine endogenous retrovirus (PERV) was discovered as a result of recent interest in using pig organs as potential donors for xenotransplantation. Cell-free PERV was shown to infect a cell line derived from human kidneys but not other human cells. When PERV-producing cells were co-cultured with human cells, a procedure which would allow direct cell-to-cell transmission of viral infection, the tropism of PERV was found to include human T & B cells, lung and muscle cells in addition to kidney cells (Patience *et al.*, 1997). This indicates that PERV may make use of direct cell-to-cell transmission to achieve a broader cell tropism.

HIV infection in patients may also be sustained by cell-to-cell spread of the virus, in particular within the lymphoid tissues (Embretson *et al.*, 1993, Pantaleo *et al.*, 1993). In addition, this route of spread of infection may allow the virus to bypass neutralizing antibodies which are

produced during HIV infection (Pilgrim *et al.*, 1997). HIV- infected cells from various sites of infection (e.g. dendritic cells) may travel to the lymphoid tissues followed by cell-to-cell spread of the virus (Cameron *et al.*, 1996, Spira & al., 1996) which facilitates viral dissemination (Pantaleo *et al.*, 1998). Cell-to-cell transmission infection has been estimated to be 10^2 - 10^3 fold more efficient than cell-free HIV infection (Dimitrov *et al.*, 1993). This was based on relating mathematical models on the rate of infection with quantitation of infectious virus per cell. In addition, observations from our laboratory with cell-to-cell transmission of infection show that this route of infection produces an earlier synchronous infection than cell-free viral infection (Li & Burrell, 1992, Kok *et al.*, 1993) (see (Sato *et al.*, 1992, Barbosa *et al.*, 1994)). The stable maintenance of HIV (as proviral DNA) within a cell would facilitate viral persistence and transmission of the virus might occur by cell-to-cell transmission of infection without virion formation in subsequent host generations.

In light of the possible significance of HIV cell-to-cell transmission of infection, a full study of the replication cycle of a virus should use models that mimic the two basic routes of infection *viz* cell-free and cell-to-cell transmission infections. In addition, an essential requirement in studying the replication cycle of a virus *in vitro* is to obtain one-step infection of the susceptible cells; in order to achieve this, a high multiplicity of infection is required. The cell-free viral infection route has the disadvantage in requiring large quantities of high titred viruses in order to obtain a one-step, synchronous infection. Such stocks of free virions can be difficult to achieve for technical reasons. Thus, use of a cell-to-cell transmission of infection system may facilitate a technical solution to achieve synchronous infection in addition to its biological significance.

In this thesis, the replication cycle of HIV was further studied using a cell-to-cell transmission infection model developed earlier in the laboratory (Li & Burrell, 1992). The H3B viral donor lymphoid cell line is a clone of H9 cells infected with HTLV-III_B. Each H3B cell contains two proviral DNA copies; however there is no detectable unintegrated HIV DNA by Southern blot hybridization. The target or recipient cells are a clone of Hut78 lymphoid line which is CD4+ (AIDS Reference and Reagent Program). Multi-nucleated giant (ballooning) cells are observed early after the co-culture of these two cell lines, presumably by interactions between gp120 and CD4 moieties on H3B and Hut78 cells respectively. Similar giant cell formations have also

been observed in the brain tissues of patients with AIDS (Teo *et al.*, 1997), in lung (Donaldson *et al.*, 1994) and adenoidal lymphoid tissues (Frankel *et al.*, 1996).

Co-culturing H3B cells and Hut78 cells yields a one-step, synchronous infection in which unintegrated HIV DNA is observed within 4h p.i.and increases to a maximum by 16 - 24h p.i. (see section 3.1.A). As H3B cells contain no detectable unintegrated HIV DNA, the detectable viral DNA must arise from a new round of infection after *de novo* reverse transcription (Li *et al.*, 1994). Other studies with cell-to-cell transmission infection system have reported similar kinetics from production of unintegrated HIV DNA (Sato *et al.*, 1992, Barbosa *et al.*, 1994). Using this cell-to-cell transmission infection model, the kinetics and pattern of HIV RNA synthesis and viral DNA integration were investigated in this thesis.

6.2 HIV RNA expression following cell-to-cell transmission of infection

The expression of HIV RNA includes a complex pattern of splicing events which produce three main classes of polyadenylated transcripts viz. the genomic length, unspliced 9 kb RNA, the 4 kb singly-spliced mRNAs and the multi-spliced 2 kb mRNAs. The control of HIV transcription is regulated by viral proteins (eg. Tat & Rev) (see sections 1.4.C.i & 1.5.C.i) as well as cellular factors (e.g. RNA polymerase II, cytokines and transcription binding factors) (see section 1.6.E). HIV transcription begins at the 5' LTR of the viral DNA where the promoter, TATAA box, the *cis*-acting sequence (TAR) for Tat transactivation and cellular transcription factor binding sites are located. The complex splicing events are in turn regulated by the Rev transactivator which translocates unspliced and singly-spliced viral RNA from the nucleus to the cytoplasm.

In the above cell-to-cell transmission system, two distinct phases of HIV RNA synthesis were observed. The first phase comprised the early induction (4 - 12 h p.i.) of 9 kb genomic length RNA; this was followed by a second and dramatic increase in all three classes of HIV transcripts at 24 - 72 h p.i.(see Chapter 3). These observations demonstrated temporal regulation of HIV gene expression. Essentially, similar results were obtained with cell-free viral infection. In contrast, using the cell-free viral infection route, Kim *et al.* showed that the earliest species of HIV RNA observed were the 2 kb multi-spliced regulatory transcripts at 16 h

p.i., followed by the 4 kb envelope transcripts and the unspliced 9 kb genomic RNA (Kim *et al.*, 1989b). In both systems unintegrated viral DNA was produced at 4h p.i. It is believed that the lack of detection by Kim *et al.* of the first phase of replication may relate to use of a lower m.o.i. as they reported only 10 - 20% of cells being infected.

In the cell-to-cell transmission infection model used, there are potentially three transcriptional templates for HIV RNA synthesis viz. newly made viral DNA, newly made and newly integrated provirus, and proviral DNA from H3B viral donor cells. The template for the first phase of HIV RNA synthesis was likely to be the newly-made, unintegrated viral DNA and this was shown by studies with inhibitors of reverse transcription (AZT, DDI or PFA; (Kok *et al.*, 1993), also see Chapter 3). Further experiments showed that this early phase of HIV RNA synthesis (see Chapter 4) was not inhibited when actinomycin D was used to irreversibly inhibit transcription from the integrated proviral DNA in viral donor cells (Kok *et al.*, 1998). The production of early phase 9 kb RNA was also not affected in growth-arrested cells, in which it was shown that integration of viral DNA did not occur (section 5.5). These results together confirmed that newly made, unintegrated viral DNA was the template for early phase 9 kb RNA transcription.

Further evidence for this proposal was obtained from 'AZT-chase' studies in which the drug was added to the co-culture mix of cells at various periods p.i. Viral RNA and DNA were extracted from these treated cells and analysed by Northern and Southern blot hybridizations respectively, which showed the decrease in the level of viral DNA (due to inhibition of reverse transcription by AZT) corresponded to the reduction in the level of viral RNA. Thus, it is proposed that the first phase of HIV RNA induction in the cell-to-cell transmission infection model uses unintegrated viral DNA as transcriptional template. Albeit minor, this pathway is yet to be fully characterized and its contribution in the overall life cycle of HIV remain to be defined.

Based on the above, an intracellular pathway, early in HIV replication, of viral DNA and RNA synthesis is proposed as shown in Fig. 6.2i. At the time of infection by cell-to-cell transmission, there is a small amount of genomic RNA in the form of a *ca.*180S complex that

Fig. 6.2i.

A proposed depiction of the concurrent synthesis of viral DNA and RNA following cell-to-cell transmission of HIV infection.

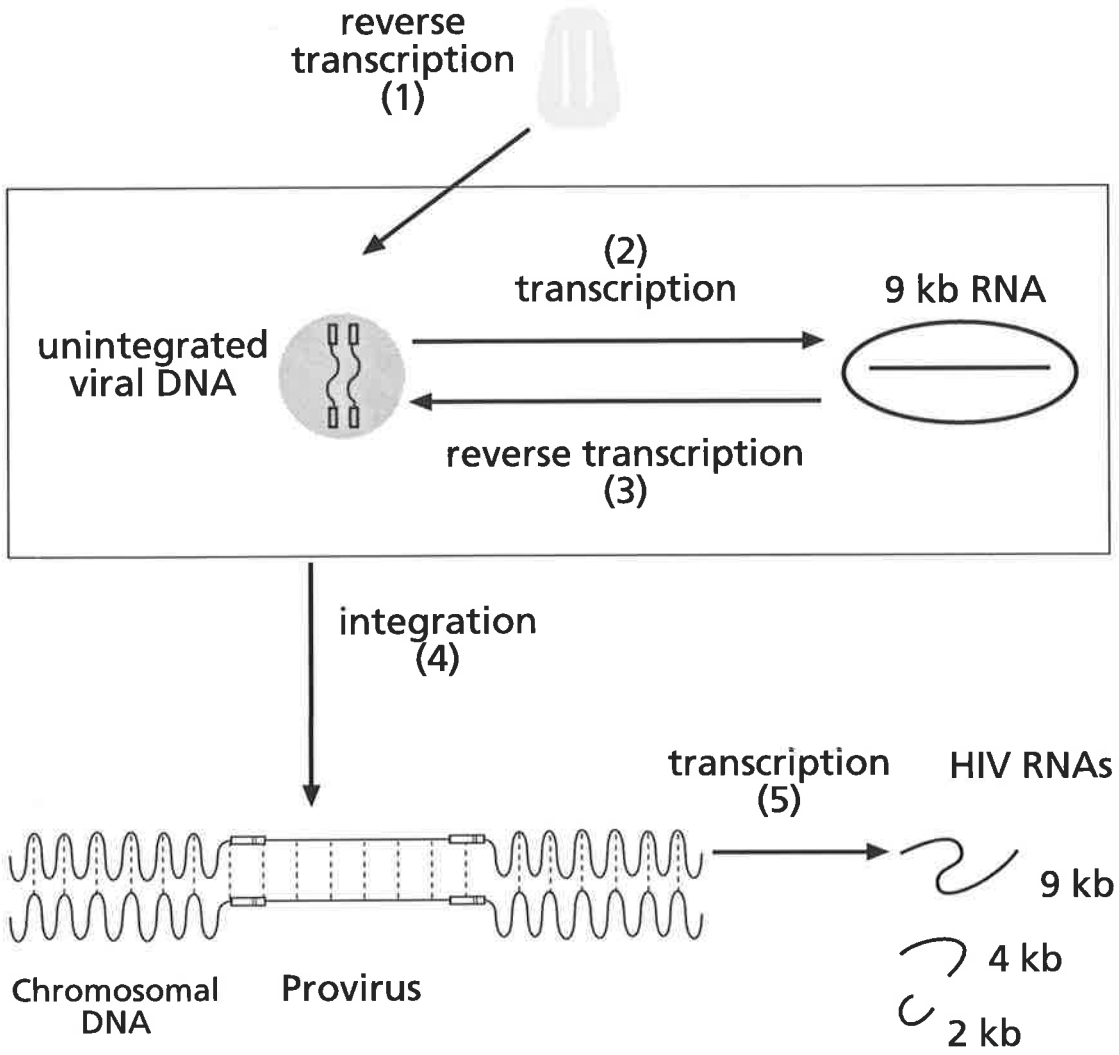
1 Upon infection of a susceptible cell, the viral genomic RNA in association with fully cleaved, mature reverse transcriptase and possibly other proteins (Li et al., 1994) from the donor cell is reverse transcribed into double-stranded DNA as part of the HIV early replication complex.

2 The newly synthesized unintegrated viral DNA, in the cytoplasm and/or the nucleus, provides the template for the first phase of viral RNA synthesis.

3 The product of the first phase RNA induction (9 kb viral RNA) is in turn reverse transcribed into more unintegrated viral DNA.

4 Concurrently, the viral DNA as part of the replication complex is integrated into the cellular DNA as the provirus.

5 The proviral DNA provides a more efficient template for the second phase of viral RNA synthesis.


reverse
transcription
(1)
(2)
transcription
9 kb RNA
unintegrated
viral DNA
reverse transcription
(3)
integration
(4)
transcription
(5)
HIV RNAs
9 kb
4 kb
2 kb
Chromosomal
DNA
Provirus

also contains fully processed viral reverse transcriptase and possibly other proteins from the persistently infected donor cells. This RNA is reverse transcribed into linear unintegrated viral DNA (Li *et al.*, 1994, Karageorgos *et al.*, 1995) which then, in association with the early HIV replication complex structure (Karageorgos *et al.*, 1993), acts as template for the first phase of viral 9 kb RNA synthesis. The newly induced viral RNA molecules may in turn be reverse transcribed into more DNA. While current models for reverse transcription predict that no more than two DNA molecules could be produced from a dimeric HIV genomic RNA (Varmus & Brown, 1989, Coffin, 1990, Li *et al.*, 1993b, Jones *et al.*, 1994), DNA as transcriptional template is capable of producing multiple copies of RNA. Quantitation of the viral nucleic acids produced during the early phase (0-12 h p.i.) of cell-cell transmission showed that on average one copy of viral DNA yielded only *ca.* two copies of viral RNA (see Table 4.7) (Kok *et al.*, 1998). This indicates that the pathway involving RNA expression from unintegrated DNA is a minor one (Fig. 6.2i)

These observations may be compared with another persistent viral infection. HIV shares two common features with Hepatitis B virus *viz* both produce persistent infections and use reverse transcription in their replication cycles. Persistence of infection and reliable transmission to new hosts are both facilitated if a virus can evade the host immune response and adopts a particular viral replication strategy which does not damage the host. With retroviruses, persistent infection is assisted by the integration of the reverse transcribed DNA in the host genome. In contrast, hepatitis B viral DNA, which is not known to code for an integrase and rarely integrates in the host, persists as a covalently closed circular form that is transcriptionally active only in the nucleus (Tuttleman *et al.*, 1986, Nassal & Schaller, 1993, Monjardino, 1998). In this regard, it may be envisaged that the concurrent synthesis of HIV RNA and unintegrated DNA observed during the first phase of viral RNA induction is a potential, albeit minor, contribution to viral pathogenesis.

6.3 HIV DNA integration following cell-to-cell transmission of infection

The molecular events of retroviral DNA integration using *in vitro* models have been extensively studied (see review by (Brown, 1997)); however there have been limited integration studies with cellular models. Earlier integration kinetic studies in relation to cellular events have been

reported with Rous sarcoma virus (Humphries *et al.*, 1981) and a recent study with visna virus (List & Haase, 1997). Following reverse transcription of the genomic RNA, integration of the viral DNA in the host genome is one of the crucial events in the replication cycle of retroviruses. *In vitro* studies have shown that the linear viral DNA is the precursor to the integrated proviral DNA and that the integrase is necessary and sufficient for this reaction (see section 5.1)

To study the time course of HIV DNA integration, extensive gel electrophoresis was used to remove unintegrated viral DNA from the chromosomal DNA (Hirt pellet) harvested at different times after mixing of H3B viral donor and Hut78 recipient cells (see Chapter 5). Integration began at 8 h p.i. as detected by Southern blot analysis of purified, KpnI restricted, chromosomal DNA. This closely follows the production of significant amounts of unintegrated viral DNA at 4 -8 h p.i. (see section 3.1.A). Integration of HIV DNA was observed to reach a maximum level by 24 h p.i. as judged by Southern hybridization signals within the time point, and was essentially complete by 72 h p.i. when the pattern of integration was less complex and the total signals were lower in the latter time point. In cells treated with aphidicolin to arrest cell cycling, no viral DNA integration was detected. The latter observation was consistent with the observation that the HIV strain used (HIV HXB2) contains a premature termination codon towards the 3' end of the viral *vpr* sequence (see section 5.5). The carboxyl end of the Vpr protein has been shown to be associated with the nuclear localization signal necessary for translocation of the pre-integration complex into interphase nuclei (Fouchier *et al.*, 1998, Nie *et al.*, 1998, Zhou *et al.*, 1998). This lack of a functional Vpr would be predicted to reduce the virus capacity to replicate in non-dividing cells.

6.4 Further studies

The two distinct phases of HIV RNA synthesis observed in the cell-to-cell transmission infection model may be further investigated to identify the exact roles of unintegrated viral DNA as potential transcriptional template (Kok *et al.*, 1993, Cara & Reitz, 1997, Kok *et al.*, 1998). Integrase mutants of HIV proviral clones have been constructed which are defective in proviral DNA formation and therefore newly-made viral DNA would not be able to integrate as this enzyme is both necessary and sufficient for viral DNA integration (Katz *et al.*, 1990, Goodarzi *et al.*, 1995, Wiskerchen & Muesing, 1995, Leavitt *et al.*, 1996). The latter approach would

obviate the need for use of aphidicolin (see section 5.5) to prevent integration of newly-made viral DNA into the host chromosomes. The mechanisms of integration blockage in growth-arrested cells may need further studies. Alternatively, the use of HIV integrase inhibitors, for example dicaffeoylquinic acid (McDougall *et al.*, 1998), cobalamin (Weinberg *et al.*, 1998), anthraquinone (Farnet *et al.*, 1996) or plant proteins (Lee-Huang *et al.*, 1995) could be used to prevent viral DNA integration. This would allow the role of unintegrated viral DNA as transcriptional template to be further analysed.

The studies in this thesis showed that during the early phase of viral replication (0-12h) following cell-to-cell transmission infection, reverse transcription and HIV RNA synthesis occur concurrently. HIV RNA synthesis is dependent on newly synthesized DNA as template while the role played by newly synthesized RNA in further reverse transcription remains to be clarified. Other issues requiring further work are the location of where the early phase viral 9 kb RNA is made, what possible enzymes and regulatory mechanisms are involved with transcription from unintegrated viral DNA template and relation with reverse transcription. Further characterization of the pre-integration complex (Karageorgos *et al.*, 1993), nuclear import (Gallay *et al.*, 1996, Whittaker & Helenius, 1998), incomplete integration structure (section 5.6) and studies of chromosomal structures (Farnet & Bushman, 1997, Miller *et al.*, 1997) during viral DNA integration would contribute to a better understanding of integration processes. Unintegrated viral DNA are initially made in the cytoplasm which also contains thousands of copies of mitochondria per cell. Studies of possible HIV DNA integration and viral RNA expression with these active cellular organelles have been limited; although it has been shown that HIV RNA levels are significantly higher in mitochondria than in cytoplasm or nucleus (Somasundaran *et al.*, 1994). The implications of the latter report with mitochondria and possible relation to viral replication/pathogenesis may produce interesting investigations. Further studies of the early events, for example viral entry, reverse transcription, integration and transcription during cell-to-cell transmission of infection would facilitate the design of antiviral therapy and vaccine production.

Appendix I

Amino acids symbols and codons

A	Ala	Alanine	GCA	GCC	GCG	GCU		
C	Cys	Cysteine	UGC	UGU				
D	Asp	Aspartic acid	GAC	GAU				
E	Glu	Glutamic acid	GAA	GAG				
F	Phe	Phenylalanine	UUC	UUU				
G	Gly	Glycine	GGA	GGA	GGG	GGU		
H	His	Histidine	CAC	CAU				
I	Ile	Isoleucine	AUA	AUC	AUU			
K	Lys	Lysine	AAA	AAG				
L	Leu	Leucine	UUA	UUG	CUA	CUC	CUG	CUU
M	Met	Methionine	AUG					
N	Asn	Asparagine	AAC	AAU				
P	Prol	Proline	CCA	CCC	CCG	CCU		
Q	Gln	Glutamine	CAA	CAG				
R	Arg	Arginine	AGA	AGG	CGA	CGC	CGG	CGU
S	Ser	Serine	AGC	AGU	UCA	UCC	UCG	UCU
T	Thr	Threonine	ACA	ACC	ACG	ACU		
V	Val	Valine	GUA	GUC	GUG	GUU		
W	Trp	Tryptophan	UGG					
Y	Tyr	Tyrosine	UAC	UAU				

Adapted from (Alberts *et al.*, 1989).

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