STUDIES ON THE EARLY EVENTS OF HUMAN IMMUNODEFICIENCY VIRUS REPLICATION

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ABSTRACT

Using a one-step cell-to-cell transmission infection model, the early events of the human immunodeficiency virus (HIV) replication cycle were investigated. Replication complexes, structures containing newly synthesized unintegrated viral DNA, were characterized using sucrose gradient sedimentation, immunoprecipitation and polymerase chain reaction (PCR). It was found that unintegrated HIV DNA in the cytoplasm of infected cells sedimented as part of a discrete complex with a sedimentation coefficient of approximately 320S. PCR detection of the DNA component of immunoprecipitated HIV replication complexes showed that the cytoplasmic complexes were associated with viral integrase, reverse transcriptase, protease, the matrix protein (p17), vpr and histones. In contrast, nuclear complexes were found to be smaller (80S) and associated only with integrase, protease and histones. Virus donor cells persistently infected with HIV were found to contain reverse transcriptase activity, part of which was associated with full-length HIV RNA in a particulate structure (180S), which may represent the complex from which the 320S cytoplasmic replication complex originates.

The kinetics of HIV reverse transcription following cell-to-cell HIV infection were investigated, using PCR quantitation of reverse transcription intermediates that had completed distinct step(s) during reverse transcription. An initial lag period of 1.5hrs was identified before the minus strand strong-stop viral DNA was first detected. The post-transfer and newly extended minus strand viral DNA was detected 2hrs post infection, whereas both the plus-strand strong-stop DNA and the fully extended minus-strand DNA were detected at 2.5hrs post infection. These data suggest that once the reverse transcription is initiated the HIV reverse transcriptase synthesizes minus-strand DNA at a rate of 250-300 bases per minute, or 4-5 bases per second, in infected cells; and that both the first template transfer and the initiation of the plus strand DNA synthesis may impose a temporal arrest to the overall reverse transcription process. The second template transfer was found to be more efficient than the first one and apparently did not impose temporal arrest on the reverse transcription process, implicating different mechanisms for the two template switches during reverse transcription.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution.

To the best of my knowledge and belief , this thesis contains no material previously published or written by another person , except where due reference is made in the text of the thesis.

In accordance with the University of Adelaide regulations, I give my consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

LITSA EVLAMBIA KARAGEORGOS

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ABBREVIATIONS

bp	base pair
CPE	cytopathic effect
Da	dalton
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
dNTP	deoxynucleotide triphosphate
dsDNA	double stranded DNA
DDW	deionized distilled water
DNA	deoxyribonucleic acid
DNAse I	deoxyribonuclease I
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
eg	for example
FBS	foetal bovine serum
g	gravity force
gp	glycoprotein
gm	gram(s)
HIV	human immunodeficiency virus
hr	hour
hrs	hours
kb	kilobase
kDa	kilodalton
Mab	monoclonal antibody
min(s)	minute(s)
ml	mililitre(s)
mRNA	messenger RNA
MW	molecular weight
nm	nanometer
nt	nucleotide
p	polypeptide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcriptase
S	Svelberg unit

SDS	sodium dodecyl sulphate (also called sodium lauryl sulphate)
sec	second
SSC	standard saline citrate; 150mM NaCl, 15mM Na citrate, pH7.1
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
tRNA	transfer ribonucleic acid
TAE	40mM Tris-HCl, 1mM EDTA, pH 8.0
TBE	50mM Tris-HCl pH 8.0, 50mM boric acid, 1mM EDTA
TCA	trichloro acetic acid
tRNA	transfer ribonucleic acid
U	unit
UV	ultraviolet

PUBLICATIONS ARISING

Publications:

Karageorgos, L., Li, P. and Burrell, C.J. (1993) Characterization of HIV replication complexes early after cell-to-cell infection. *AIDS Res. Hum. Retroviruses* **9**: 817-823.

Li, P., Stephenson, A.J., Brennan, P.A., Karageorgos, L., Kok, T., Kuiper, L.J., Swift, J. and Burrell, C.J. (1994) Initiation of reverse transcription during cell-to-cell transmission of HIV infection uses pre-existing reverse transcriptase. (in press) *J. Gen. Virol.*

Karageorgos, L., Li, P. and Burrell, C.J. (1994) Stepwise analysis of reverse transcription in a cell-to-cell HIV infection model: kinetics and implications. (submitted for publication) *Virology*

Poster/Paper Presentations:

Poster at the 1991 ASM:- " Characterization of early Human Immunodeficiency Viral (Type 1) Intermediates." L. E. Karageorgos , P. Li and C. J. Burrell

Paper / Talk and Poster at the 1992 Australian Society of HIV Medicine Conference in Sydney:- "Characterization of HIV Replication Intermediates in Acutely Infected Cells." L. E. Karageorgos , P. Li and C. J. Burrell

Poster at the 1992 Annual Meeting of Laboratory of Tumor Cell Biology , NIH. Bethesda, Maryland, U.S.A. "HIV Replicative Intermediates in Acutely Infected Cells." L. Karageorgos, P. Li & C.J. Burrell

Paper / Talk at the 1993 Australian Society of HIV Medicine Conference in Melbourne "Reverse Transcription Factories in HIV Cell-to-Cell Infection." L. E. Karageorgos , P. Li and C. J. Burrell

Poster at the 1993 First National Conference on Human Retroviruses and Related Infections , Washington D.C. " Reverse Transcription Factories in HIV Cell-to-Cell Infection." L. E. Karageorgos , P. Li and C. J. Burrell

CHAPTER 1

INTRODUCTION

1.0 Preface

Due to the rapid progress of research into the replication cycle of the human immunodeficiency virus type 1, much of the work presented in this thesis has already been published (see Publications Arising). These publications are cited below in the relevant sections.

This introductory chapter will be divided into several parts: a general background of the human immunodeficiency virus will be discussed first, followed by an overview of the replication cycle of HIV. Molecular events involved in the replication of the human immunodeficiency virus will be discussed in detail later in the chapter.

1.1 THE HUMAN IMMUNODEFICIENCY VIRUS

1.1.1 The History of AIDS

Human immunodeficiency virus (HIV) is the aetiologic agent responsible for the fatal disease of acquired immunodeficiency syndrome (AIDS). The most prominent feature of AIDS is a severe immune suppression, as a result of, among other possible mechanisms, the functional impairment and depletion of CD4+ T-helper cells. The pronounced depression of cellular immunity results in the infected individuals contracting opportunistic infections and malignancies (Fauci, 1988). In addition, neurological symptoms and dementia may develop in some patients (Price *et al.*, 1988). HIV-1 is spread by sexual contact, exposure to infected blood or blood products, and perinatal transmission from mother to child (Curran *et al.*, 1988).

First described in 1981, AIDS is believed to be the result of a new infection of humans that began in central Africa. HIV most probably originated from an animal virus among monkey species, in particular chimpanzees, and that may have crossed into humans once or a number of times in central Africa. From Africa , HIV spread to Haiti among Haitian guestworkers returning from Zaire in the 1970's , and then to the US, the Caribbean, and South America, particularly among homosexual men and Hispanic intravenous drug users. In a short time HIV then moved from the US to Europe and Australasia via homosexual men and IDUs (Gallo, 1987; Piot *et al.*, 1988; Crofts, 1992).

The disease was first observed in homosexual males but spread rapidly into other groups , including intravenous drug users and recipients of contaminated blood or blood products. The pattern of disease transmission observed for AIDS strongly suggested that the causative agent was a blood-borne viral pathogen. In 1983 a novel retrovirus was isolated from the lymphocytes of AIDS patients that has since become known as human immunodeficiency virus type 1 (HIV-1) (Barre-Sinoussi *et al.*, 1983; Popovic *et al.*, 1984).

The virus was initially named lymphadenopathy-associated virus (LAV) by Montagnier's French group (Barre-Sinoussi *et al.*, 1983), human T-lymphotropic virus type III (HTLV-III) by Gallo's group (Popovic *et al.*, 1984), and AIDS-related virus (ARV) by Levy and coworkers (Levy *et al.*, 1984). In 1986 a unifying nomenclature for immunodeficiency viruses was proposed and the virus was renamed human immunodeficiency virus, abbreviated HIV. In 1986, a second and less virulent human immunodeficiency virus was identified in West Africa which also causes AIDS but cross-reacts serologically to a limited extent only with HIV in screening tests (Clavel *et al.*, 1986; Clavel *et al.*, 1987). It therefore was designated HIV-2, and the first was subsequently renamed HIV-1. In the rest of this thesis, all descriptions and discussions of 'HIV' refer to HIV-1 only.

1.1.2 Classification as a Retrovirus

1.1.2.1 Retroviruses

HIV is a member of the lentivirus subfamily of retroviruses. This classification is based on genome organization as well as pathogenic features of the virus and virion morphology. Retroviruses are RNA containing viruses that replicate through a double-stranded DNA intermediate by virtue of a viral coded RNA-dependent DNA polymerase (reverse transcriptase). The family of retroviridae is divided into three subfamilies : (1) Oncovirinae are tumour-inducing agents, and include all oncogenic retroviruses. (2) Lentivirinae are agents that cause slow , non-neoplastic diseases and include Visna-Maedi Virus, Caprine Arthritis Encephalitis Virus (CAEV), Equine Infectious Anaemia Virus (EIAV), and immunodeficiency viruses of humans, simians, felines and probably other species. (3) Spumavirinae consist of the "foamy" viruses that induce persistent infections without any clinical disease but cause vacuolization of cells in culture.

1.1.2.2 Simple and Complex Retroviruses

Retroviruses have customarily been divided into three subgroups, (see section 1.1.2.1) on the basis of the pattern of disease and nucleic acid sequence homology. This classification, however, failed to reflect increased knowledge and understanding of the retrovirus replication cycle. Hence, an alternative classification based on viral genomic organization and gene regulation was introduced (Cullen, 1991). This classification divides retroviruses into simple and complex classes. Complex viruses include all members of the lentivirus and spumavirus subfamilies as well as the HTLV-I-related viruses, while the simple classification includes all other members of the previous oncovirus subfamily.

Complex retroviruses encode at least five distinct gene products. These are the products of *gag*, *pol*, and *env* genes, also seen in the simple retroviruses, as well as two regulatory proteins (eg. Tat and Rev in HIV, see section 1.2.4.2). Simple

retroviruses lack the regulatory proteins. Complex retroviruses are defined not only by the complexity of their genomic organization but, more specifically, by the particular pattern of viral gene regulation that depends on the action of two regulatory proteins, for example Tat and Rev, in the case of HIV-1. These proteins regulate the ordered expression of the proviral genome.

Gene expression of the complex retroviruses (eg. HIV-1), is distinguished from that of the simple retroviruses (eg. MLV) by several criteria: (i) Complex retroviruses encode a third, multiply spliced class of viral transcripts, encoding two nuclear regulatory proteins, in addition to the singly spliced (*env*) and unspliced (*gag-pol*) mRNAs seen in the simple retroviruses. (ii) The first of these regulatory proteins is a sequence-specific *trans* activator of LTR-driven gene expression. (iii) The second regulatory protein acts to repress the extent of viral mRNA splicing, leading to reduced levels of multi-spliced RNAs and increased levels of unspliced RNAs. (iv) The combined action of these two proteins divides gene expression of complex retroviruses into two temporal phases, a pattern not seen in the simple retroviruses.

1.1.3 Structural Organization of HIV

1.1.3.1 The Virion Structure of HIV

Like other lentiviruses, the mature HIV virion contains a ribonucleoprotein core particle surrounded by an outer protein shell. The overall diameter of the HIV virion is approximately 110nm, containing a ribonucleoprotein core of nearly 100nm in length and 50nm tapering to 40nm in width (Arnold and Arnold, 1991). This club-shaped inner core consists of the capsid proteins (CA, p24) and nucleocapsid proteins (NC, p7p9) which are associated with two copies of the plus-strand HIV genomic RNA (Figure 1.1). The viral RNA genome is transcribed from the provirus, by the host DNA-dependent RNA polymerase II and hence has the post-translational modifications found in cellular mRNAs (5' cap, 3' polyA tract) (Coffin, 1982). The two copies of this HIV

RNA genome are associated, presumably by base-pairing near their 5' ends, in a 70S complex. Also associated with the HIV RNA within the core are the viral enzymes reverse transcriptase, integrase and protease. This ribonucleoprotein assembly is surrounded by an icosahedral outer protein shell consisting of the HIV matrix protein (MA), p17 (Gelderblom *et al.*, 1987; Marx *et al.*, 1988). This outer protein shell is, in turn, surrounded by a lipid bilayer envelope stubbed with knoblike protrusions consisting of the outer surface glycoprotein, gp120, in association with the hydrophobic transmembrane-protein, gp41 (Figure 1.1). The HIV lipid bilayer is also studded with various host proteins, including Class I and Class II major histocompatibility antigens, acquired during virion budding.

1.1.3.2 Genome Structure of HIV

At the genome organization level , all retroviruses encode three distinct virus-encoded genes, consisting of the *gag* , *pol* and *env* genes (Varmus and Brown, 1989) (Figure 1.2). The *gag* gene encodes the virion structural core proteins, while *pol* encodes enzymes required for reverse transcription and integration. A viral protease, required for the post-translational processing of the precursor Gag and Gag-Pol ploy proteins, may be encoded in *pol* or may be part of *gag*. The *env* gene encodes the viral envelope glycoproteins. These three genes are generally arranged in the same order (5'-gag-pol-env-3').

Important features of all retroviral RNA are the short direct repeats at each end (usually 20-80 nucleotides long) called R. The non-coding region next to R is designated U5 at the 5' end and U3 at the 3' end of the viral RNA. The arrangement of the viral RNA is hence R-U5--genes--U3-R. Reverse transcription generates a double-stranded DNA copy of the RNA genome that is slightly larger than the genomic viral RNA. This increase in size is due to the duplication of the U3 and U5 regions at the ends of the viral DNA during reverse transcription (refer to section 1.3 and Chapter

Figure 1.1 Model of the structure of the HIV virion, showing relative locations of the components based on our current knowledge of the structure.

MA=p17= matrix protein

CA=p24= capsid protein

NC=p7/p9= nucleocapsid protein

PR=p11= protease

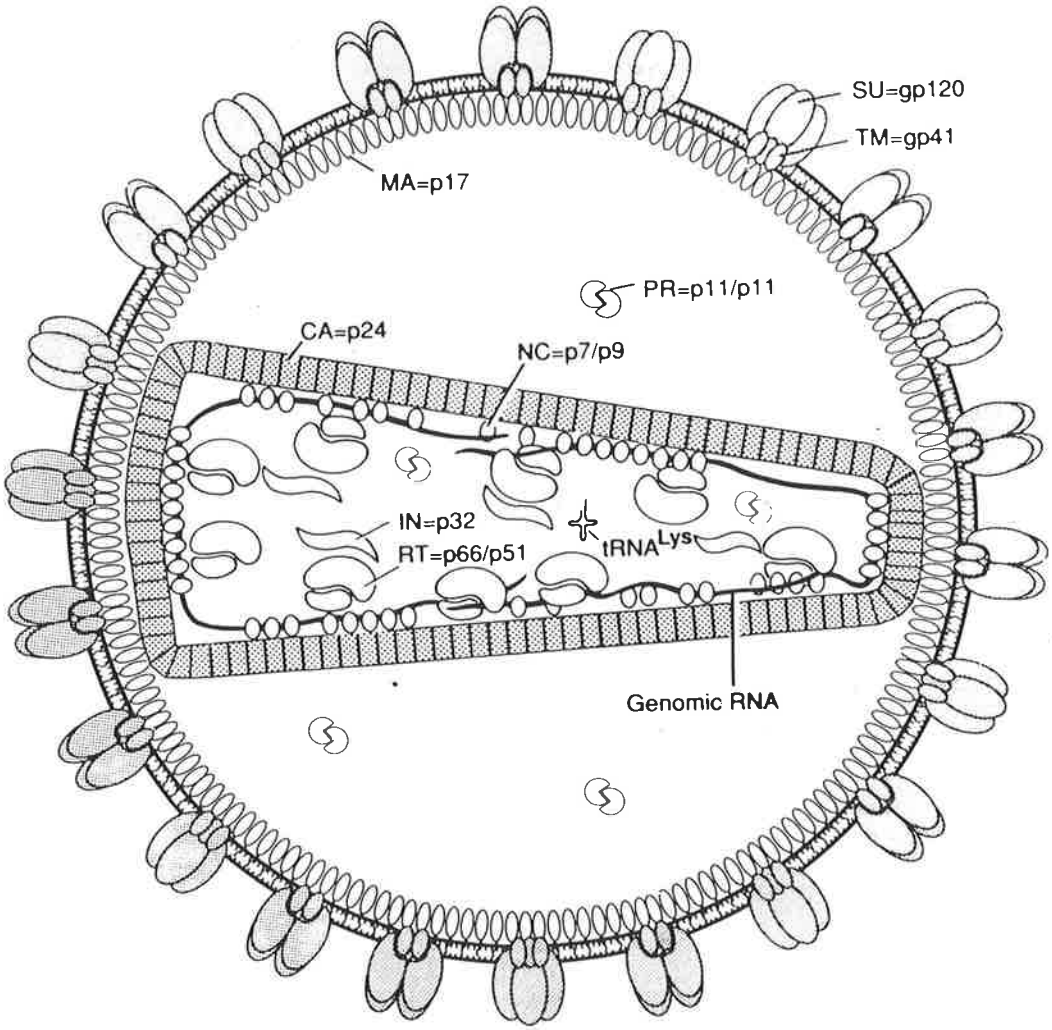
RT=p66/p51= reverse transcriptase

IN=p32= integrase

SU=gp120= envelope protein (surface glycoprotein)

TM=gp41= transmembrane protein

Adapted from Arnold, E. and Arnold, G.F. (1991) *Adv Vir Res* **39**:1.



=MA=p17
=CA=p24
=NC=p7/p9
=PR=p11/p11
=RT=p66/p51

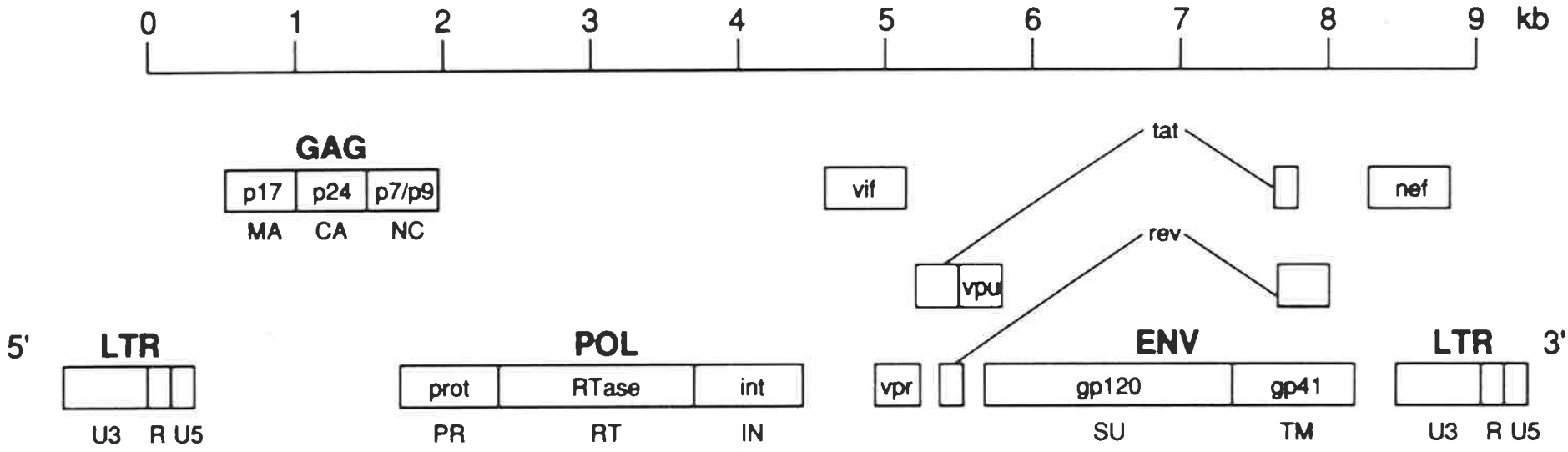
=IN=p32
=SU=gp120
=TM=gp 41
=tRNA Lys

Figure 1.2 Schematic layout of the HIV-1 genome.

The names of the mature protein products are indicated, as are the standardized two-letter abbreviations for the virion proteins (Leis *et al.*, 1988).

Adapted from Arnold, E. and Arnold, G.F. (1991) *Adv Vir Res* **39**:1.

HIV-1 Genome


0
1
2
3
4
5
6
7
8
9
kb
GAG
p17
p24
p7/p9
MA
CA
NC
tat
vif
nef
rev
vpu
5'
LTR
U3
R U5
POL
prot
RTase
int
PR
RT
IN
vpr
ENV
gp120
gp41
SU
TM
LTR
3'
U3
R U5

4 for further details). This duplication generates the long terminal repeats (LTRs) found at each end of the viral DNA. The final arrangement of the linear unintegrated DNA and integrated provirus is therefore U3-R-U5---genes---U3-R-U5 (Figure 1.2), with the LTRs composed of three regions U3, R and U5. The U3 and R regions of the LTR contain promoter and enhancer elements required for efficient transcription by cellular RNA polymerase II. The *cis*-acting regulatory signals found in the LTRs specify the start site for RNA transcription at the beginning of R and modulate the amounts of HIV RNA synthesized. Sequences important for efficient mRNA polyadenylation are also contained within the 3' LTR.

Just downstream of the U5 region at the 5' end of the viral RNA is a region known as the primer binding site (PBS). This region is complementary to the specific tRNA involved in the initiation of reverse transcription. Also involved in reverse transcription is a purine rich sequence, known as the polypurine tract (PPT), that primes the synthesis of the plus-strand of DNA. This sequence is found at the 5' end of the 3' LTR for all retroviruses and also in the centre of the genomes of lentiviruses, including HIV (refer to section 1.3 for more details on reverse transcription).

In the case of HIV, *gag* codes for p17 (matrix protein , MA), the core proteins p24 (capsid protein , CA), and p7/9 (nucleocapsid , NC) , which are cleaved from a p55 precursor protein by the viral protease. The *pol* gene encodes for three viral enzymes: protease (PR , p11), RNA-dependent DNA polymerase (reverse transcriptase , p51/66) and integrase (IN , p31), which are cleaved from a precursor polyprotein p160, also by the viral protease (Figure 1.2). The envelope proteins are encoded by the *env* gene which is initially expressed as a precursor glycoprotein gp160 and subsequently cleaved, probably by a cellular protease, into gp120 (or SU, surface) and gp41 (or TM, transmembrane) glycoproteins. gp120 is the outer envelope protein which forms the knobs on the virus surface. gp41 is the transmembrane envelope protein by which gp120 is anchored via a non-covalent bond, to the viral membrane. gp120 binds to the

CD4 receptors of T helper lymphocytes and other cells and mediates adsorption of HIV to the cell surface.

HIV is a complex retrovirus, encoding for at least six other genes, in addition to the structural genes *gag*, *pol* and *env* (Figure 1.2; Cullen and Greene, 1990). These additional genes encode three regulatory proteins (Tat (trans-activator) and Rev (regulator of virion protein), that are essential for viral replication, and Nef (negative regulatory factor), shown to be required for *in vivo* replication and pathogenicity), two proteins believed to be involved in virus maturation and release, (Vif (virion infectivity factor) and Vpu (viral protein U)), and Vpr (viral protein R), believed to be involved in the early stages of HIV infection. The function of all these proteins is discussed in more detail in section 1.2.4.

1.1.4 HIV Infection *in vivo*

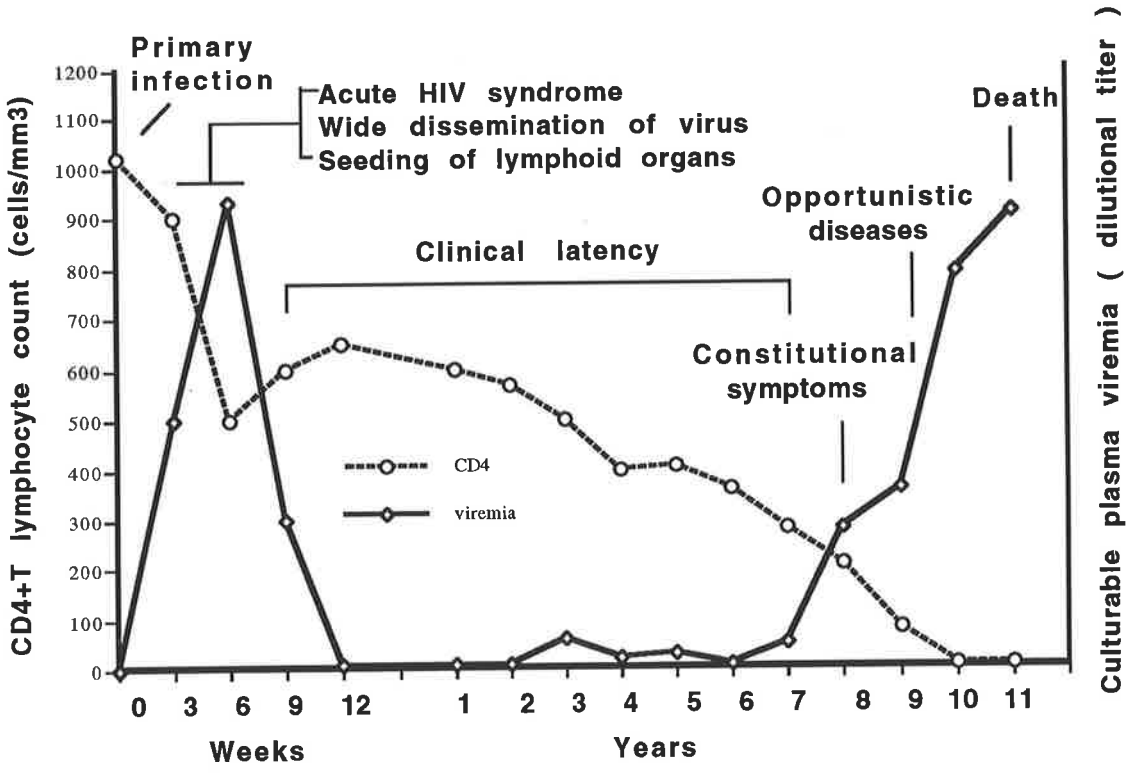
The development of AIDS is the result of a chronic progressive infection with HIV (Fauci and moderator, 1991; Pantaleo *et al.*, 1993). Although the course of HIV infection may vary widely among HIV-positive individuals, a common pattern of development has been recognized (Figure 1.3). Primary infection with HIV is followed by an acute phase of virus growth, viraemia, then the development of strong cellular and humoral immune responses to the virus. A prolonged period (average of 10 years) of clinical latency follows, during which the patient is usually asymptomatic. Finally clinical disease develops, sometimes intially with intermittent episodes, and progression to death eventually takes place (Fauci and moderator, 1991).

During the early viraemic period of HIV infection, high levels of HIV replication markers are observed; viral p24 antigen can be easily detected and virus can be readily isolated from the blood (Clark *et al.*, 1991; Daar *et al.*, 1991). This initial high level of HIV *in vivo* is associated with a sharp and selective depletion of CD4-bearing helper

Figure 1.3 Typical course of HIV infection.

During the early period after infection there is widespread dissemination of virus and a sharp decrease in the number of CD4+ T cells in peripheral blood. An immune response to HIV ensues, with a decrease in detectable viraemia followed by a prolonged period of clinical latency. The CD4+ T-cell count decreases during the following years, until it reaches a critical level below which there is a substantial risk of opportunistic diseases.

Adapted from Fauci, A.S. (1991) *Ann. Intern. Med.* **114**: 678.



A.S. Fauci Science 1993 262: 1011

T-lymphocytes, that may be related to a direct cytopathic effect, indirect mechanisms including immune-mediated cell lysis and possibly apoptosis. A second consequence of the initial infection is the infection and seeding of the lymphoid organs with HIV , providing a continuing site of persistent viral replication (Pantaleo *et al.*, 1991; Embretson *et al.*, 1993; Pantaleo *et al.*, 1993). During the stage of clinical latency, the number of CD4+ cells rebounds, remaining within the normal range, or slowly decreases over time. Levels of infectious virus in the blood are also markedly lower during this stage, but still detectable in most patients, from time to time. It has been demonstrated that significant replication persists in lymph node follicles (Embretson *et al.*, 1993; Pantaleo *et al.*, 1993). Within the lymph nodes, infection may spread by cell-to-cell transfer, rather than by cell-free virus; such situations high-light the need for the further understanding of the cell-to-cell transmission of HIV infection (discussed further in section 1.7).

Progression to clinically apparent disease is characterized by a dramatic reduction of CD4+ lymphocytes and the development of opportunistic infection, AIDS encephalopathy, or characteristic malignancies such as Kaposi's Sarcoma (KS). A marked increase in levels of infectious virus in the blood is also usually associated with the development of AIDS (Coombs *et al.*, 1989; Ho *et al.*, 1989). Phenotypic differences have been detected in HIV strains isolated at diffent stages of HIV infection. HIV isolates from late-stage infection replicate in CD4+ T cells better than in monocytes/macrophages, in contrast to early stage isolates (Cheng-Mayer *et al.*, 1988; Fenyö *et al.*, 1989; Tersmette *et al.*, 1989; Miedema *et al.*, 1990). In addition, these late-stage isolates replicate more rapidly and to a higher level in culture (rapid-high), and ~50% of these late isolates lead to the formation of syncytia in culture (SI, or syncytium-inducing isolates) (Fenyö *et al.*, 1988; Tersmette *et al.*, 1988; Tersmette *et al.*, 1989). In contrast, virus isolated during the asymptomatic phase is more often non-syncytium inducing (NSI) (Tersmette *et al.*, 1989). This change in viral phenotype may be responsible for the higher levels of HIV replication observed late in disease.

1.1.5 How does HIV cause Immune Deficiency ?

Although our knowledge and understanding of HIV has greatly increased in the past few years, the issue of how the virus actually destroys the immune system, is still unclear and controversial. A major feature of AIDS is the reduction in the number of circulating CD4+ T-lymphocytes; however, the mechanism by which it does this is uncertain. The simplest explanation for loss of CD4 cells is direct killing by HIV via lysis or syncytia formation, as a result of the emergence of virulent cytopathic HIV variants over time. Such direct killing is believed insufficient to explain, in full, the dramatic decrease in CD4 cells observed in AIDS, although massive covert infection of CD4+ cells in the lymphoid organs has recently been recognized. The existence of indirect mechanisms for damage to the immune system have been proposed: (i) the HIV envelope protein gp120 itself could nonspecifically activate lymphocytes, rendering the cells sensitive to apoptosis (Gougeon and Montagnier, 1993); (ii) antigen-presenting cells (macrophages and dendritic cells) produce aberrant cytokine signals, causing changes in T cell responses ; (iii) replenishment of mature T cells from precursor cells may be impaired directly or indirectly as a result of infection; and (iv) other infections activate HIV by stimulating the lymphocytes that harbour the proviruses (all reviewed in Weiss, 1993). Almost all these models involve unbalanced immune activation as a prelude to immune collapse. The proposal of so many varied models illustrates the complexity of HIV infection and the lack of consensus on how the virus causes AIDS.

However, regardless of which mechanism HIV causes AIDS, the key requirement for each model, is the replication of HIV. Thus, in this thesis, the aim is to investigate and further understand the molecular events involved in the early stages of HIV infection.

1.2 Replication Cycle of HIV

1.2.1 Replication Cycle Overview

Shortly after entering the cytoplasm of a host cell, the single-stranded RNA genome is converted into a double-stranded DNA copy by the viral enzyme reverse transcriptase. This double-stranded DNA molecule then migrates into the nucleus where it is integrated into the host chromosome. Once integrated, the provirus can be transcribed into RNA which can be used either as mRNA for the synthesis of viral proteins or as genomic RNA for progeny viruses (refer to Figure 1.4).

1.2.2 Viral Attachment , Fusion and Entry

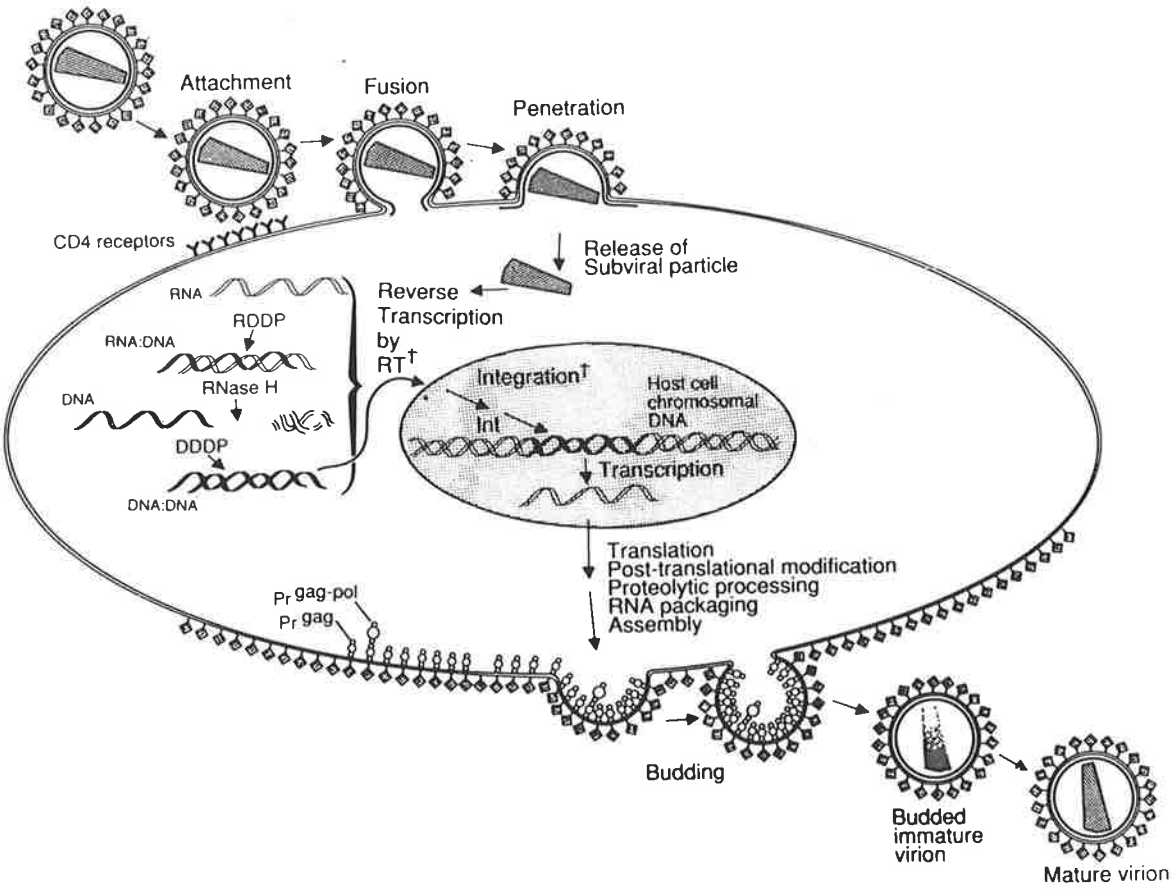
The first step in the virus replication cycle is the binding of the virion to the surface of a target cell. The major cellular receptor for HIV is CD4. CD4 was first postulated to be the receptor for HIV after early studies showed that (i) antibodies to CD4 blocked viral infection and virus-mediated cell fusion (syncytium formation) (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; McDougal *et al.*, 1985), and (ii) the viral envelope glycoprotein , gp120 , remained bound to CD4 after solubilization of cell-bound viral particles (McDougal *et al.*, 1986; Lasky *et al.*, 1987). Confirmatory evidence for the receptor function of CD4 was obtained from transfection studies in which human cells lacking CD4 (CD4- cells) , which are normally resistant to HIV infection , became susceptible when transfected with a cDNA encoding CD4 gene (Maddon *et al.*, 1986).

CD4 is a surface glycoprotein found on a subset of T-lymphocytes as well as on monocytes-macrophages and other cell types. The HIV envelope glycoprotein, gp120, binds to CD4 as indicated by immunoprecipitation studies where gp120 and CD4 were coprecipitated by antibodies directed against either of the proteins (McDougal *et al.*, 1986). A region near the carboxy terminus of gp120, spanning amino acids 397-439, has been shown to be required for binding to CD4 by studying viruses with different mutations introduced into this region (Kowalski *et al.*, 1987; Lasky *et al.*, 1987). The deletion of 12

Figure 1.4 Simplified diagram of the HIV replication cycle.

After atttachment of virus particles to the CD4 receptor, the virus enters the host cell and the outer lipid envelope of the virus is removed. The single-stranded virion RNA is converted into a double-stranded DNA molecule by the viral enzyme reverse transcriptase. This double-stranded DNA molecule migrates into the cytoplasm where it is integrated into the host chromosome. Once integrated it can be transcribed into mRNA which can be used either for the production of viral proteins or as genomic RNA for progeny virions.

Adapted from Arnold, E. and Arnold, G.F. (1991) *Adv Vir Res* **39**:1.


Attachment
Fusion
Penetration
CD4 receptors
Release of
Subviral particle
RNA
RDDP
RNA:DNA
RNase H
DNA
DDDP
DNA:DNA
Reverse
Transcription
by
RT†
Integration†
Int
Host cell
chromosomal
DNA
Transcription
Translation
Post-translational modification
Proteolytic processing
RNA packaging
Assembly
Pr gag-pol
Pr gag
Budding
Budded
immature
virion
Mature virion

amino acids, as well as a single amino acid substitution in this region, resulted in a complete or substantial loss of binding to CD4 (Lasky *et al.*, 1987). There are four recognized variable domains in the extracellular regions of CD4, denoted V1-V4. The binding site for gp120 has been mapped to a portion of the first variable (V1) domain (amino acid residues 16-84) (Clayton *et al.*, 1988; Jameson *et al.*, 1988; Landau *et al.*, 1988; Peterson and Seed, 1988). Additionally, amino acid residues of the second variable domain (V2) may contribute to gp120-binding (Clayton *et al.*, 1988; Landau *et al.*, 1988). After receptor binding has occurred, the transmembrane envelope glycoprotein, gp41, induces fusion between the viral envelope and host cell membranes (Gallaher, 1987; Kowalski *et al.*, 1987). A stretch of hydrophobic amino acids, at the amino terminal of gp41, is believed to be involved in viral membrane fusion (White *et al.*, 1983; Kowalski *et al.*, 1987; Felser *et al.*, 1989).

The sequence of gp120 in different isolates has revealed an extraordinary degree of variability (up to 30%) in amino acid sequence (Capon and Ward, 1991). This variability is highly localized to five hypervariable regions. Within the third hypervariable (V3) region of gp120, a cluster of amino acid residues flanked by cysteine residues constitutes a major immunodominant epitope (Modrow *et al.*, 1987) and constitutes the major neutralizing domain of HIV. Antibodies to V3 and mutations in this region block syncytium formation and neutralize viral infectivity, but do not affect CD4 binding (Jacks *et al.*, 1987; Skinner *et al.*, 1988; Freed and Risser, 1991; Page *et al.*, 1992). In addition, amino acid substitutions in the V3 domain resulted in moderate to marked decreases in virus infectivity and fusion activity (Freed *et al.*, 1991; Page *et al.*, 1992). Their findings suggest that the V3 domain is involved in mediating fusion but not attachment to CD4.

The entry of HIV following CD4 binding was initially presumed to occur by a pH-dependent mechanism involving internalization of the virus by receptor-mediated endocytosis into acidic endocytic vesicles (Maddon *et al.*, 1986). However, later studies

with agents that block viral entry via endocytotic pathways suggested that HIV enters cells via a pH-independent process, involving the direct fusion of the viral envelope with the cell membrane (Stein *et al.*, 1987; McClure *et al.*, 1988). In addition, cells that contain mutations in the cytoplasmic domain of the CD4 cDNA that block internalization of the CD4 molecules, remain susceptible to HIV infection, and in the same study it was shown that exposure of CD4+ cells to HIV does not result in endocytosis of the CD4 molecule (Maddon *et al.*, 1988). These studies indicated that although CD4 undergoes endocytosis, this pathway is not required for HIV infection. Thus HIV entry proceeds mainly via direct fusion of the viral envelope with the cell membrane.

After entry into the host cell, the virion is assumed to undergo uncoating. Little was known about this uncoating process at the commencement of this study; however it was assumed that uncoating resulted in the loss of the envelope proteins and the virion membrane, and lead to the formation of a viral 'nucleoprotein complex' that was competent to initiate the process of reverse transcription (see section 1.5 , and Chapters 3 & 5).

Recently it has been suggested that the CD4 molecule is essential for binding HIV particles, but is not sufficient for efficient viral entry and infection (Callebaut *et al.*, 1993). A further cofactor was identified as dipeptidyl peptidase IV (DPP IV), a T cell activation antigen, also known as CD26. This molecule has been hypothesized to act as a serine protease, possibly cleaving the V3 region of the gp120 envelope glycoprotein of HIV. Coexpression of human CD4 and CD26 in murine cells rendered them permissive to infection. However, recent experimental approaches by a number of investigators, based on the report by Callebaut *et al.*, were unable to confirm the finding of Callebaut *et al.* and concluded that CD26 does not confer fusion competence to CD4-expressing cells (Alizon and Dragic, 1994; Broder *et al.*, 1994; Camerini *et al.*, 1994; Patience *et al.*, 1994).

Besides CD4-positive T-lymphocytes, a second important target cell population for HIV infection is that of monocytes and macrophages (Gartner *et al.*, 1986; Popovic and Gartner, 1987; Embretson *et al.*, 1993; Collin and Gordon, 1994). These cells express low levels of CD4 on their surface. Monocytes may act as a reservoir of virus and may disseminate virus to various organs in the body (Vaishnav and Wong-Staal, 1991). HIV may also infect a wide range of CD4- cells including the Langerhans cells of the skin and dendritic cells (Niedecken *et al.*, 1987); cells in the brain such as microglial cells, astrocytes, and endothelial cells (Cheng-Mayer *et al.*, 1987); B lymphocytes, cells of the intestinal mucosa (Nelson *et al.*, 1988); and cells of the cervical endothelium (Pomerantz *et al.*, 1988) . In this case of infection of CD4- cells, receptors other than CD4 might be involved.

1.2.3 Synthesis and Integration of Proviral DNA

Like all retroviruses, after HIV has entered the cytoplasm of a susceptible cell, the single-stranded HIV RNA is reverse transcribed into linear double-stranded DNA by the viral RNA/DNA-dependent DNA polymerase. Reverse transcriptase is primed by $tRNA_3^{Lys}$ molecules and involves the synthesis of minus-strand DNA using the incoming viral RNA as template, digestion of the original RNA genome when it is in an RNA:DNA heteropolymer duplex, and synthesis of the plus-strand DNA using synthesized minus-strand DNA as template (the process of reverse transcription is discussed in detail in section 1.3). The exact coordination of these events is not clear, but it appears that the entire synthesis of unintegrated viral DNA is carried out in a nucleoprotein complex (section 1.4.1).

This unintegrated viral DNA, presumably still associated with the nucleoprotein complex, then migrates into the nucleus where it integrates into the host genome. How the nucleoprotein complex precisely moves from the cytoplasm to the nucleus is unclear. It has been shown that the HIV-1 nucleoprotein complex is transported from

the cytoplasm to the nucleus of the host cell by an active transport process which requires ATP but which is independent of cell division (Bukrinsky *et al.*, 1992). Isolated nucleoprotein complexes can mediate the integration of viral DNA into heterologous DNA targets *in vitro*, suggesting that these complexes may contain all of the functions necessary for viral integration (Brown *et al.*, 1987; Ellison *et al.*, 1990; Farnet and Haseltine, 1990). The structural and enzymatic functions involved in the integration of the viral DNA are not yet fully understood. It is known that the integration process does depend on specific sequences at the ends of the viral DNA molecule and on the viral protein integrase brought into the cell by the virion (Farnet and Haseltine, 1991) (integration is discussed further in section 1.4.2). In a lytic infection, the integrated viral DNA, or provirus, is then transcribed into full-length RNA by the host cell RNA polymerase II, to be used either as mRNA for the production of viral proteins or as genomic RNA for progeny virions. Alternatively, HIV-1 may establish a latent or persistent form of infection where the viral genetic information (provirus) remains latent until there are proper signals for viral mRNA synthesis (refer to section 1.5). In addition, once integrated, the provirus is considered as a stable genetic element of the host genome which can be inherited by daughter cells through mitosis.

At the beginning of this study, little was known about the reverse transcription-replication complexes (or "preintegration complexes") and the reverse transcription process of HIV. Both are the major topics of the experimental part of this thesis.

1.2.4 Expression of Viral Genes

1.2.4.1 Control of Expression

For simple retroviruses, the expression of integrated provirus follows the same general rules of eukaryotic gene expression and is governed exclusively by cellular proteins (Coffin, 1990). However, like other complex retroviruses, HIV transcription is also controlled by viral regulatory proteins. Transcription is a complex process involving

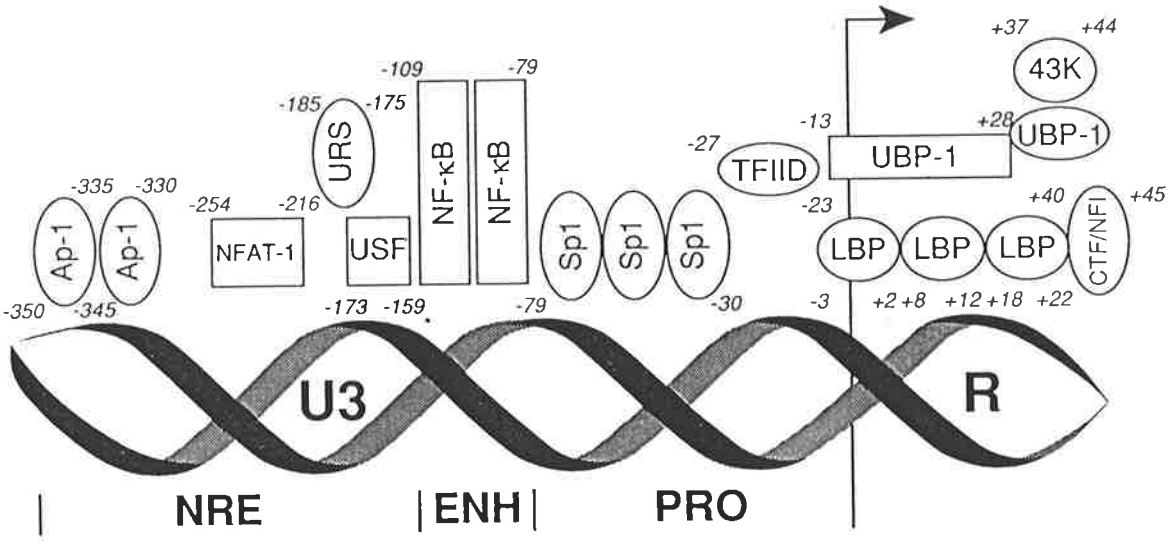
interplay between *cis*-acting regulatory sequences present in the viral LTR and *trans*-acting cellular transcription factors as well as viral regulatory proteins.

The most important control region of the HIV proviral genome is the LTR. The HIV LTR contains complicated enhancer / promoter sequences with multiple control elements. Host transcription factors act upon the integrated HIV provirus, mainly within the HIV LTR promoter element. A number of sequences important for the binding of cellular factors involved in basal and inducible RNA transcription have been defined. These include the negative regulatory element (NRE) , the sites for NF-kappaB, Spl and TATA box. In addition to the corresponding proteins (NF-kappaB, Spl, TFIID) (Nabel and Baltimore, 1987; Harrich *et al.*, 1989; Lu *et al.*, 1989; Sharp and Marciniak, 1989), an enhancer-binding protein (EBP-1) (Wu *et al.*, 1988) and two cellular proteins binding downstream of the transcription start (CTF / NF1 , LBP-1) (Jones *et al.*, 1988; Wu *et al.*, 1988) have been identified (see Figure 1.5). The region of the NF-kappaB binding sites is referred to as the core enhancer. The viral regulation protein, Tat, transactivates viral transcription by binding to mRNAs via a sequence called the tat responsive element (TAR), which is located in the R region of the viral RNA. Rev is a sequence-specific RNA-binding protein, that binds within the RRE (Rev response element) which is located within the envelope region of the viral RNA.

Like other complex retroviruses, HIV-1 shares the property of encoding two classes of viral mRNAs that can be distinguished on the basis of their temporal expression (Cullen, 1991). An early class of viral transcripts consists of the multiple spliced, ~2kb mRNA species that encode the viral regulatory proteins Tat and Rev and a third protein termed Nef. The late class of viral mRNAs consists of the unspliced (~9.2kb) and singly spliced (~4.3kb) transcripts. The full-length, unspliced ~9.2kb RNA species can act as both new genomic RNA and *gag* or *gag-pol* mRNA, encoding virion structural proteins. The 4.3kb species encodes the envelope proteins and the auxiliary proteins Vif , Vpr , and Vpu (discussed further in section 1.3.4.3).

Figure 1.5 Organization of the HIV-1 long terminal repeat (LTR). Binding sites of various factors are shown along with the coordinates of recognition sequences. NRE=negative regulatory element; ENH=enhancer; PRO=promotor. The approximate boundaries of NRE, ENH, and PRO are depicted by vertical lines. The arrow indicates the site of transcription initiation.

Adapted from Vaishnav, Y.N. and Wong-Staal, F. (1991) *Annu. Rev. Biochem.* **60**:577.


-350
-345
-335
-330
Ap-1
Ap-1
-254
-216
NFAT-1
-185
-175
URS
-109
-79
NF-κB
NF-κB
-173
-159
USF
-79
-30
Sp1
Sp1
Sp1
-27
TFIID
-13
-23
UBP-1
+37
+44
43K
+28
UBP-1
+40
+45
CTF/NFI
LBP
LBP
LBP
-3
+2
+8
+12
+18
+22
U3
R
NRE
ENH
PRO

1.2.4.2 Early Gene Expression

Analyses of one-step HIV-1 growth curves revealed that the initial population of viral mRNA molecules reaching the cytoplasm consisted of fully-spliced, 2kb viral transcripts (Kim *et al.*, 1989a). These viral mRNAs encode the regulatory proteins Tat, Rev and Nef.

The first of these early gene products to exert its effects in regulating HIV gene expression is the Tat protein. The Tat (trans-activator of transcription) protein acts as a potent transactivator, influencing the expression of all viral genes (Arya *et al.*, 1985; Sodroski *et al.*, 1985). Tat binds to an RNA stem-loop structure termed the "transactivation response (TAR) element", located within the R region all HIV-1 mRNAs (Sharp and Marciniak, 1989; Pavlakis and Felber, 1990). The precise function of Tat is highly controversial, but several lines of evidence suggest that it acts primarily at the level of transcription initiation and/or elongation (Cullen, 1986; Laspia *et al.*, 1989). The binding of Tat to the TAR region of the HIV transcripts results in the dramatic increase of HIV-LTR directed gene expression, thereby establishing a strong positive feedback loop that can lead to very high levels of HIV-1 specific RNA and protein synthesis (Arya *et al.*, 1985; Sodroski *et al.*, 1985; Somasundaran and Robinson, 1988). In particular, this action of Tat results in the accumulation of a critical level of the Rev protein which, in turn, binds to the RRE of mRNAs, facilitating the transportation of the 4.3 kb and 9.2 kb transcripts, encoding the structural proteins, out of the nucleus (Felber *et al.*, 1989; Hanly *et al.*, 1989; Malim *et al.*, 1989b) and reducing the cytoplasmic levels of multiple spliced regulatory mRNAs. By regulating the transport of viral mRNAs out of the nucleus, Rev controls the relative balance between multiply spliced and unspliced classes of RNAs found in the cytoplasm (Chang and Sharp, 1989; Emerman *et al.*, 1989; Hadzopoulou-Cladaras *et al.*, 1989; Malim *et al.*, 1989a; Malim *et al.*, 1989b), and the production of structural proteins. This action of Rev divides HIV gene

expression into two temporal phases, an early phase, primarily producing the regulatory proteins, and a later phase, where the regulatory as well as structural proteins are produced (Cullen and Greene, 1989; Kim *et al.*, 1989a; Pavlakis and Felber, 1990). Therefore, Rev mediates the establishment of an equilibrium between viral structural and regulatory protein synthesis.

Another regulatory protein expressed early in the course of HIV infection is the Nef (negative factor) protein. The function of Nef protein has been controversial. Unlike Tat and Rev, the Nef gene product is not required for HIV replication in culture. At first, Nef appeared to be involved in the negative regulation of HIV gene expression and viral replication via the HIV-LTR (Luciw *et al.*, 1987; Ahmad and Venkatesan, 1988; Cheng-Mayer *et al.*, 1989). Later reports failed to confirm these negative effects on either progeny virus production or viral gene expression levels (Hammes *et al.*, 1989; Kim *et al.*, 1989b; Bachelerie *et al.*, 1990). Recent studies (Miller *et al.*, 1994; Spina, 1994) have demonstrated that Nef acts as a positive regulatory factor for viral replication in primary blood lymphocytes and monocytes / macrophages. It has also been shown that the Nef protein obtained from primary clinical isolates of HIV down-regulates surface CD4 expression in T cells (Anderson *et al.*, 1993; Mariani and Skowronski, 1993; Greenway *et al.*, 1994). Hence the precise role of the Nef gene product in the HIV replication cycle remains uncertain. In contrast to the uncertainty surrounding the role of Nef in tissue culture, it has been demonstrated with the simian immunodeficiency virus (SIV) that Nef expression appears necessary for the development of immunodeficiency (AIDS) and clinical pathogenesis (Daniel *et al.*, 1992). Deletions of *nef* gene sequences did not affect SIV replication in tissue culture (either positively or negatively), however Rhesus monkeys injected with a *nef*-deleted clone failed to develop AIDS and the nef$^-$ virus failed to replicate efficiently *in vivo* (Kestler III *et al.*, 1991). Animals injected with wild-type SIV all exhibited high levels of SIV replication and developed AIDS. Furthermore, it has recently been demonstrated that Rhesus monkeys vaccinated with live SIV deleted in *nef* were completely protected against challenge by intravenous inoculation of

live pathogenic SIV (Daniel *et al.*, 1992). In addition, recent studies in an alternative *in vivo* model involving SCID-hu mice (severe combined immunodeficient mice), demonstrated again that Nef was required for *in vivo* replication and pathogenicity (Jamieson *et al.*, 1994). In these studies, *nef* mutants of HIV-1 that were replication competent were shown to be attenuated for growth and cytopathicity *in vivo*.

1.2.4.3 Late Gene Expression

For the assembly of infectious HIV-1 virions, viral enzymes and viral structural proteins must be produced. The viral core proteins and viral enzymes are encoded by the unspliced *gag* and *gag-pol* mRNAs and viral envelope proteins are encoded by the singly spliced *env* mRNA. The *gag* gene codes for various structural components of the virus particle, including the major capsid protein (p24), the matrix protein (p17), and nucleocapsid proteins (p7 / p9). These Gag proteins are translated initially as a 55kDa precursor known as $Pr55^{gag}$, from the genome length viral mRNA. The *pol* gene codes for protease (p11), reverse transcriptase (p66/51) and integrase (p31). In common with other retroviruses, the *pol* gene is also translated from the genomic length viral mRNA to produce a polyprotein Pr160, by a novel ribosomal frame-shifting mechanism (Jacks *et al.*, 1987). The *gag* and *pol* genes overlap by 241 nucleotides and the *pol* gene is in -1 reading frame relative to the *gag* reading frame. The Pol proteins are produced when approximately 1 in every 40 *gag* translation products is extended into the *pol* region via a translational frameshift that allows read-through of entire *pol* gene, producing a 160kDa Gag-Pol precursor ($Pr160^{gag\text{-}pol}$) (Jacks *et al.*, 1987; Hatfield and Oroszlan, 1990). The *env* genes code for the exterior coat proteins. These coat proteins are synthesized as a gp160 precursor that is subsequently cleaved by a cellular protease to produce gp120 and gp41. In contrast, the Gag and Pol proteins are cleaved by the virally encoded protease (Kohl *et al.*, 1988).

In addition to Gag, Pol, and Env, HIV encodes three additional late gene products, Vpr, Vif and Vpu, which have been shown to affect the efficiency of viral spread. The first of these, Vpr, (viral protein R) is a 15-kDa protein (96 amino acids). Vpr is the only regulatory product of HIV packaged within the virion. Vpr accelerates the replication and cytopathic effect of HIV in CD4+ T-cells, with the most pronounced effect exerted early in infection (Ogawa *et al.*, 1989; Cohen *et al.*, 1990). The presence of Vpr protein in virions suggests that it may function at some early steps in the virus replication cycle, such as the formation and integration of provirus or the initial transcription from the provirus. Though Vpr lacks a classical nuclear localization signal (Hanover, 1992), the carboxyl-terminal portion of the protein is rich in basic amino acids. A truncation mutation, which removes the carboxyl-terminal 19 amino acids, was found to impair Vpr localization in the nucleus and it was also shown that attachment of the C-terminal 19 amino acid Vpr sequence onto β-galactosidase directs this protein to the nucleus (Lu *et al.*, 1993). Therefore it was proposed that the nuclear localization domain of Vpr may allow the targeting of the viral preintegration complex to the nucleus (Lu *et al.*, 1993). Vpr was one of the viral proteins found to be associated with full-length HIV DNA in the cytoplasmic replication complex as shown and discussed in Chapter 3.

The *vif* gene encodes a 23 kDa cytoplasmic protein designated as virion infectivity factor. Vif is reported to increase the infectivity of HIV particles as much as 100- to 1000-fold (Fisher *et al.*, 1987; Strebel *et al.*, 1987) and may also enhance cell-to-cell virus transmission (Fisher *et al.*, 1987; Sakai *et al.*, 1991). Vif is present in infected cells but is not associated with the mature virus particle (Arya and Gallo, 1986; Sodroski *et al.*, 1986; Fisher *et al.*, 1987; Sakai *et al.*, 1991) and therefore it is difficult to explain its profound effect on virus infectivity. Rev function is required for Vif expression (Garrett *et al.*, 1991; Schwartz *et al.*, 1991). It has been demonstrated that the Vif protein affects the late phase of the virus life cycle (Gabuzda *et al.*, 1992). These together with the finding that Vif enhances viral infectivity during virus production but does not affect

transcription, translation, or virus release (Sodroski *et al.*, 1986; Fisher *et al.*, 1987; Strebel *et al.*, 1987) suggest that Vif may be important during the processing of virion proteins, virion assembly, or virion maturation. It has also been proposed that Vif may play a role in the processing or conformational maturation of the HIV envelope glycoproteins (Guy *et al.*, 1991).

Vpu is an 81 amino acid amphipathic integral membrane protein with at least two different biological functions: (i) enhancement of virus particle release from the plasma membrane of HIV infected cells (Geraghty and Panganiban, 1993) and (ii) degradation of the virus receptor CD4 in the endoplasmic reticulum (Willey *et al.*, 1992; Chen *et al.*, 1993), resulting in enhanced intracellular transport and processing of gp160 (Willey *et al.*, 1992). The precise mode of action of Vpu is uncertain, since these two biological functions of Vpu seem to be independent (Schubert and Strebel, 1994), occurring at different sites within a cell.

1.2.5 Assembly and Release of Mature Virus

The late events in the HIV replication cycle involve the assembly of virus components, release of the immature virus particles from the cell via the process of budding and extracellular maturation of the virion. These late events are poorly understood. Retroviral assembly is a unique process in which two viral RNA molecules are packaged, and the products of *gag* and *pol* are incorporated into immature virions in the form of their polyprotein precursors during assembly and are proteolytically cleaved during or after budding to form the mature virus particles.

The process of assembly of HIV particles appears to follow the same pathway as that of type-C retroviruses. The interactions between the Gag proteins, the plasma membrane, and the virion RNA control the process. The process of assembly is initiated by the aggregation of Pr55gag and Pr160gag-pol molecules under the plasma

membrane mediated by the N-terminal part of the Gag protein. Both the Gag and Gag-Pol polyproteins of HIV bear an N-terminal myristic acid residue that is essential for virion assembly (Gottinger *et al.*, 1989).

The genomic RNA is brought to the site of assembly by the nucleocapsid domain of the Gag/Gag-Pol precursor. The HIV genome contains a specific packaging signal within the RNA leader region, between U5 and the *gag* gene, and recognition of this sequence again requires the integrity of a zinc-finger motif within the nucleocapsid protein of Gag (Lever *et al.*, 1989; Aldovini and Young, 1990; Gorelick *et al.*, 1990). The p24 capsid protein, also derived from Gag, forms a protein shell surrounding the nucleocapsid and therefore determines the tubular shape of the core (Gelderblom *et al.*, 1987). The cleavage of the Gag and Gag-Pol polyproteins is believed to occur concurrently with the morphological maturation of virions in which the core condenses to form an electron-dense cylindrical structure. Mutations in the HIV protease gene were shown to abolish proteolytic processing of capsid precursor Pr55gag (Kohl *et al.*, 1988; Gottinger *et al.*, 1989; Peng *et al.*, 1989). Electron microscopy has revealed that the assembly and budding steps were not affected in these mutants, but the virions produced resembled immature core particles, suggesting that the protease mediates a postbudding morphological maturation of the core structure (Gottinger *et al.*, 1989; Peng *et al.*, 1989). Moreover, the mutant particles were non-infectious (Kohl *et al.*, 1988; Peng *et al.*, 1989).

The envelope glycoproteins, synthesized initially as a precursor gp160, are incorporated into virions by a separate pathway. The precursor is cleaved intracellularly, and the products, gp120 and gp41, are inserted into the plasma membrane (Willey *et al.*, 1988). The association of immature nucleocapsids with regions of cell membrane containing envelope glycoproteins is probably mediated by the interaction between the p17 (MA) domain of Gag precursor and Env protein complex.

Therefore, in the assembly of HIV and other retroviruses, it is proposed that there are two different groups of molecules that have to transverse very different pathways within the cell. The *gag* gene products are translated on free polyribosomes in the cytoplasm, then transported through uncharacterized pathways directly to the inner side of the plasma membrane where they cause a membrane extrusion. The viral glycoproteins on the other hand, encoded by the *env* gene, are translated on membrane bound polysomes on the rough endoplasmic reticulum and then transported through the vesicular transport pathway of the cell, through the secretory pathway to the golgi and then onto the plasma membrane. A specific interaction between Gag and Env proteins is then thought to result in them appearing at the same point on the plasma membrane, such that envelope can be incorporated into virus particles. Gag products alone are sufficient for assembly into virus-like particles but the glycoproteins are required for the infectivity of the progeny virus. The immature virus particle that forms undergoes a maturation step, involving proteolysis and rearrangement, to give the mature form. It is these mature virus particles that are then able to continue the infection process.

1.3 Retroviral Reverse Transcription

The feature that distinguishes retroviruses from other viruses is the replication of single-stranded RNA genome through a double-stranded DNA intermediate. This copying of single-stranded RNA into double-stranded DNA is known as reverse transcription. A number of models have been proposed for the mechanism of reverse transcription (Gilboa *et al.*, 1979; Panganiban and Fiore, 1988; Coffin, 1990; Charneau and Clavel, 1991; Lee and Coffin, 1991; Pulsinelli and Temin, 1991; Peliska and Benkovic, 1992; Li *et al.*, 1993; Jones *et al.*, 1994). The synthesis of the first strand of DNA is primed by a cellular tRNA. Generally, a stretch of 18 nucleotides at the 3' end of the tRNA is complementary to the primer binding site (PBS) located just downstream of the U5 region at the 5' end of the viral RNA. Different retroviruses use different tRNAs as primers; $tRNA^{Trp}$ and $tRNA^{Pro}$ are used by avian and murine (except mouse mammary

tumour virus) retroviruses, and $tRNA^{Lys}$ isoacceptors are used by mouse mammary tumour virus and human retroviruses. HIV uses $tRNA_3^{Lys}$ (Jiang *et al.*, 1993), one of the three major $tRNA^{Lys}$ isoacceptors in mammalian cells. Once the tRNA primer has bound to the PBS, reverse transcription is initiated and the first (or minus) strand is extended through the U5 region and R to the 5' end of the RNA at which point the enzyme runs out of template. This species is called the minus-strand strong-stop DNA (Haseltine *et al.*, 1977; Shine *et al.*, 1977).

In addition to DNA polymerase activity, reverse transcriptase also contains RNase H activity. RNAse H degrades the RNA part in an RNA:DNA hybrid, as the RNA is reverse transcribed. This process exposes the newly synthesized minus-strand strong-stop DNA as single-stranded. This strong-stop DNA contains a sequence complementary to the R region that is also located at the 3' end of the genomic RNA, allowing the strong-stop minus DNA to be transferred and bind to the 3' end of the viral RNA, in order to allow DNA synthesis to continue. This transfer of templates is referred to as the first template switch (or jump) (Swanstrom *et al.*, 1981). This first jump switches the growing point of the minus-strand DNA from the 5' end of one RNA molecule to the 3' end of either the same RNA molecule (intramolecular), or to the second genomic RNA found in the virion (intermolecular). At first it was reported that the first strand transfer was an intermolecular event, involving both molecules of RNA (Panganiban and Fiore, 1988), but later it was shown by Hu and Temin, (1990) that the first strand transfer could be either intra- or inter-molecular. Recently, however, Jones *et al.*, (1994) showed that in the absence of recombination, one molecule of retroviral RNA was sufficient for reverse transcription, suggesting that both the template switches can occur intramolecularly. There is experimental evidence that suggested that both inter-strand and intra-strand transfers may occur *in vivo* (Hu and Temin, 1990). Studies of mutant proviruses in which the 5' and 3' copies of R can be distinguished, show that it is not necessary to copy the entire R sequence adjacent to U5 before the template switch occurs (Lobel and Goff, 1985). The minus-strand is then extended in a continuous

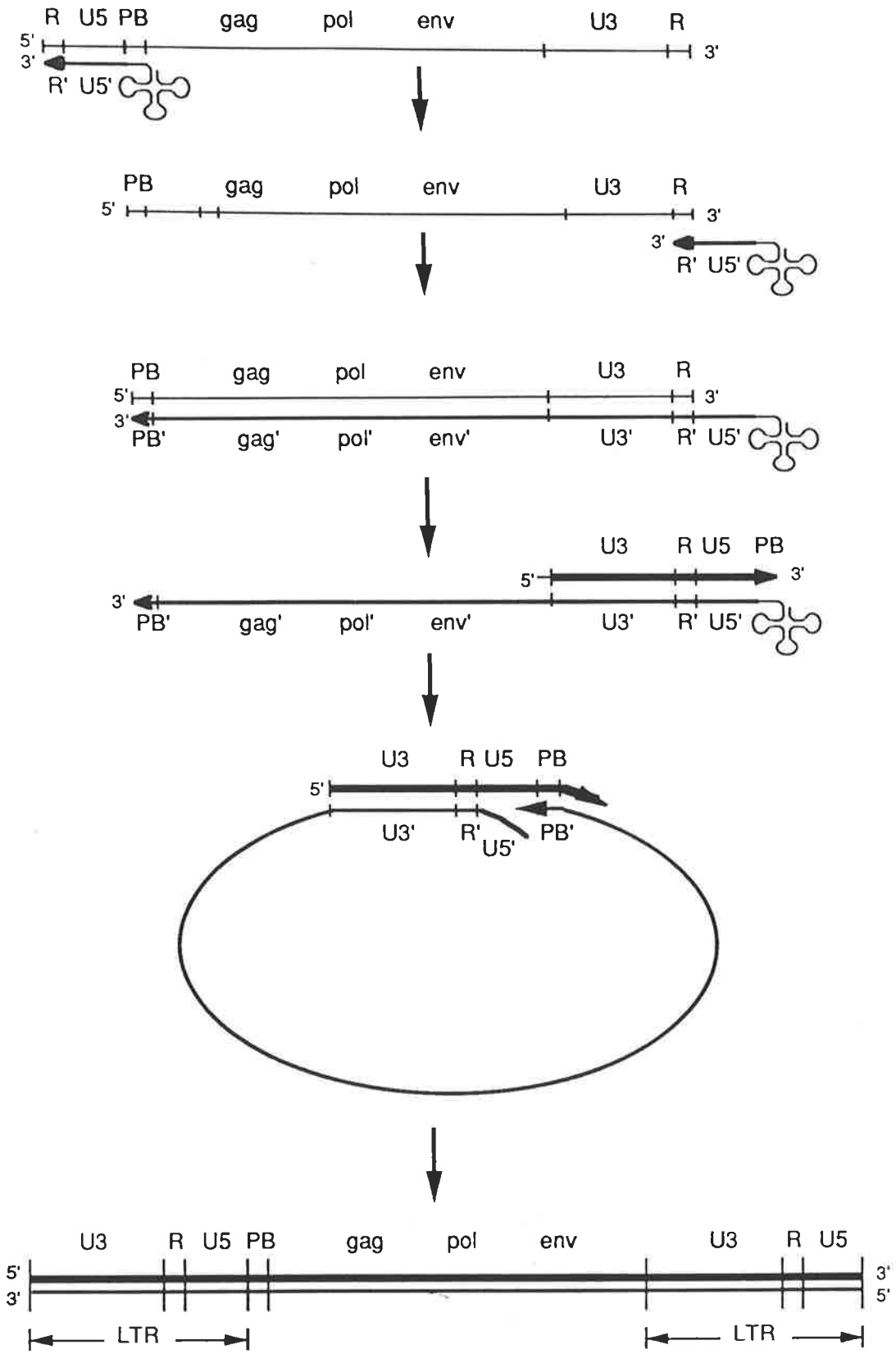
fashion until at least part of the PBS (intramolecular jump) or R (intermolecular) has been copied. This template switch is further discussed and investigated in Chapter 4 of this thesis.

Once the minus-strand has been extended past the U3 region on the viral RNA template, the intermittent exonuclease action of RNAse H generates a purine-rich oligoribonucleotide (polypurine tract, PPT) that primes the synthesis of the second (plus) strand precisely at the point that corresponds to the 5' end of the LTR (Omer *et al.*, 1984; Smith *et al.*, 1984; Taylor and Sharmeen, 1987). The plus-strand is extended through U3, R, and U5 by copying minus-strand DNA, and then to the end of the PBS by copying the first 18 nucleotides of the tRNA which is still linked to the minus DNA, until the polymerase encounters the first modified base in the tRNA, which it cannot use as a template, and synthesis of the (+) strand DNA segment stops. This plus-strand product is called the plus-strand strong-stop DNA and it contains precisely one copy of the LTR sequence plus 18 nucleotides of PBS. The tRNA primer is believed to be removed by RNAse H catalysed cleavage.

Apart from the debate over whether the first template transfer is intramolecular and/or intermolecular, the process of reverse transcription to this stage of DNA synthesis, where the plus-strand strong-stop DNA is synthesized, is commonly agreed by all proposed models. However the later steps in the process are unclear.

Currently the most widely accepted model for reverse transcription was originally proposed by Gilboa *et al.*, (1979) and then modified by Coffin (1990) (see Figure 1.6). In this model of reverse transcription, the (+)PBS sequence at the 3' end of plus-strand strong-stop DNA and the (-)PBS sequence at the 3' end of the long, nascent minus-strand DNA hybridize, following removal of the tRNA primer by RNAseH. This allows the plus-strand to be extended to the 5' end of the minus-strand template, and the minus-strand to be extended to the 5' end of the plus-strand. Hence, in this model

Figure 1.6 **Model for retroviral reverse transcription** originally proposed by Gilboa *et al.*, (1979), as modified by Coffin, (1990). Thin lines depict RNA, medium lines depict negative-strand DNA, and thick lines depict positive-strand DNA. Negative-sense sequences are also indicated by a "prime" (‘). Adapted from Coffin, J.M. (1990) Retroviridae and their replication p: 1437-1500 in Virology, Ed. B.N. Fields, Raven Press.


R U5 PB
gag
pol
env
U3 R
5'
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PB
gag
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U3 R
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gag'
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env'
U3' R' U5'
U3 R U5 PB
5'
U3'
R'
U5'
PB'
U3 R U5 PB
gag
pol
env
U3 R U5
5'
3'
3'
5'
LTR
LTR

the DNA circularizes to allow DNA synthesis to be completed. However, there has never been conclusive evidence for the existence of partially double stranded circular viral DNA before the appearance of the linear viral DNA.

The second PPT sequence located in the centre of the genome of lentiviruses including HIV, provides a second origin of plus-strand synthesis (Charneau and Clavel, 1991; Charneau *et al.*, 1992). It has been shown that HIV reverse transcriptase is able to catalyze strand-displacement synthesis on double-stranded DNA template *in vitro* (Huber *et al.*, 1989). In the model proposed by Li *et al.*, (1993) (Figure 1.7), DNA synthesis initiated using this second PPT sequence, or a second initiation event at the 3' PPT would result in a new strand of DNA which can then displace the plus-strand strong-stop DNA. This displaced plus-strand strong-stop DNA can hybridize to the 3' end of the minus DNA, thus mediating the second template transfer, and allowing the completion of the synthesis of the plus-strand of DNA. Apart from mediating the second template switch, it has also been suggested that this displaced plus-strand strong-stop DNA may also be used as a template by the tRNA for synthesis of its complementary minus-strand, resulting in a double-stranded strong-stop DNA species ~650 base pairs in length, which is readily detected in acutely infected cells (as shown in Figure 1.7H) (Li *et al.*, 1993). This second transfer appears to take place only in an intramolecular reaction (Hu and Temin, 1990). The site of initiation of the (+) and (-) strands and the subsequent removal of the RNA primers define both boundaries of the LTRs and the ends of the completed linear viral DNA. The final product is a linear DNA duplex containing a single copy of all sequences present in the viral RNA, plus duplications of the U3 and U5 domains.

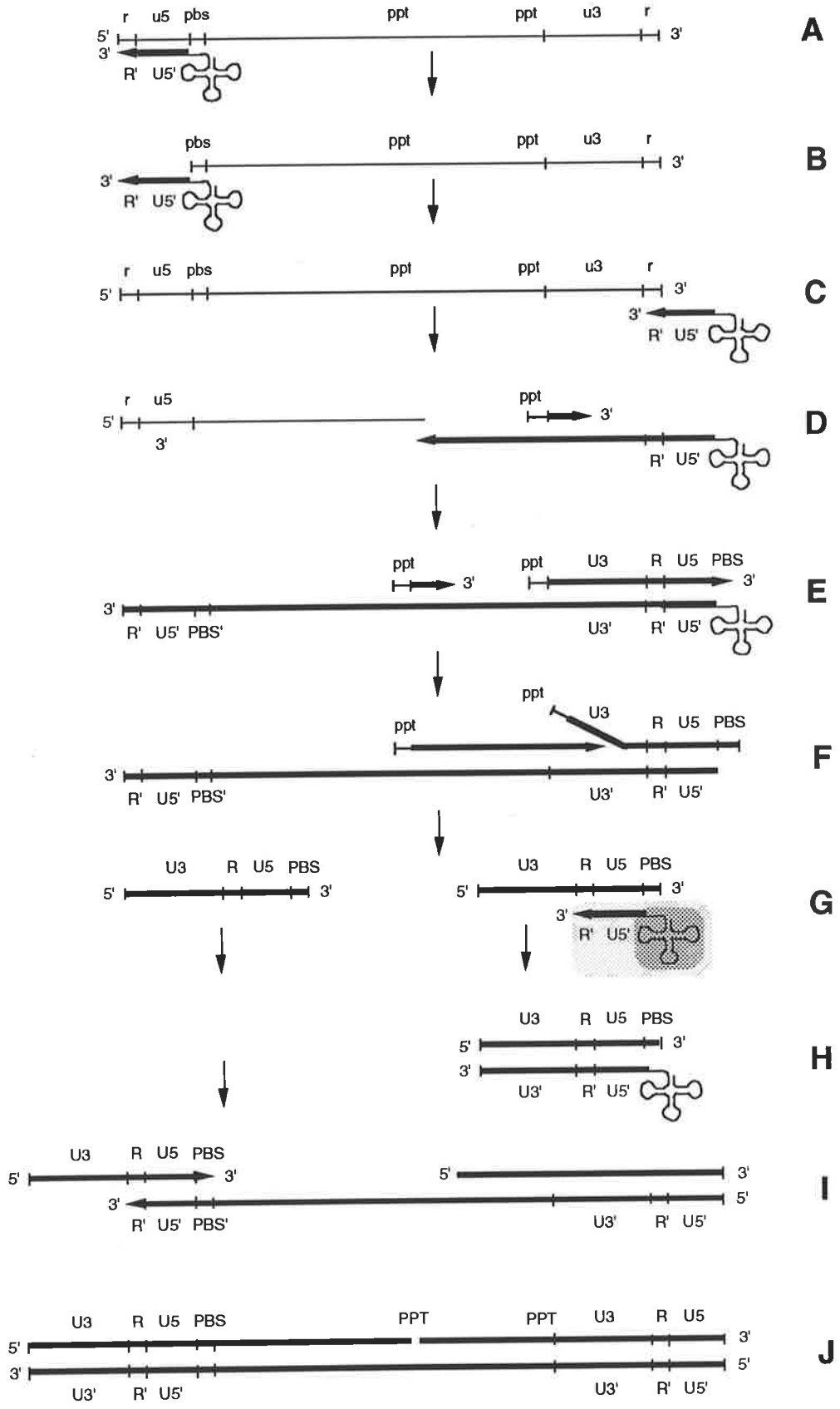
The linear DNA is the precursor to the integrated provirus and to two circular forms that contain either one or two long terminal repeats (LTRs). Circles with a single LTR are thought to arise either by some mechanism involving homologous recombination between the LTRs present on the linear viral DNA molecule (Shank *et al.*,

Figure 1.7 Model for HIV reverse transcription.

Thin lines represent RNA, thick lines represent DNA, and  represents tRNA.

(A) Synthesis of minus strong-stop DNA. (B) First template switch. (C) Synthesis of plus strand DNA from the right and central PPT sites. (D) The plus strand strong-stop DNA (from the right PPT site) is being displaced. (E) Displaced plus strand strong-stop DNA. (E)->(F) Synthesis of ds strong-stop DNA. (E)->(G) Second template switch. (H) Full length ds linear viral DNA with a gap at the central PPT site. Shaded areas in (E) indicate that cellular tRNA may provide the 3' end hydroxyl group. * and ** in (A), (B) and (C) indicate the two different genomic RNA molecules that would be in the same virion.

Model modified from Li, *et al.* (1993) *Virology* **194**:82-88.


A
r u5 pbs ppt ppt u3 r
5'
3'
R' U5'
B
pbs ppt ppt u3 r
C
r u5 pbs ppt ppt u3 r
D
r u5
ppt
E
ppt ppt U3 R U5 PBS
R' U5' PBS'
U3'
F
G
U3 R U5 PBS
H
I
J
PPT PPT

1978b; Gilboa *et al.*, 1979) or from a circular intermediate in the formation of the linear molecule during reverse transcription (Dina and Benz, 1980; Junghans *et al.*, 1982). Circular forms with two LTRs appear to be formed by blunt-end ligation of the linear double-stranded DNA molecule (Shank and Varmus, 1978a; Varmus and Brown, 1989). Whether this ligation is catalyzed by cellular or viral enzymes is unclear. Autointegration (intra-molecular integration), presumably mediated by the viral integrase, is also believed to give rise to 2-LTR circular DNA molecules (Shoemaker, *et al.*, 1981; Lee and Coffin, 1990; Farnet and Haseltine, 1991). *In vivo* results indicate that circular molecules formed by ligation or recombination are more abundant than products of autointegration (Bukrinsky *et al.*, 1993; Randolph and Champoux, 1993). There is no known function for these circular forms of the viral DNA. The accumulation of circular molecules has been implicated with HIV cytotoxicity (Pauza *et al.*, 1990; Tang *et al.*, 1992). At first circular DNA was believed to be a precursor to integration; however, antibodies to HIV integrase failed to precipitate the 2-LTR circular DNA within a nucleoprotein complex, indicating that the 2-LTR circular DNA is not associated with integrase and therefore unlikely to integrate (Bukrinsky *et al.*, 1993).

1.4 Retroviral Replication Complexes and Integration

1.4.1 Replication Complexes

Reverse transcription results in a linear double-stranded DNA molecule in the cytoplasm of the host cell. In a study of murine leukaemia virus (MLV), the native state of newly synthesized viral DNA in acutely infected cells was examined (Bowerman *et al.*, 1989). The viral DNA and integration activity co-purified during velocity sedimentation, gel filtration, and density equilibrium centrifugation, indicating that the MLV viral DNA was in a nucleoprotein complex with a sedimentation coefficient of ~160S. The complexes appeared to contain all the components required for successful integration into target DNA. Immunoprecipitation studies demonstrated that the viral capsid protein, $p30^{gag}$, was associated with MLV nucleoprotein complexes.

The viral DNA within this structure was shown to be accessible to nucleases and it was suggested that this integration-competent nucleoprotein complex was derived from and similar to the core of extracellular virions. These results suggested that newly synthesized retroviral DNA within infected cells remains associated with the protein machinery responsible for integration into the host genome. This finding was consistent with the proposal that retroviruses bring into infected cells all the activities needed for reverse transcription and integration (Varmus and Brown, 1989).

At the commencement of this PhD study very little was known about these early events in HIV infection. It was presumed that like the MLV nucleoprotein complexes, the unintegrated HIV DNA would also be associated with all the necessary viral proteins and functions required for nuclear localization and integration, but this had not been shown. Subsequently, newly synthesized HIV DNA isolated from the cytoplasm of infected cells was shown to be part of a nucleoprotein complex capable of integrating into heterologous DNA targets *in vitro* (Ellison *et al.*, 1990; Farnet and Haseltine, 1990). This ability to integrate suggested that these complexes may contain all the enzymatic machinery and functions necessary for viral integration. Due to their ability to mediate integration, the HIV nucleoprotein complexes were referred to as "pre-integration complexes".

In 1991, Farnet and Haseltine reported that unintegrated HIV DNA was associated only with the viral enzyme integrase, in the form of a nucleoprotein preintegration complex. In this study (Farnet and Haseltine, 1991), cytoplasmic extracts were prepared from cells infected with metabolically radiolabelled virions of HIV. Viral DNA-containing complexes were purified from these cytoplasmic extracts by gel filtration chromatography and sucrose gradient sedimentation. It was shown that these nucleoprotein complexes, contained integrase as the only viral protein detectable by immunoprecipitation and gel electrophoretic analyses. Since the viral proteins had been radioactively labelled, they could be detected by fluorography after gel electrophoresis.

The purified nucleoprotein complexes were also shown to be capable of integrating into heterologous DNA targets *in vitro*. In this study, Farnet and Haseltine failed to investigate the structure and protein composition of complexes isolated from the nucleus and assumed that since the cytoplasmic complexes were capable of integrating into target DNA, they were the precursors to integration.

Later in 1993, coinciding with the publication of and in agreement with our results (see Chapter 3), Bukrinsky *et al.* reported that in addition to integrase, the viral proteins reverse transcriptase and matrix were also associated with unintegrated HIV DNA (Bukrinsky *et al.*, 1993). In their study, HIV nucleoprotein complexes were isolated from cytoplasmic and nuclear extracts of CD4+ cells after acute virus infection. Gentle hypotonic cell-lysis conditions were adopted in this study in order to maintain the integrity of the HIV nucleoprotein complex and to prevent dissociation of its components during isolation. Both cytoplasmic and nuclear complexes were shown to have a density of 1.36g / ml and also *in vitro* integration activity. Immunoprecipitation and PCR detection of the immunoprecipitated DNA demonstrated that the viral matrix protein (p17), integrase and reverse transcriptase, but not capsid (p24) proteins, were associated with both cytoplasmic and nuclear complexes. In the study by Farnet and Haseltine discussed above, infected cells were lysed in the presence of detergent (0.5% Triton X-100), conditions that, according to Bukrinsky *et al.*, disrupted the association of the matrix protein (p17) from the viral nucleic acids (Bukrinsky *et al.*, 1993). The presence of detergent and hence the dissociation of reverse transcriptase and p17, was offered by Bukrinsky *et al.* as a possible explanation for the discrepancy between the conflicting results.

As I shall discuss in more detail in Chapter 3, unintegrated DNA from our cell-to-cell infection was found to be associated with the viral proteins integrase, reverse transcriptase, p17, protease, Vpr and as well as histones, in a nucleoprotein complex with a sedimentation coefficient of 320S (Karageorgos *et al.*, 1993). Transportation of the

replication complex from the cytoplasm into the nucleus was also shown to be accompanied with a reduction in size from 320S to 80S. In addition, nuclear replication complexes were shown to lack the viral proteins reverse transcriptase and p17, and to contain only integrase, protease and histones.

1.4.2 Integration

Transport of the replication complex into the nucleus is followed by another major step in the life cycle of a retrovirus, integration. Reverse transcription of the viral RNA genome results in a blunt-ended double-stranded DNA molecule with terminal repeats on each end. The linear form of the viral DNA, and not one of the circular forms, is believed to be the direct precursor to the integrated provirus, based on the following observations (Fujiwara and Mizuuchi, 1988; Brown *et al.*, 1989). (i) The structure of the Moloney murine leukaemia virus (Mo-MLV) DNA integration intermediate was studied using a cell-free reaction with exogenous target DNA (Fujiwara and Mizuuchi, 1988). In these integration intermediates, the 3' ends of the viral DNA are joined to the target DNA, while the 5' ends of the viral DNA remain free. The 5' ends of the LTR sequences in these intermediates were sequenced and found to be identical to those found in the unintegrated linear double-stranded viral DNA, demonstrating that the linear form of Mo-MLV DNA could integrate directly without prior circularization. (ii) Similarly, the sequence of the initial covalent product of an *in vitro* integration reaction was analysed and compared with the sequence of the ends of the unintegrated linear viral DNA molecules of murine leukemia virus (MLV) (Brown *et al.*, 1989). Again the boundaries of the integrated provirus matched the ends of the linear viral DNA, indicating that the direct precursor to the integrated MLV provirus was a linear molecule. (iii) In addition, integration of linear HIV DNA into heterologous DNA targets, occurred in cytoplasmic extracts that contained no detectable circular forms of viral DNA (Farnet and Haseltine, 1990).

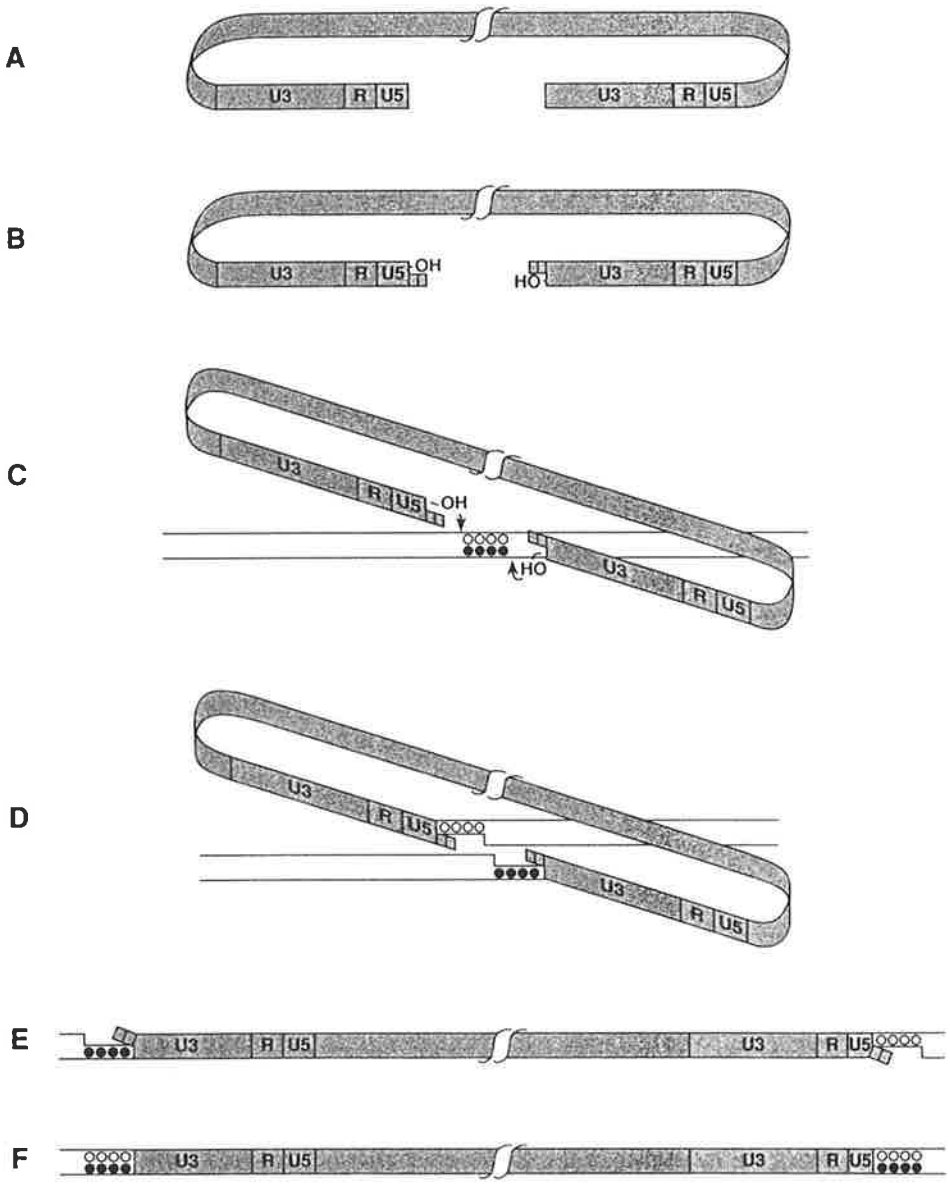
Retroviral DNA integration involves a coordinated set of DNA cutting and joining reactions. The integration reaction can be divided into three steps (Figure 1.8). In the first step, the virally encoded integration protein, integrase, recognizes sequences at the ends of the linear molecule and cleaves two bases from the 3' ends of both viral DNA strands, resulting in a free hydroxyl group on each recessive 3' end (Fujiwara and Mizuuchi, 1988; Brown *et al.*, 1989). This nicking of a dinucleotide from each 3' end of the viral DNA takes place within the nucleoprotein complex while the complex is within the cytoplasm of the infected cell (Fujiwara and Mizuuchi, 1988; Brown *et al.*, 1989). Once inside the nucleus the nucleoprotein complex attaches to host DNA. The integrase enzyme introduces a staggered cut in the target DNA giving rise to overhangs with a phosphorylated 5' end. These 5' phosphorylated ends are joined to the recessed 3' hydroxyl ends of the linear viral DNA by the integrase enzyme. Evidence suggests that only the integrase protein is required for this reaction. First, retroviruses defective in integration protein do not carry out this two-base cleavage reaction (Roth *et al.*, 1989) and secondly, purified recombinant integration protein can cleave these two bases from model DNA substrates (Craigie *et al.*, 1990).

In the final step, the host and viral DNA are joined together with the DNA repair mechanism. After removal of the two mismatched nucleotides at each 5' terminus of viral DNA, cellular enzymes may fill the single-strand gap and ligate the remaining ends to generate the fully integrated provirus. The energetics of this ligation reaction are not completely clear. Synthetic substrates with the two bases already removed are efficiently joined to DNA targets by integration protein, which indicates that the energy released during the cleavage of the viral ends is not used in the reaction (Craigie *et al.*, 1990). That leaves only the cleavage of the host DNA or the integration protein itself as a source of energy. One possibility is a high energy DNA:protein intermediate in the reaction that would store the energy from the phosphate bond (or from the protein itself) to be used later in the joining reaction (Katzman *et al.*, 1991). Alternatively, it has been shown that both the viral DNA cleavage reaction and DNA strand transfer reaction

Figure 1.8 Integration of retroviral DNA.

The linear form of unintegrated retroviral DNA is shown in (A). The integration protein cleaves two bases from the 3' ends of both viral DNA strands while the nucleoprotein cores are still in the cytoplasm, which leaves a free OH on each 3' end. This nucleoprotein complex migrates to the nucleus where the integration protein, integrase, catalyses an attack by these 3'OHs on viral DNA (C). In the case of MLV, the attack is made on host DNA at two sites on opposite strands four bases apart. Other retroviruses attack positions in the host genome five or six bases apart. Evidence suggests that the reaction is concerted, so that (C) and (D) represent successive stages in the reaction. Simply pulling on the ends of host DNA converts (D) to (E). DNA repair converts the mismatched gaps at the ends of the provirus to double strand. Note that the positions of the attack on host DNA in (C) are what define the size of the repeat in the host sequences that flank the integrated provirus (F).

Adapted from Whitcomb, J.M. and Hughes, S.H. (1992) *Annu. Rev. Cell Biol.* **8**:275.


A
U3
R
U5
U3
R
U5
B
U3
R
U5
OH
HO
U3
R
U5
C
U3
R
U5
OH
HO
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R
U5
E
U3
R
U5
U3
R
U5
F
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U5

are accompanied by inversion of the chirality of participating phosphorothioate groups, such that, as the host DNA strand is broken, it is immediately joined to viral DNA, and hence proposed that each of these reactions proceeds by a one-step mechanism, not involving a covalent protein-DNA intermediate (Engelman *et al.*, 1991).

The resulting integrated provirus is shorter than its linear double-stranded precursor by the number of bases removed by the integrase protein prior to integration, and is flanked by a short direct repeat of host DNA.

1.5 HIV Latency

After entry into the CD4+ cell, HIV-1 may establish a latent or persistent form of infection. A latent HIV infection involves the integration of proviral DNA into host genetic material with periods of no viral expression. Viral latency is poorly understood. This 'cellular latency' is quite distinct and different from 'clinical latency' which refers to the interval between infection and clinical disease in an individual.

The stage of the cell cycle at the time of infection greatly influences retrovirus replication. HIV and other retroviruses are capable of infecting and persisting in quiescent cells without producing progeny virions (Humphries and Temin, 1972; Humphries and Temin, 1974; Harel *et al.*, 1981; Zack *et al.*, 1988; Stevenson *et al.*, 1990; Zack *et al.*, 1990; Zack *et al.*, 1992). However, activation of these cells by antigens, mitogens or cytokines can induce progeny virus production. Incomplete species of HIV DNA have been detected in quiescent cells. Zack *et al.* demonstrated that HIV-1 can efficiently enter quiescent primary human lymphocytes and initiate reverse transcription, but that the reverse transcription process is not completed in these cells, presumably because host factors needed for complete reverse transcription are absent from the resting T cells. Mitogenic stimulation of these quiescent cells harbouring this partial reverse transcript induces completion of the reverse transcription process and is capable

of leading to progeny virus production (Zack *et al.*, 1990; Zack *et al.*, 1992). Therefore this may be a form of latent HIV infection; however this latent intermediate form was shown to be labile, suggesting that there may be a mechanism for clearance of the virus *in vivo*. Stevenson *et al.*, (1990) also showed that T cell activation was not required for the initiation of HIV infection, however this study showed that reverse transcription was complete in the infected resting T cells and the unintegrated HIV DNA was maintained extrachromosomally with a block to integration. In addition, these extrachromosomal HIV genomes were shown to be transcriptionally active, although, integration was required for the production of infectious virus. Subsequent T cell activation allowed integration of extrachromosomal forms and led to a productive viral life cycle. In this study, extrachromosomal forms of viral DNA were found to persist for several weeks after infection of resting T cells and to maintain their ability to integrate and act as a template for infectious virus, following T cell activation. These *in vitro* studies therefore support the role of resting T cells as a reservoir for HIV.

The above studies indicated that some non-activated cells can harbour the HIV genome in an unintegrated state without evidence of virus replication. This type of silent infection in cells may be considered as a form of latency, although it differs from classical virus latency, in which the full viral genome is integrated into the host chromosome but expression is suppressed.

Hoxie *et al.*, (1985), in one of the first *in vitro* studies of HIV cellular latency, reported that long-term cultures of naturally infected human CD4+ lymphocytes did not express much virus until several weeks after culture, when high levels of HIV were spontaneously produced, followed by cell death. A different system related to latency is provided by U-1 and ACH-2 cell lines, which are stably infected cell lines that produce relatively low levels of HIV (Folks *et al.*, 1986; Folks *et al.*, 1987; Poli *et al.*, 1990; Pomerantz *et al.*, 1990; Michael *et al.*, 1991). After treatment with mitogens, these cells can be activated to produce greatly increased levels of progeny virus. However,

whether these *in vitro* models have relevance to an *in vivo* situation has yet to be determined.

Clinical latency, the interval between infection and clinical disease (discussed in section 1.1.4), is quite different from the latent state within the cell. The factors influencing this clinical condition are not only cellular, but also involve the immunologic response of the host against the virus. Clinical latency can be present at the time when virus replication is active in the host. Thus the interrelationship between cellular and clinical latency has not been well defined.

1.6 Control of HIV Infection

No cure or vaccine exists for HIV infection. Currently the only effective means of controlling HIV-1 is to avoid infection in the first place. A number of interventions aimed at decreasing HIV transmission, for example health education, surveillance and contact tracing, public health measures targeted to high risk groups etc., are being implemented with variable degrees of success. To complement these interventions, the availability of safe, effective, and affordable HIV vaccines and antivirals would be highly desirable for the control of the HIV pandemic.

1.6.1 Antivirals

With the knowledge obtained from studying the structure of HIV and its replication cycle, specific drug design for anti-HIV therapy is possible. The replication cycle of HIV is complex, presenting a relatively large number of potential targets for chemotherapeutic intervention. One problem with antiviral drugs in general, is that virus life cycles are so closely connected with cellular processes that selectivity is difficult to achieve. There are some steps in the HIV life cycle that are unique. Table 1 shows inhibition strategies targeted at various stages in the replication cycle of HIV.

Table 1.1 Steps in the replication cycle of HIV to which inhibitors have been targeted.

Steps in Viral Life-Cycle	Potential Inhibitors
Attachment	solubleCD4 CD4-Ig CD4-toxins, dextran sulfate, Abs
Uncoating	Hypericin
Reverse Transcription	Nucleoside Analogus Foscarnet , TIBO etc.
RNAseH Degradation	Illimaquinone , AZTMP
DNA synthesis of second strand	None
Migration to Nucleus	None
Integration	None
Latency	None
Viral Trancription	Ro24-7439 , TAR decoys
RNA Nuclear Transport	RRE decoys, Transdominant mutant Rev
RNA stability and Protein Synthesis	Antisense Molecules, GLQ 223 Ribozymes
Protein Glycosylation	Glucosidase Inhibitors
RNA Packaging & Virion Assembly	Myristic Acid Analogs Transdominant mutant Gag Antisense/ribozyme
Release of Virus	Interferons
Maturation	Peptide & Peptidyl, Mimetic Protease Inhibitors
other	Immunomodulators

Nucleoside analogues, directed at the reverse transcription step, have shown some success. The best known of these is AZT (azidothymidine). Once AZT has entered the cell, it is then phosphorylated to its monophosphate form by cellular thymidine kinase, to the diphosphate form by thymidylate kinase, and finally to the triphosphate form where it exists in low concentrations (Furman, *et al.*, 1986). AZT triphosphate competes effectively with the natural substrate (dTTP) for the HIV reverse transcriptase. Its incorporation into the growing HIV DNA strand results in termination of DNA elongation, due to it lacking a 3' hydroxyl group and hence preventing the formation of subsequent 3' to 5' phosphodiester bonds.

Two other similar agents are ddC (dideoxycytidine) and ddI (dideoxyinosine). Like AZT, ddC activity depends on intracellular conversion to the 5'-triphosphate form by cellular enzymes (Cooney *et al.*, 1986). The activation of ddI involves its phosphorylation by cellular kinase and addition of an amino group to form a dideoxyadenosine monophosphate. This intermediate is further phosphorylated by cellular enzymes to dideoxyadenosine triphosphate which binds to the HIV reverse transcriptase and , with a mechanism similar to AZT triphosphate and ddC triphosphate, inhibits HIV DNA chain elongation (Ahluwalia *et al.*, 1987). The main problem with these nucleoside drugs is the emergence of resistant virus strains.

Other directions for antiviral therapy include inhibition of virus attachment by using recombinant CD4 or sulphated polysaccharides. Approaches at limiting virus replication have also involved molecular procedures that inhibit the viral protease. Antisense oligonucleotides and their thio-derivatives have also been proposed. Also, with a better understanding of the early events in the replication cycle of HIV, antivirals can be designed and targeted at these critical early stages. As seen in Table 1, serious attempts to block HIV replication at the final stages of reverse transcription, at the

stage of transporting the replication complex from the cytoplasm to the nucleus, or at the stage of integration have not been successful.

HIV gene therapy is currently receiving particularly intense study. Gene therapy is simply defined as the transfer of new genetic material to cells of an individual with resulting therapeutic benefit to the individual. The most promising feature of gene therapy in HIV is the possibility of delivering a gene or gene product precisely where it is needed, at or in the cell. For example, the main target cell for HIV, the CD4 lymphocyte, can be removed from the body, engineered , and returned to the same individual. The general principle of HIV gene therapy strategies is to use competitive HIV gene products (nucleic acid or protein) to specifically inhibit HIV replication. Protein-based intracellular immunization strategies involve the expression in HIV-susceptible cells of transdominant negative mutant forms of HIV regulatory or structural proteins, such as transdominant Gag and Rev (Green *et al.*, 1989; Trono *et al.*, 1989; Malim *et al.*, 1989a). Nucleic acid-based systems involve engineering lymphocytes to overexpress ribozymes, antisense RNA or RNA decoys for the key HIV regulatory proteins Tat and Rev (Sullenger *et al.*, 1990; Sullenger *et al.*, 1991). Although significant conceptual and technical hurdles remain to be overcome before gene therapy for HIV infection is achieved, progress in this field is likely to be rapid in the next few years.

1.6.2 Vaccines

Antigenic variation among different HIV-1 strains, especially in the envelope gene, is likely to present a considerable problem for vaccine development. In addition, transmission of HIV directly by cell-to-cell fusion may provide a means for ongoing virus spread within the body in the presence of antibody. A large number of different vaccine strategies are being explored with HIV, including experimental vaccines based on synthetic peptides, subunit antigens produced by genetic engineering, live recombinant vectors, whole inactivated virus and live attenuated vaccines. Model systems

include Simian immunodeficiency virus (SIV) in various species of monkeys, other animal retrovirus systems, and HIV in man.

One popular approach has involved a vaccine based on envelope proteins produced by genetic engineering. The reasons for using a subunit of the virus as the basis of a vaccine is due to the overwhelming requirement for safety. Such envelope based vaccines are currently being tested in Phase I/II trials in human volunteers, in the USA (Schwartz, 1993).

A recent encouraging report used attenuated SIV strains , defective in the *nef* gene (Daniel *et al.,* 1992). Vaccination with these SIV *nef* deletion mutants protected monkeys against challenge with wild-type pathogenic strains of SIV. Similar results using SIV deletion mutants have since been reported in other studies (Mehtali *et al.*, 1993; Stott *et al.*, 1993). Although preliminary, this gives hope that live and attenuated HIV-1 vaccines may show greater promise than subunit vaccines.

1.6.3 Education

Multi-million dollar public health awareness campaigns have been launched in the past few years to educate specific 'target groups' as well as the general public about HIV and AIDS. Specific "target groups" include those individuals thought to be at greatest risk of infection, such as young people, homosexual and bisexual men, and prostitutes and their clients. Repeated messages about 'safe-sex' through the mass media, and promotion and availability of condoms, have improved the public's attitudes and has lead to a sexual behaviour change. There is growing evidence that many people from a striking diversity of cultures, on different continents, have managed to adopt safer sexual behaviour, including having fewer sex partners, choosing nonpenetrative forms of sex and, the best documented change, using condoms (Merson, 1993).

In addition, needle exchange programmes have led to the education of intravenous drug users. Such programmes have been focussed towards stopping the spread of HIV via needle and syringe sharing.

1.7 Cell-to-Cell HIV Infection

Productive infection with HIV may be initiated either by infection of susceptible cells with free virions, or by cell-to-cell transmission (Gupta *et al.*, 1989; Li and Burrell, 1992; Li *et al.*, 1992). Cell-to-cell spread is a common mode of virus transmission which has been documented for many enveloped viruses, such as herpes viruses, rhabdoviruses, and retroviruses (Hooks *et al.*, 1976; Dietzschold *et al.*, 1985; Lodmell and Ewalt, 1987; Gupta *et al.*, 1989). HIV infected cells expressing the HIV-1 envelope protein can fuse with uninfected cells expressing the CD4 receptor on the cell membrane, thus allowing HIV to spread from one cell to another rapidly without releasing fully formed particles (Sato *et al.*, 1992). This involves transfer of viral nucleoprotein complexes, with subsequent *de novo* reverse transcription (Li *et al.*, 1992; Karageorgos *et al.*, 1993 and also discussed in more detail in Chapters 3 and 5).

In other studies, cell-to-cell transmission of virus in the presence of neutralizing antibodies has been demonstrated (Gupta *et al.*, 1989). It has been shown that HIV can be transmitted from monocytes or lymphocytes to epithelial cells during such close contact that neutralizing antibodies do not block the transfer (Phillips and Bourinbaiar, 1992). In this study, complete virus particles were shown to be transferred and virus particles were shown to be present at the site of cell-to-cell contact by electron microscopy. Time-lapse photography has also been used to show the transfer of virus particles from an infected lymphocyte to several different epithelial cells during short intervals of contact (Pearce-Pratt and Phillips, 1993).

In HIV infection *in vivo*, the role of cell-to-cell transmission is not known. It has been demonstrated that significant replication persists in lymph node follicles (Embretson *et al.*, 1993; Pantaleo *et al.*, 1993). It is possible that the slow spread of HIV in an infected individual, which may continue for many years, is primarily sustained by cell-to-cell virus transmission. HIV-induced cell-to-cell fusion and transmission would be likely to be seen in organs such as the lymph nodes and spleen, where high concentrations of lymphocytes are found. In adults, ~98% of CD4+ cells in the body are located in the bone marrow and peripheral lymphoid organs, while only a small percentage (~2%) of total CD4+ cells are found circulating in the peripheral blood (McCune, 1991). Within these lymphoid organs, CD4+ cells are in close proximity with each other, allowing easy cell-to-cell spread of HIV. It is also possible that cell-to-cell infection occurs *in vivo*, during transmission of infection, between individuals as a result of the transfer of infected cells between sexual partners or as a result of intravenous drug use.

In this study, a one-step cell-to-cell transmission model for synchronized HIV infection (Li and Burrell, 1992) has been employed to investigate the early events in HIV replication, to compare specifically the similarities and differences between a cell-free virus infection and a cell-to-cell virus infection. This cell-to-cell HIV infection model uses H3B cells, a laboratory clone of H9 cells persistently infected with HIV, as virus donor cells and HUT78 cells, a $CD4^+$ T-cell line, as recipients. The donor cells, rather than free virions, were shown to be responsible for initiation of infection in this system and any possible contributions made by small amounts of cell-free virus were insignificant (Li and Burrell, 1992). The kinetics of unintegrated HIV DNA synthesis in this cell-to-cell infection model are comparably rapid to those observed after a high multiplicity infection with cell-free virus (Li and Burrell, 1992). The kinetics observed in this cell-to-cell system are likely to have been facilitated by (i) high multiplicity of infection; (ii) bypassing the initial stages of free virus attachment, penetration and uncoating; and (iii) transmission of intermediate components of the replication cycle in

addition to virion components. Evidence for synchronicity of this infection comes from the observation that by 12 hrs after mixing, ~90% of the input HUT-78 cells and H3B cells were incorporated in giant cells which were p24 positive by immunofluorescence, with a one-step release of progeny virus at 24 hrs after cell mixing (Li and Burrell, 1992). The cell-to-cell transmission model was studied herein because (i) it provides a convenient reproducible method to achieve high multiplicity, synchronous infection; and (ii) it is thought to represent an important process in the pathogenesis of infection *in vivo*.

1.8 Aims and Scope of Thesis

The aims of this thesis were to investigate the early events in the HIV replication cycle, in particular the events and mechanisms involved in the synthesis of unintegrated viral DNA following synchronized cell-to-cell transmission of viral infection.

General knowledge and understanding of the early molecular events of HIV replication following infection progressed rapidly during the period of this study (1990-1994). Prior to 1990, little was known, at the molecular level, about the details of HIV reverse transcription. Studies on type C retroviruses had demonstrated that full-length unintegrated viral DNA was organized with viral capsid protein in a 160S nucleoprotein complex and contained all enzymatic functions necessary for integration *in vitro* . Such replication complexes had not been documented for HIV. Isolation and characterization of the HIV early replication complex(es) in terms of their structural and functional properties, as well as their association with viral and cellular proteins, could help clarify the mechanisms involved in HIV reverse transcription and integration. Furthermore, although the basic steps of reverse transcription had been known for some time from studies of type C retroviruses and some aspects of these had been confirmed for HIV, a detailed kinetic study was lacking. Thus, it could be hoped that better understanding of these early events in HIV replication might provide important new

insights into HIV infection and pathogenesis, and strengthen the rational basis for design of antiviral drugs.

This thesis describes three specific aspects of HIV replication following synchronized, one-step cell-to-cell transmission of infection:

(i) characterization of the HIV DNA-containing replication complexes in the cytoplasm and in the nucleus of the infected cells (Chapter 3).

(ii) description of the kinetics of various stages of HIV reverse transcription (Chapter 4).

(iii) investigations of HIV RNA-containing complexes in the virus donor cells which, upon initiation of infection of recipient cells, would lead to the synthesis of unintegrated full-length HIV DNA and the formation of the replication complexes (Chapter 5).

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL CULTURE AND VIRUS INFECTION

2.1.1 Media

Most cell culture work was performed using RPMI-1640 (Cytosystems) medium supplemented with 10% heat-inactivated foetal bovine serum (FBS ; CSL) , 12ng/ml (final concentration) penicillin and 160ng/ml (final concentration) gentamycin. FBS was heat-inactivated by heating the FBS at 56°C for 30 minutes. Both penicillin and Gentamycin were supplied by the IMVS media kitchen.

For the poliovirus work, Dulbecco's Modified Eagles Medium (DMEM; Gibco) supplemented with 1% FBS and antibiotics (as above) was used.

2.1.2 Cells

HUT-78 cells were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. H9 cells and H9 cells persistently infected with HTLV-IIIB were originally from Dr. R. Gallo's laboratory. H3B cells are a laboratory clone of H9 cells persistently infected with the HTLV-IIIB strain of HIV. The HTLV-IIIB persistently infected cells were cloned by seeding single cells into microtiter plate wells containing uninfected H9 cells. One clone was named H3B. The H3B cells were >95% HIV p24 antigen positive as judged by immunofluorescence, contained an average of two copies of integrated HIV provirus per cell, contained no unintegrated DNA detectable by Southern analysis and secreted 0.01 $TCID_{50}$ virus per hour per cell to the culture supernatant. H3B cells were employed in this study as the virus donor cells in the cell-to-cell transmission model of infection, as well as the producer cells for virus inoculum used in the cell-free virus infection model (Li and Burrell, 1992).

For the poliovirus work, BGM (Buffalo Green Monkey Kidney Epithelial) cells were acquired from the Virus Detection Unit, Division of Medical Virology , IMVS.

2.1.3 Routine Cell Maintenance

Both HUT-78 and H3B cells were maintained in 150 cm^2 flasks (Costar) in RPMI-1640 growth medium supplemented with 10% FBS and antibiotics (see section 2.1.1). Cells were subcultured three times a week at an initial density of 2×10^5 / ml. For long term storage, cells were stored in RPMI-1640 containing 50% FBS and 10% dimethylsulphoxide (DMSO; Univar) under liquid nitrogen.

2.1.4 Preparation of HIV Virus Inoculum

To prepare cell-free virus inoculum, H3B cells were maintained at a cell density of 5×10^7 cells / ml at 37°C. Supernatant was harvested hourly by centrifugation at 930 rpm for 3 mins, and stored at 4°C. Cells were then resuspended in an equal volume of fresh medium and incubated at 37°C. The hourly harvests were pooled, chilled on ice, recentrifuged at 3500 rpm for 10 minutes, and then stored at -70°C. On average, the virus titre of this inoculum was 10^5 to 10^6 $TCID_{50}$/ml.

2.1.5 Poliovirus Labelling and Isolation

Poliovirus (type-1 Sabin strain) was labelled by incorporating ^{3}H-uridine into the RNA genome and ^{35}S-methionine into viral proteins. Poliovirus (~ 10^8 $TCID_{50}$/ ml) was originally acquired from the Fairfield Infectious Diseases Hospital, Victoria, Australia. BGM cells were grown at 37°C in 750 cm^3 roller bottles (Falcon) , until 90%-100% confluent. They were infected at a multiplicity of 10 pfu / cell in a total of 50 ml of methionine-free media (1xRPMI 1640 medium with 2g/L sodium bicarbonate , without methionine and glutamine ; ICN). After 1.5 hours incubation at 37°C, 2 µCi/ml ^{3}H-uridine (Amersham) and 2 µCi/ml ^{35}S-methionine (Amersham) was added.

After a further 6.5 hours incubation, when the CPE (cytopathic effect) was 3-4+, the cells were harvested. The medium was decanted and saved , the cells washed twice with phosphate buffered saline (PBS) (with phenol-red) for 15 minutes and then 50 ml of 2mM EDTA in PBS was added and the cells incubated at 37° C on the roller for 20 minutes. Once the cells became opaque, they were dislodged from the roller bottle by tapping and transferred to 50 ml tubes along with the original saved media. The cells were spun at 2000rpm for 15 minutes, washed twice with PBS, spinning at 2000 rpm for 10 minutes each time. The cells were resuspended in 20 mM Tris-HCl, pH7.5, subjected to 3 cycles of freeze / thawing at -70°C and then centrifuged at 2000 rpm for 15 minutes to pellet the cell debris. The supernatant was collected and the poliovirus pelleted by centrifuging at 40 000 rpm for 1 hour in a SW-41 rotor. The pellet was resuspended in 4 mls of 20 mM Tris-HCl, pH7.5 by repeated syringing through a 23 gauge needle, followed by a 10 minutes sonication. After spinning at 2000rpm for 15 minutes the supernatant was collected and the virus pelleted again by spinning at 40 000 rpm for 1 hour in a SW-41 rotor. The poliovirus pellet was resuspended in 400µl of 20mM Tris-HCl, pH7.5.

The poliovirus stock was then purified further by caesium chloride (CsCl) centrifugation. 2.2ml of CsCl (ρ=1.3) was mixed with 2.2ml CsCl (ρ=1.4) along with the 400 µl poliovirus suspension and centrifuged at 68 000rpm for 16 hours at 4°C. The gradient was fractionated (200µl fractions) and a sample of each fraction was placed onto Whatman 3MM filter paper, air dried, placed into scintillation vials along with 1ml of OptiPhase 'HiSafe'3 scintillation cocktail (LKB Scintillation Products) and counted in a Packard 1900 TR Liquid Scintillation Analyzer. The fractions containing the ^{3}H-labelled poliovirus were dialysed overnight at 4°C against 20 mM Tris-HCl, pH7.5.

2.1.6 Cell-to-Cell Model of Transmission of HIV Infection (Li and Burrell, 1992)

HUT-78 cells and H3B cells were routinely subcultured at an initial density of 1 x 10^6 /ml, one day before mixing. For cell-to-cell transmission of infection, H3B cells were spun down at 930rpm for 3 minutes, washed once with serum free RPMI 1640 at 37°C to remove extracellular virions , and resuspended in growth medium. These H3B cells were then co-cultured with washed HUT-78 cells at a ratio of 1:4 (H3B:HUT-78 cells). Routinely, cytoplasmic and nuclear extracts from experiments involving 8x10^7 uninfected HUT-78 cells and 2x10^7 H3B cells were prepared for analysis by sucrose gradient centrifugation. In chapter 4 for the kinetics of reverse transcription, cells were mixed at a ratio of 2:1 (H3B:HUT-78), in order to ensure a tightly synchronized infection.

2.1.7 Cell-free HIV Virus Infection

Infection of HUT-78 cells with cell-free virus was carried out at a nominal multiplicity of 0.5 $TCID_{50}$ of virus per cell using a centrifugal enhancement technique (Pietroboni *et al.*, 1989). Routinely, 5x10^7 HUT-78 cells were spun down and resuspended in a smaller volume (20 ml) of media, counted, and treated with 0.001% DEAE-dextran at 37°C for 30 minutes. After spinning at 930 rpm for 3 minutes, the supernatant was pipetted off and 1.25 x 10^7 $TCID_{50}$ of virus was added and incubated at room temperature for 30mins, with occasional shaking. The culture was then centrifuged at 2650 rpm for 25 minutes at 20°C and the supernatant removed. An equal amount of virus was added again for 30 minutes at room temperature and spun again as before. These cells were then washed 3 times in serum-free RPMI and then resuspended in 100ml of RPMI-1640 plus 10% FBS.

2.2 ISOLATION, DETECTION AND ANALYSIS OF NUCLEIC ACIDS.

2.2.1 HIRT Extraction of Extrachromosomal DNA (Hirt, 1967)

At appropriate times after infection, cells were harvested by low-speed centrifugation (930 rpm for 3 mins), washed with phosphate-buffered saline (PBS) ,and extrachromosomal DNA was separated from chromosomal DNA by the HIRT procedure. Cell pellets were resuspended in 160 µl buffer I (5mM Tris pH 7.7, 10mM EDTA) and 20 µl stock Proteinase K (10mg /ml ; Merck). Then 200 µl buffer II (5 mM Tris pH7.7, 10mM EDTA, 1.2% SDS) was added, the tube inverted slowly 10 times and incubated at 37°C for 15 minutes. Finally, 100ml 5M NaCl was added, the tube was inverted again 10 times, and stored at 4°C for 4 hours or overnight. Samples were then spun at 18000g for 45 minutes at 2°C. The Hirt supernatants were extracted twice with phenol-chloroform-isoamylalcohol (25:24:1) and the DNA was precipitated by ethanol. The DNA was then resuspended in 100µl TE and treated with 100 µg/ml RNAseA (Boehringer Mannheim) for 1 hour at 37°C, extracted with phenol-chloroform-isoamylalcohol and precipitated by ethanol.

2.2.2 Preparation of Cytoplasmic and Nuclear Extracts from Infected Cells.

Six hours after infection by cell-to-cell HIV infection, or 8 hours after cell-free virus infection, cells were harvested by low speed centrifugation (930 rpm for 3 mins) and then washed once with cold buffer A [10mM Tris hydrochloride (pH 7.4), 150mM KCl, 5mM $MgCl_2$, 1mM dithiothreitol (DTT), 20mg/ml aprotinin (Sigma Chemical Co.)]. Cells were then lysed in buffer A containing 0.05% (vol/vol) Triton X-100 for 15 minutes at room temperature. Lysates were centrifuged at 1000xg for 3 mins at 4°C to pellet nuclei and the supernatant was centrifuged for a further 10 mins at 8000xg. The resulting supernatant, referred to as the cytoplasmic extract, was adjusted to 0.5% triton X-100, with or without prior treatment with RNAse A (20µg/ml) for 30mins at room temperature. Finally the cytoplasmic extract was adjusted to 8%

(wt/vol) sucrose and frozen. The nuclear pellet was resuspended in cold buffer A, spun again at 1000g for 3 mins at 4°C and resuspended in hypotonic buffer A [10mM Tris hydrochloride (pH 7.4), 50mM KCL, 5mM $MgCl_2$, 1mM DTT, 20mg/ml aprotinin]. The nuclei were then freeze-thawed twice before homogenization with a Dounce homogenizer. The nuclear extract was adjusted to 0.5% Triton X-100, clarified with a 10 min spin at 8000xg and finally adjusted to 8%(wt/vol) sucrose and frozen.

2.2.3 Phenol Extraction and Ethanol Precipitation of Nucleic Acids.

Phenol for use in extractions was prepared according to Maniatis *et al.*, (1982) (see section 2.6.1). Crude nucleic acid samples were extracted by adding two volumes of 25:24:1 phenol:chloroform:isoamyl alcohol. The mixture was then vortexed, centrifuged at 10 000g for 5 minutes and the aqueous phase collected into a clean tube. This extraction was repeated for a second time. Nucleic acids were then precipitated by the addition of a 1/10 volume of 3M Na Acetate and 2.5 volumes of 100% ethanol, followed by an incubation at -20°C for 1 hour or overnight. The nucleic acid was pelleted by centrifugation at 18 000g for 20 minutes at 4°C. The pellet was then washed once with 70% ethanol, spun again at 18000g for 10 minutes at 4°C and the nucleic acid dried under vacuum (in a spin drier VR-1 Hetovac) for 10 minutes. Finally, the DNA samples were resuspended in TE and the RNA samples in sterile double distilled water (DDW).

2.2.4 DNA Extraction from Sucrose Fractions for Southern Analysis

Sucrose gradient fractions were collected and individually digested with 1mg/ml proteinase K (Merck) in 10mM EDTA / 0.5% SDS for 1hr at 55°C, then extracted twice with phenol / chloroform / isoamylalcohol (25:24:1), ethanol precipitated , washed with 70% ethanol, dried, resuspended in 10mM Tris-HCl (pH 7.6) / 10mM EDTA (pH 8) and treated with RNAse A (20µg/ml ; Boehringer Mannheim) for 1 hr at 37°C before

analysis by agarose gel electrophoresis and Southern blotting according to standard methods(Maniatis *et al.*, 1982) (see sections 2.2.10 and 2.2.11).

2.2.5 DNA Extraction from Sucrose Fractions for PCR

A portion (100µl) of the individual sucrose gradient fractions was mixed with 150µl of buffer A, treated with RNAse A (100µg/ml; Boehringer Mannheim) for 30 mins at 37°C, and then digested with 1mg/ml proteinase K (Merck) in 10mM EDTA / 0.5% SDS for 1hr at 55°C. Next, the samples were extracted twice with phenol / chloroform / isoamyl-alcohol (25:24:1) and the DNA was precipitated by ethanol. The DNA was resuspended in 5µl of TE and amplified by PCR (see section 2.2.8)

2.2.6 RNA Isolation from Sucrose Fractions for PCR

A portion (100µl) of the individual sucrose gradient fractions, and also of the initial crude cytoplasmic extract, was mixed with equal volume of urea/SDS solution (7M urea, 1% SDS, 0.35M NaCl, 10 mM EDTA, 10mM Tris-HCl (pH 7.5)) and 200µl phenol / chloroform / isoamylalcohol (25:24:1). After vortexing, the samples were placed at -70°C until required or spun at 13000 rpm for 5 minutes at 4°C. The aqueous layer was collected and the RNA was precipitated by ethanol (Gough, 1988). The RNA was then resuspended in 100µl of DNAse I buffer (0.1M sodium acetate (pH5.0) / 5mM $MgCl_2$ / 1mM DTT), treated with 10U/ml DNAse I (GIBCO BRL) at 37°C for 1 hour, extracted with phenol/chloroform/ isoamylalcohol (25:24:1) and precipitated again with ethanol. Next the RNA was converted to cDNA in standard reverse transcriptase reaction and then amplified by PCR, as described in section 2.2.9.

2.2.7 Oligonucleotides

Oligonucleotides used as primers in the PCR reactions were synthesized by Bresatec, Pty. Ltd. (Adelaide, South Australia). A number of primer pairs were used to detect HIV DNA by PCR (see text). The sequences and nucleotide positions of the

oligonucleotides were based on the sequence of HXB2 clone (April, 1990 version (Myers *et al.*, 1990)) as follows:

GAG -P1	5'-CCC AGT AGG AGA AAT T-3'	(nt 1556-1571)
GAG -P2	5'-CTT ATG TCC AGA ATG C-3'	(nt 1630-1644)
gag 1 (+)	5'-GAT GAC AAA TAA TCC ACC-3'	(nt 1535-1552)
gag 2 (-)	5'-AGT TTT ATA GAA CCG GTC-3'	(nt 1680-1697)
gag 3 (-)	5'-TGC ACA CAA TAG AGG GTT GC-3'	(nt 1035-1054)
PBS 2 (+)	5'-GCG CCC GAA CAG GGA CC-3'	(nt 638-624)
PBS 1 (-)	5'-GGT CCC TGT TCG GGC GC-3'	(nt 638-624)
R1 (+)	5'-CAA TAA AGC TTG CCT TGA GTG-3'	(nt 526-546)
R2 (-)	5'-CAG ACG GGC ACA CAC TAC-3'	(nt 553-570)
R3 (+)	5'-GGT TAG ACC AGA TCT GAG CC-3'	(nt 464-483)
$U_{3.1}$ (+)	5'-GGA AGG GCT AAT TCA CTC C-3'	(nt 2-20)
$U_{3.1}$ (-)	5'-GGA GTG AAT TAG CCC TTC C-3'	(nt 2-20)
U3.3 (+)	5'-GCT GCT TTT TGC CTG TAC TG -3'	(nt 435-454)
U5 (-)	5'-TTT CCA CAC TGA CTA AAA GG-3'	(nt 605-624)

Primers were 5'-labelled with γ-^{32}P-ATP using a standard kinase labelling reaction , as described in section 2.2.14.

2.2.8 DNA PCR reaction

The PCR reaction was carried out in a Perkin-Elmer thermocycler with reaction conditions as described by the manufacturer. The standard reaction conditions per PCR reaction were as follows (total 50µl): 22.8µl DDW, 5µl 10 x buffer (670mM Tris-HCl pH8.8, 166mM $(NH4)_2SO_4$, 2mg/ml gelatin, 4.5% Triton-X-100 ; Bresatec), 8µl 25mM $MgCl_2$, 1µl 10mM dATP, 1µl 10mM dCTP, 1µl 10mM dGTP, 1µl 10mM dTTP (dNTP from Perkin Elmer Cetus), 0.2µl Taq polymerase (5.5U/µl), 2.5µl of each primer (50ng/µl) and 5µl of DNA.

Dedicated PCR pipettes and tips were used to dispense the PCR reagents while a positive displacement pipette and tips were used to dispense the DNA only. Clean gloves were worn at all times. All dispensing was performed in a Bio-safety cabinet with a UV light.

Generally more than 10 reactions were run at one time, therefore all the reaction components, apart from the DNA , were mixed together in a master mix tube and aliquoted (45µl) into PCR tubes. Two drops of mineral oil (Faulding Paraffin Liquid) was added to each tube. Finally the DNA (5µl) was dispensed into each tube. The tubes were centrifuged briefly and placed in a thermocycler (Perkin Elmer Cetus DNA Thermal Cycler).

The thermocycling temperatures used were 94°C for 1 min, 37°C for 1 min, and 72°C for 1 min for a total of 13 , 15 , 17 or 20 cycles as specified in the text. The amplified products were analysed on 8% polyacrylamide gels , which were dried and subjected to autoradiography. In some cases , dried gels were exposed to Phosphor-Imager analysis (Molecular Dynamics , programme ImageQuant).

2.2.9 RNA-RT PCR and Detection by Southern Hybridization

The RNA purified from the sucrose fractions in section 2.4.6, was mixed with 40ng of each specific HIV gag primer (primers gag1 and gag2) in a final volume of 11µl, boiled for 5 minutes and then placed on ice for a further 5 minutes. Reaction buffer (11µl) containing 50mM Tris-HCl (pH 8.3), 50mM KCl, 5mM $MgCl_2$, 10mM DTT, 10mM of each dNTP and 2U of Rous-Associated Virus-2 reverse transcriptase (Amersham) was then added and the reaction incubated at 37°C for 1 hour. Twenty microlitres of this reaction was used directly in the PCR amplification reaction (20 cycles, thermocycling conditions as described in section 2.2.8).

The PCR products (15µl of the PCR reaction) were resolved on 8% polyacrylamide gels (section 2.2.12) and transferred electrophoretically (via Bio-Rad Trans-Blot) onto Hybond-N^+ membrane (Amersham) at 50mA for 1.5 hours in 0.3xTBE buffer (Tris-HCl / boric acid / EDTA). The nucleic acid was fixed to the membrane with 0.4M NaOH for 30 mins and washed with 6xSSC for 1 minute. Prehybridization, hybridization and washing of filters was performed in a Robbins Scientific Hybridization Incubator model 310. The membranes were prehybridized in 10mls of prehybridization buffer (25xDenhardts, 6xSSC, 100µg/ml ssDNA, 0.5% SDS) at 42°C overnight. An internal oligonucleotide probe (GAG-P2) end-labelled with γ-^{32}P-ATP by standard methods using T4 polynucleotide kinase (section 2.2.14) was added along with 10 mls of hybridization buffer (2x Denhardts, 6x SSC and 0.5% SDS) and incubated at 42°C for 1 hour. The membrane was washed six times for 15 minutes with 5xSSC/0.5% SDS and subjected to autoradiography.

2.2.10 Agarose Gel Electrophoresis

DNA was separated in horizontal 0.8% agarose gels using a BRL model H5 electrophoresis system and a Bio-Rad model 500/200 power supply. The gels were prepared by dissolving Ultra-Pure DNA grade agarose (Bio-Rad) in 1 x TAE (see section 2.6.1) by boiling in a microwave oven. The gel mixture was cooled to 60°C prior to pouring into gel plates and allowed to set for 1 hour before placing in the gel tank. DNA samples were mixed with 0.2 volumes of 6 x gel loading buffer (see section 2.6.1), heated at 65°C for 5 minutes and placed on ice for a further 5 minutes, prior to loading onto gel. The samples were then electrophoresed at 80 volts for 4 hours. Gels were stained with ethidium bromide (0.5 µg /ml in DDW) for 15 minutes, rinsed in DDW, and photographed using an ultraviolet transilluminator (Ultra-Lum) and Hoefer's Photoman with a Polaroid DS34 Direct Screen Instant Camera containing 667 film.

2.2.11 Southern Hybridization

DNA for Southern blot hybridization analysis was separated by agarose gel electrophoresis (section 2.2.10) and transferred to Nitrocellulose (0.45mm , Scheicher& Schuell). First, the gel was treated twice for 15 minutes with 0.25 M HCl, rinsed in DDW, treated twice for 15 minutes with denaturing solution (0.5M NaOH, 1.5M NaCl), rinsed with DDW, and finally treated twice for 20 minutes with neutralizing solution (1.5M NaCl, 1M Tris pH7.4). The gel was then assembled into a BRL DNA Blot Transfer System and the DNA was transferred overnight to the Nitrocellulose membrane in 20 x SSC, by capillary diffusion. The membrane was baked at 80°C for 2 hours in a vacuum oven.

Prehybridization, hybridization and washing of filters was performed in a Robbins Scientific Hybridization Incubator model 310. The filter was soaked in 6 X SSC for 10 minutes before being prehybridized in 6 x SSC, 5 x Denhardts, 100µg / ml ssDNA and 0.5% SDS for 4 hours at 65°C. The prehybridization fluid was then drained and replaced with 10 mls of fresh hybridization fluid (6 x SSC, 5 x Denhardts, 0.5% SDS, 100µg/ml ssDNA, 10 mM EDTA) containing approximately 10^6 cpm/ml of ^{32}P-labelled probe and hybridized overnight at 65°C. The probe used was a ^{32}P-labelled Sac I fragment of HTLV-IIIB DNA derived from plasmid pBH10 (Hahn *et al.*, 1984). The probe was prepared using the Amersham Mega-prime DNA labelling kit (see section 2.2.13).

The membrane was then washed twice in 2 xSSC, 0.5% SDS for 5 minutes at 65°C, twice in 2 x SSC, 0.1% SDS for 15 minutes at 65°C and finally three times in 0.1 x SSC, 0.1% SDS for 30 minutes at 65°C. The membrane was air dried, wrapped in plastic film and exposed to Kodak XAR film in an x-ray cassette, containing an intensifying screen, at -70°C for times varying from 4 hours to 2 days. X-ray films were developed using an Ilfospeed 2240 automated developer.

2.2.12 Polyacrylamide Gel Electrophoresis (PAGE)

Non-denaturing PAGE gels were used to resolve both ^{32}P-labelled PCR products (as from section 2.2.8) and unlabelled PCR products that were detected by Southern hybridization (section 2.2.11).

A stock solution of 33% acrylamide / 0.9% bis-acrylamide was prepared by dissolving 33 g of acrylamide (BDH) and 0.9 g of N,N'-methylenebisacrylamide (BDH) in DDW to final volume of 100ml. All weighing was done using gloves and face mask. The stock was stored in dark bottles at 4°C. Fresh 10% ammonium persulphate (Bio-Rad) was prepared each week by dissolving 0.1g in 1 ml of DDW and then storing at 4°C. TEMED (N,N,N,',N'-tetramethylethylene-diamine) (BDH) was purchased ready for use.

An 8% PAGE gel was assembled by mixing 1.2 mls of 33% acylamide / 0.9% bis-acrylamide, 2.75ml DDW, 1ml 5 x TBE, 50 µl 10% ammonium persulphate and finally 3.5 µl of TEMED. The mixture was poured between the glass plates of a mini-Protean II cell vertical electrophoresis system (Bio-Rad). Gels were allowed to set for 1 hour before use. Gels were electrophoresed at 100V for 1 - 1.5 hours. After electrophoresis the gel plates were separated.

Gels with ^{32}P-labelled PCR products were transferred to 3MM Chr chromatography paper (Whatman) and dried for 45 minutes on a Bio-Rad model 5833 gel dryer, connected to a Hetovac VR-1 vacuum apparatus. These gels were then exposed to Kodak XAR film in an x-ray cassette at -70°C for 2-16 hours. X-ray films were developed using an Ilfospeed 2240 automated developer.

Gels separating PCR products to be detected by southern hybridization, were stained with ethidium bromide and photographed as in section 2.2.10. The DNA was

then transferred to Hybond -N+ nylon transfer membrane (Amersham) as described in section 2.2.9.

2.2.13 Random Primer Labelling of DNA

The probe used was a ^{32}P-labelled Sac I fragment of HTLV-IIIB DNA derived from plasmid pBH10 (Hahn *et al.*, 1984). The probe was prepared using the Amersham Mega-prime DNA labelling kit , according to the manufacturer's instructions. Basically 200ng of the 8.9Kb SacI insert was added to 5µl of primer solution, containing random nonamer primers in an aqueous solution , and made up to 20µl with DDW. This mixture was boiled for 5 minutes and then snap cooled on ice for 5 minutes. To this, 5 µl of reaction buffer, 4µl of each dATP, dGTP, dTTP, 5µl of ^{32}P αdCTP (10µCi/µl ; Bresatec) and 2µl of DNA polymerase (Klenow fragment) were added and incubated for 30 minutes at 37°C. The probe was purified by spun column technique (section 2.2.15) before use. Specific activity of the probe was ~2 x 10^8 cpm/µg of DNA.

2.2.14 5'-end Labelling of Oligonucleotides

Oligonucleotides to be used as primers in PCR reactions (as in section 2.2.8) or as probes in southern hybridizations (as in section 2.2.11) were 5'-labelled with γ-^{32}P-ATP (Bresatec) using T4 polynucleotide kinase (Bresatec) in a standard kinase labelling reaction (Maniatis *et al.*, 1982). Routinely , 100ng-500ng of oligonucleotide was mixed with 5µl γ-^{32}P-ATP (10µCi/µl ; Bresatec), 2 µl 10 x PNK buffer (0.5M Tris-HCl pH7.6, 0.1M $MgCl_2$, 10mM DDT, 10mM β-mercaptoethanol), 1 µl T4 polynucleotide kinase (5U/µl ; Bresatec) and made up to a final volume of 20µl with DDW. This mixture was then incubated at 37°C for 1 hour , after which the labelled oligonucleotides were purified from unincorporated γ-^{32}P-ATP by spun column chromatography (section 2.2.15, below). Labelled oligonucleotides were used immediately or stored at -20°C.

2.2.15 Spun Column Chromatography

Spun column chromatography was used to separate ^{32}P-labelled DNA probes from their radioactive precursors. Sephadex (Pharmacia) was used as the gel matrix in this study. Sephadex G-25 (medium) (Pharmacia) was used to purify ^{32}P-labelled oligonucleotides used as primers in PCR and probes for Southern hybridization, while Sephadex G-50 (medium) (Pharmacia) was used to purify full-length probes prepared by the Mega-prime labelling method (as in section 2.2.13). Preparation of the Sephadex suspension is described in technical appendix (section 2.6.1).

To prepare the column, the bottom of a 1 ml disposable syringe (Terumo) was plugged with a small amount of sterile siliconized glass wool. The syringe was filled with the Sephadex suspension, until the syringe was completely full. This syringe was placed into a disposable 10ml plastic tube (Johns) and centrifuged at 350g (2500 rpm) for 4 minutes at room temperature in a swingout-bucket rotor (#2147) in a Heraeus Biofuge 17RS centrifuge. The column was then washed with 100 µl of STE and recentrifuged. The spun column was then placed in a fresh disposable tube containing a decapped microfuge tube. The DNA probe sample was loaded onto the column and the empty reaction tube that had contained the probe was rinsed with 20µl of STE and this was also loaded onto the column. The column was then recentrifuged as above, collecting the eluate in the microfuge tube at the bottom of the syringe. Using forceps , the decapped microfuge tube, which contained the eluted purified probe, was carefully removed and capped.

The radioactively labelled probes or the ^{32}P-labelled oligonucleotides were either used immediately or stored at -20°C until needed.

2.3 ULTRACENTRIFUGATION

2.3.1 Preparation of Sucrose Solution

A stock sucrose solution of 66% w/w was prepared by dissolving 171 grams of sucrose in 90 ml of buffer A at room temperature (Cline and Ryel, 1971). This solution was stored at 4°C. Desired sucrose concentrations were then prepared according dilution charts (Griffith, 1986). For this study the following concentrations were used: 15%, 25%, 30%, 40%, 45%, 60% (w/w). Aprotinin (20mg/ml) and DDT (1mM) were added just before pouring gradients. Desired sucrose concentrations were verified by checking the refractive index of the sucrose solution, and the concentrations corrected by adding either more buffer A or sucrose. Table 2.1 (Technical Appendix 2.6.2) shows the mixing proportions of sucrose (for total of 10 ml) and corresponding refractive indices.

2.3.2 Rate-Zonal Sucrose Gradient Centrifugation

Continuous sucrose gradients (15%-30%), for rate-zonal sedimentation, were poured using a Hoefer Scientific SG Series 15ml Gradient Maker and a SEM magnetic stirrer. Prior to pouring the 10ml 15%-30% sucrose gradient, 20mg/ml aprotinin, 1mM DDT and Triton X-100 to a final concentration of 0.5% were added to the sucrose solutions and a 60% sucrose cushion (500µl) was placed at the bottom of the tube (Beckman polyallomer centrifuge tubes (14 x 89mm)).

For sedimentation analysis of the HIV replication complexes in the cytoplasm and nucleus of infected cells, cytoplasmic or nuclear extracts (1ml) were layered onto the 10ml gradient of 15% to 30% sucrose in buffer A-0.5% Triton X-100 and centrifuged at 35000 rpm for various times at 4°C in a Beckman SW-41 rotor. Fractions (1ml) were collected from the bottom of the gradient and DNA was isolated from these fractions as described in section 2.2.4. and 2.2.5.

2.3.3 Isopycnic Centrifigation

For determination of the density of the HIV cytoplasmic replication complex, isopycnic centrifugation was carried out by modifying the procedure described by Caliguiri and Tamm, (1970). A discontinuous sucrose-density gradient was formed by layering sucrose solutions of different concentrations made in buffer A without Triton in the following order, 0.75ml of 60%, 1.75ml of 45%, 1.75ml of 40%, 2.5ml of cytoplasmic extract containing 30% sucrose, 1.75ml of 25% and 0.75ml of buffer A on top. Gradients were centrifuged at 26,000 rpm (86,000g) for 16hrs at 4°C in a Beckman SW-41 rotor.

2.3.4 Calculation of Sedimentation Coefficient

The sedimentation coefficient of the HIV replication nucleoprotein complex was estimated by the method of McEwen (1967), using ^{3}H-uridine labelled poliovirus as an internal marker (160S) in the sucrose gradient to verify the calculations. The method for estimating the sedimentation coefficient of a particle that has banded in a sucrose gradient is as follows.

Obtain Zo for rotor and gradient from formula:

$$Zo = Z_1 r_2 - Z_2 r_1 / r_2 - r_1$$

where Z_1 = minimum % w/w of sucrose gradient

Z_2 = maximum % w/w of sucrose gradient

r_1 = minimum radial distance (cm) from centrifugal axis to meniscus of gradient

r_2 = maximum radial distance (cm) from centrifugal axis to bottom of tube

Zo = solute concentration corresponding to extrapolation of a linear gradient distribution to zero radius

In these studies, 10 ml 15%-30% sucrose gradients were poured in Beckman Polyallomer centrifuge tubes (14 x 89 mm) with a 60% sucrose cushion (500µl). The

gradients were centrifuged in a Beckman Ultracentrifuge, using a SW-41 rotor. Therefore

$Z_1 = 15\%$

$Z_2 = 30\%$

$r_1 = 7.74cm$

$r_2 = 15.31cm$

hence $Zo = (15 \times 15.31) - (30 \times 7.74) / 15.31 - 7.74$

$= 229.65-232.2 / 7.57$

$= -0.03$

$Zo = 0$

Hence for these studies Zo=0 and in the case of DNA-containing HIV replication complexes , we used the table for densities of 1.3 (generally used for most proteins, and some plant and bacterial viruses) (see Table 2.2, section 2.6.2). For RNA complexes we used a density of 1.5 (recommended for RNA and ribosomes) (see Table 2.3 in section 2.6.2). Temperature = 4°C. Using these tables we can obtain the Time Integral (I) value for sucrose at the meniscus of the gradient and at the separated zone for the particle.

$\Delta I = I$ (sucrose % of banding position) - I(15%)

The Sedimentation Coefficient (s) is given by the equation = $\Delta I / \omega^2 t$ (Griffith, 1986).

where $\omega^2 = (0.10472 \times RPM)^2$

$RPM = 35\ 000$

$\omega^2 = (0.10472 \times 35\ 000)^2$

$\omega^2 = 1.3 \times 10^7\ sec^{-2}$

A sedimentation coefficient of $1x10^{-13}$ seconds is called a Svedberg unit or simply a Svedberg (S).

2.4 IMMUNOPRECIPITATION

An immunoprecipitation-PCR technique was used to identify viral and cellular proteins associated with the newly synthesized viral DNA. Sucrose gradient fractions containing the replication complexes were analysed by immunoprecipitation using antisera to viral proteins or histones (see below). If a particular protein was present then the entire replication complex would be precipitated by the specific antiserum. DNA PCR was then used to detect the immunoprecipitated complex by the presence of the DNA component (details below).

2.4.1 Antibodies

The following sera were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS , National Institute of Allergy and Infectious Diseases: sheep antiserum to HIV-1 p17 (catalogue no. 286, M. Phelan) ; rabbit antiserum to HIV-1 protease C-terminal peptide (catalogue no. 226, B. Korant) ; human polyclonal immunoglobulin G to HIV-1 reverse transcriptase (catalogue no. 187, J. Laurence) ; rabbit antiserum to HIV-1 integrase (catalogue nos. 756 , 757, 758, D. P. Grandgenett) ; monoclonal antibody to HIV-1 p24 core protein (catalogue no. 389 , P. Yoshihara) ; rabbit antiserum to HIV-1 Vpr (catalogue no. 808, B. Cullen) ; rabbit antiserum to HIV-1 Vif (catalogue no. 809, B. Cullen) ; rabbit antiserum to Vpu (catalogue no. 969 , F. Maldarelli and K. Strebel). Monoclonal antibody to HIV p24 (VIC6) was obtained from Dr. A. Hohmann of the Flinders Medical Centre , Adelaide. Mouse antiserum to HIV-1 p24 was also purchased from Diagnostic Technology (product code 5603). Monoclonal antibody to histones (H1 and core histones H2a , H2b , H3 and H4) was purchased from Biogenesis Ltd., Bournemouth, England (catalogue no. 4975-0204). Monoclonal antibody to RNA Polymerase II (175kD subunit) was purchased from Biodesign International, Kennebunkport, ME, USA (catalogue no. H54122M).

2.4.2 Immunoprecipitation of HIV Replication Complex

Sucrose fractions (200µl) containing the peak of the viral HIV replication complex, as detected by DNA PCR, were diluted 2-fold in buffer A/0.5% Triton-X100 and incubated with 2µl of neat antiserum overnight (16 hrs) at 4°C on a rotating platform. Immune complexes were then precipitated by incubation with 40µl of protein A-Sepharose CL-4B (Pharmacia) for 1-2 hrs at 4°C. Precipitated complexes were washed four times in a buffer containing 10mM Tris-HCl (pH 7.4), 150mM NaCl and 1% Triton X-100.

The DNA component of any viral replication complex that had been immunoprecipitated was recovered by resuspending the protein A -Sepharose pellet in buffer A and digesting with proteinase K (1mg/ml) / 10mM EDTA / 0.5% SDS for 1hr at 55°C, followed by phenol/ chloroform extraction. The DNA that had co-precipitated with HIV replication complexes was then detected using PCR as described in section 2.2.8, for a total of 20 cycles. The amplified products were analysed on 8% polyacrylamide gels, which were dried and subjected to autoradiography (section 2.2.12).

2.5 MICROCOCCAL NUCLEASE DIGESTION OF THE REPLICATION COMPLEXES

Chicken red blood cell nuclei were chosen as a positive control for generating eukaryotic nucleosome repeat patterns by digestion with micrococcal nuclease. Chicken red blood cells were obtained from chickens kept in the IMVS animal house. Chicken red blood cell nuclei were prepared according to the method of Horz and Zachau (1980). This involved pelleting the chicken red blood cells at 2000 rpm for 5 minutes at room temperature, washing the pelleted cells 3 times in 1 x SSC and then freezing them at -70°C. When required the chicken red blood cells were thawed, washed 3 times in buffer A, with the inclusion of 1mM $CaCl_2$, and digested at 1-

1.2 mg DNA / ml with 60 units / ml of micrococcal nuclease for 5, 10, 15, 20 and 25 minutes at 37° C. The resulting nucleosome repeat length of DNA was found to be 200bp-210bp as seen by ethidium bromide staining (in agreement with Horz and Zachau, 1980). The optimal incubation time at 37°C was 20 minutes.

The micrococcal nuclease (Pharmacia) used in these experiments was dissolved at 1mg / ml (8U/µl) in double-distilled water as described by the manufacturer. Aliquots of 50µl were frozen and stored at -20°C. Aliquots were thawed only once.

Cytoplasmic and nuclear extracts from a cell-to-cell infection ($1x10^8$ cells, as used routinely for sucrose gradient analysis, see section 2.2.2) were adjusted to 1mM $CaCl_2$, prewarmed to 37°C and then digested with 60 units / ml of micrococcal nuclease at 37°C. Before the addition of micrococcal nuclease and 5, 10, 15 and 20 minutes after the addition of micrococcal nuclease, an aliquot was taken out and the reaction stopped by adding EDTA to 20mM , SDS to 0.5% and proteinase K to 1mg/ml, followed by incubation at 50°C overnight. DNA was extracted twice with phenol-chloroform , ethanol precipitated, washed with 70% ethanol and dissolved in 20µl TE. Purified HIV DNA (1ng) was also digested with 60U of micrococcal nuclease. DNA samples were mixed with 6 x loading buffer, loaded onto a 1.5% agarose gel and run at 40V in 1 x TBE overnight at 4°C. DNA was detected by Southern hybridization as described in section 2.2.11.

2.6 TECHNICAL APPENDIX:

2.6.1 Buffers and Solutions

33% Acylamide : 0.9% Bis acrylamide:

Dissolve 33 g of acrylamide (BDH), 0.9 g of N,N'-methylenebisacrylamide (BDH) and DDW to 100ml. Store solution in dark bottles at 4°C.

10% Ammonium persulphate:

To 0.1g of ammonium persulphate, add DDW to 1 ml. Store at 4°C for about a week.

1M Dithiothreitol (DTT):

Dissolve 3.09 g of DTT in 20 ml of 0.01 M sodium acetate (pH 5.2). Sterilize by filtration. Dispense into 1 ml aliquots and store at -20°C.

Ethidium Bromide (Sigma):

10mg/ml dissolved in DDW. Store at 4°C in a light-proof bottle. Caution ethidium bromide is mutagenic.

Gel Loading Buffer(6x):

0.25% Bromophenol Blue (BDH), 0.25% Xylene Cyanol (Labchem), 15% Ficoll 400 (Sigma) in DDW. Store at 4°C.

Hybridization Washes:

Wash I = 2 x SSC , 0.5% SDS.

(for 500ml = 50ml 20 x SSC , 12.5ml 20% SDS, DDW)

Wash II = 2 x SSC , 0.1% SDS

(for 500ml = 50ml 20 x SSC , 2.5 ml 20% SDS , DDW)

Wash III = 0.1 x SSC , 0.1% SDS

(for 500ml = 2.5ml 20 x SSC , 2.5ml 20% SDS , DDW)

PBS (Phosphate-buffered saline):

Dissolve 8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4 and 0.24g of KH_2PO_4 in 800ml of DDW. Adjust the pH to 7.4 with with HCl. Make up to 1 lire with DDW. Aliquot and sterilize by autoclaving.

Phenol:

Crystalized phenol (Wacko) was melted at 68°C, 8-hydroxyquinoline added to final concentration of 0.1%, and the melted phenol was washed three times with 1M Tris-HCl pH8.0, then repeated washes in 0.1M Tris-HCL pH 8.0, 0.2% β-mercaptoethanol until the pH of the aqueous phase reaches 7.6. Phenol was stored under 0.1M Tris-HCl pH8.0, 0.2% β-mercaptoethanol at 4°C until required.

Phenol: Chloroform: Isoamyl alcohol (25:24:1):

Phenol (25 volumes) was mixed with chloroform (24 volumes) (BDH) and isoamyl alcohol (1 volume) (BDH). This mixture was used in phenol extraction procedures.

Protein A -SepharoseCL-4B:

Place a small amount of dry Protein A-Sepharose in a 10ml tube and add 4x the volume of sterile PBS (phosphate-buffered saline). Allow the beads to rehydrate, inverting the tube a number of times. Spin at 2000 rpm for 2 minutes, remove PBS and wash protein A-Sepharose 3x with PBS, finally resuspending the protein A-Sepharose in 2.5 volumes of PBS. Store at 4°C.

5 x RT buffer:

250mM Tris pH 7.5 , 250mM KCl , 50mM $MgCl_2$

For 100 ml = 25ml 1M Tris pH 7.5 , 25ml 1M KCl , 5 ml 1M $MgCl_2$, 45 ml DDW. Sterilize by autoclaving.

Sephadex:

Sephadex G-25 and G-50 were prepared by slowly adding the desired grade to sterile DDW in a 100 ml bottle. (5gm of Sephadex G-50 yields 80 ml of slurry). This swollen resin was washed several times with sterile DDW to remove soluble dextran. The DDW was then removed and the resin equilibrated in STE (10mM Tris pH 7.6, 1mM EDTA, 100mM NaCl) , autoclaved and stored at 4°C.

20% SDS (sodium dodecyl sulphate):

Dissolve 200g of SDS in 900ml of DDW. Heat to 68°C to assist dissolution. Adjust pH to 7.2 by adding a few drops of HCl. Adjust the volume to 1 litre with DDW. A mask and gloves were worn when weighing and preparing SDS.

Siliconized Glass Wool:

Glass wool was placed in a glass beaker and covered with Coatasil (Ajax Chemicals) and allowed to soak in. This was all done in a fumehood. The glass wool was then wrung and placed on a piece of foil and baked at 150°C for 3 hours. Pour Coatasil back into bottle for reuse. The glass wool was then washed in 100% ethanol, wrung, placed on foil and dried at 150°C for 1 hour. The siliconized glass wool was then placed in clean jars and autoclaved.

Siliconizing Hybridization Tubes:

About 100ml of Coatasil (Ajax Chemicals) was added to dry hybridization tubes, the lids placed back onto the tubes and the Coatasil swirled around a couple of times. The Coatasil was then poured back into its bottle and the hybridization tubes allowed to dry overnight in the fume hood. The hybridization tubes were washed three times with sterile DDW before use.

Southern hybridization - Gel Treatment:

Solution I = 0.25 M HCl

Solution II (Denaturing solution) = 0.5M NaOH , 1.5 M NaCl

(For 1 litre = 50 ml 10M NaOH , 300ml 5M NaCl , 650ml DDW)

Solution III (Neutralizing solution) = 1.5M NaCl , 1M Tris pH7.4

(For 1 litre = 300ml 5M NaCl , 500ml 2M Tris pH 7.4 , 200ml DDW)

20 x SSC:

175.3 g NaCl , 88.2 g Na_3 Citrate , DDW to 1 litre (pH=7.0)

STE :

100mM NaCl , 10mM Tris pH7.6 , 1mM EDTA

For 1 litre = 20ml 5M NaCl , 5 ml 2M Tris pH7.6 , 5ml 0.2M EDTA , 970 ml DDW.

Autoclave to sterilize.

50 x TAE:

242 g Tris , 57.1 ml Glacial Acetic Acid , 100ml 0.5M EDTA pH 8.0 , DDW to 1 litre.

(1xTAE = 40mM Tris-acetate , 1mM EDTA).

5 x TBE:

54 g Tris , 27.5 g boric acid , 20 ml 0.5M EDTA pH8.0 , DDW to 1 litre.

1 x TBE = 0.9M Tris-borate , 0.2mM EDTA).

TE:

10mM Tris pH 8.0 , 1mM EDTA. Sterilise by autoclaving.

1M Tris:

Dissolve 121.1 g of Tris base in 800 ml of H_2O. Adjust the pH to the desired value by adding concentrated HCL. eg. pH : HCL

7.4	70ml
7.6	60ml
8.0	42ml

Adjust the volume of the solution to 1 litre with H_2O , aliqot and sterilize by autoclaving.

2.6.2 Tables

Tables 2.1, 2.2 and 2.3 follow.

Table 2.1 shows the mixing proportions of sucrose (for total of 10 ml) and corresponding refractive indices.

Adapted from O.M. Griffith, (1986) Techniques of Preparative, Zonal, and Continuous Flow Ultracentrifugation. *Beckman Instruments, Inc.*

Desired sucrose concentration (w/w)	66% sucrose	buffer A	Refractive Index
15%	1.7ml	8.3ml	1.3557
25%	3.2ml	6.8ml	1.3723
30%	4ml	6ml	1.3811
40%	5.5ml	4.5ml	1.3997
45%	6.35ml	3.65ml	1.4096
60%	8.9ml	1.1ml	1.4418

Table 2.2 Values of Time Integral for Sucrose Gradient Centrifugation

Temperature = 5°C Particle Density 1.30

Adapted from O.M. Griffith, (1986) Techniques of Preparative, Zonal, and Continuous Flow Ultracentrifugation. *Beckman Instruments, Inc.*

Temperature 5.0°C Particle Density 1.30

WT. PCT. SUCROSE	ZD=5	ZD=0	ZD=-5	ZD=-10	ZD=-15	ZD=-20	ZD=-25	ZD=-30	ZD=-40	ZD=-60	ZD=-100
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	0.0000	0.0000	0.2648	0.1437	0.0987	0.0752	0.0607	0.0509	0.0385	0.0259	0.0156
2	0.0000	0.0000	0.5296	0.2873	0.1973	0.1503	0.1213	0.1018	0.0769	0.0517	0.0312
3	0.0000	0.5930	0.7450	0.4195	0.2927	0.2249	0.1827	0.1538	0.1169	0.0790	0.0479
4	0.0000	1.1860	0.9603	0.5516	0.3881	0.2995	0.2440	0.2058	0.1568	0.1062	0.0646
5	0.0000	1.5655	1.1484	0.6768	0.4820	0.3746	0.3066	0.2594	0.1985	0.1351	0.0825
6	0.0000	1.9449	1.3364	0.8020	0.5758	0.4497	0.3691	0.3130	0.2402	0.1640	0.1003
7	1.1347	2.2408	1.5084	0.9233	0.6695	0.5261	0.4335	0.3688	0.2841	0.1948	0.1196
8	2.2693	2.5367	1.6804	1.0446	0.7632	0.6024	0.4979	0.4245	0.3279	0.2255	0.1389
9	2.8487	2.7903	1.8431	1.1645	0.8581	0.6809	0.5649	0.4829	0.3744	0.2585	0.1598
10	3.4280	3.0439	2.0058	1.2843	0.9530	0.7594	0.6318	0.5412	0.4208	0.2915	0.1806
11	3.8521	3.2740	2.1639	1.4047	1.0502	0.8410	0.7021	0.6029	0.4704	0.3271	0.2034
12	4.2762	3.5041	2.3219	1.5251	1.1474	0.9225	0.7723	0.6645	0.5200	0.3627	0.2262
13	4.6302	3.7214	2.4788	1.6478	1.2482	1.0081	0.8466	0.7302	0.5733	0.4014	0.2512
14	4.9841	3.9387	2.6356	1.7705	1.3490	1.0936	0.9208	0.7958	0.6265	0.4400	0.2761
15	5.3017	4.1502	2.7942	1.8974	1.4547	1.1842	1.0001	0.8663	0.6842	0.4823	0.3037
16	5.6193	4.3617	2.9528	2.0242	1.5604	1.2748	1.0794	0.9367	0.7418	0.5246	0.3313
17	5.9184	4.5727	3.1158	2.1571	1.6725	1.3717	1.1648	1.0130	0.8047	0.5712	0.3620
18	6.2175	4.7837	3.2788	2.2899	1.7846	1.4686	1.2501	1.0893	0.8676	0.6177	0.3926
19	6.5093	4.9986	3.4490	2.4307	1.9047	1.5733	1.3430	1.1727	0.9369	0.6694	0.4269
20	6.8011	5.2135	3.6191	2.5715	2.0247	1.6780	1.4358	1.2560	1.0061	0.7211	0.4612
21	7.0940	5.4366	3.7993	2.7226	2.1549	1.7923	1.5377	1.3479	1.0829	0.7790	0.5000
22	7.3863	5.6597	3.9794	2.8737	2.2850	1.9065	1.6395	1.4398	1.1597	0.8368	0.5387
23	7.6879	5.8953	4.1729	3.0379	2.4276	2.0326	1.7524	1.5420	1.2457	0.9021	0.5828
24	7.9889	6.1309	4.3664	3.2021	2.5702	2.1586	1.8652	1.6442	1.3317	0.9674	0.6268
25	8.3049	6.3837	4.5771	3.3827	2.7282	2.2990	1.9916	1.7591	1.4290	1.0418	0.6774
26	8.6209	6.6364	4.7877	3.5632	2.8862	2.4394	2.1180	1.8740	1.5262	1.1161	0.7280
27	8.9590	6.9119	5.0202	3.7642	3.0633	2.5977	2.2611	2.0045	1.6372	1.2017	0.7866
28	9.2970	7.1873	5.2526	3.9652	3.2403	2.7559	2.4041	2.1350	1.7482	1.2872	0.8451
29	9.6654	7.4922	5.5127	4.1920	3.4414	2.9364	2.5679	2.2850	1.8764	1.3866	0.9137
30	10.0338	7.7971	5.7728	4.4188	3.6424	3.1169	2.7317	2.4349	2.0046	1.4859	0.9823
31	10.4427	8.1401	6.0681	4.6781	3.8735	3.3254	2.9216	2.6092	2.1544	1.6028	1.0635
32	10.8515	8.4830	6.3634	4.9374	4.1046	3.5339	3.1114	2.7835	2.3041	1.7197	1.1447
33	11.3135	8.8750	6.7038	5.2382	4.3741	3.7780	3.3345	2.9889	2.4814	1.8588	1.2420
34	11.7754	9.2669	7.0442	5.5390	4.6436	4.0221	3.5575	3.1942	2.6586	1.9979	1.3392
35	12.3077	9.7232	7.4435	5.8939	4.9631	4.3125	3.8238	3.4400	2.8716	2.1661	1.4576
36	12.8399	10.1794	7.8427	6.2488	5.2825	4.6029	4.0900	3.6857	3.0846	2.3343	1.5759
37	13.4655	10.7205	8.3194	6.6749	5.6676	4.9542	4.4130	3.9846	3.3447	2.5408	1.7222
38	14.0910	11.2615	8.7961	7.1009	6.0526	5.3055	4.7360	4.2835	3.6048	2.7472	1.8684
39	14.8429	11.9171	9.3772	7.6227	6.5262	5.7390	5.1356	4.6542	3.9286	3.0056	2.0524
40	15.5947	12.5726	9.9582	8.1445	6.9997	6.1724	5.5351	5.0248	4.2523	3.2639	2.2364
41	16.5216	13.3865	10.6837	8.7989	7.5957	6.7196	6.0409	5.4950	4.6644	3.5945	2.4732
42	17.4484	14.2003	11.4091	9.4533	8.1917	7.2667	6.5466	5.9651	5.0765	3.9251	2.7100
43	18.6219	15.2375	12.3383	10.2949	8.9608	7.9748	7.2027	6.5763	5.6141	4.3583	3.0221
44	19.7953	16.2746	13.2675	11.1365	9.7299	8.6829	7.8587	7.1874	6.1516	4.7915	3.3342
45	21.3234	17.6330	14.4902	12.2481	10.7489	9.6236	8.7323	8.0027	6.8711	5.3740	3.7561
46	22.8514	18.9914	15.7128	13.3596	11.7679	10.5642	9.6058	8.8180	7.5906	5.9565	4.1779
47	24.9082	20.8296	17.3744	14.8756	13.1617	11.8541	10.8062	9.9405	8.5841	6.7644	4.7661
48	26.9649	22.6677	19.0360	16.3916	14.5555	13.1440	12.0065	11.0630	9.5776	7.5723	5.3542
49	29.8450	25.2542	21.3833	18.5401	16.5364	14.9814	13.7199	12.6680	11.0024	8.7358	6.2055
50	32.7251	27.8407	23.7305	20.6886	18.5172	16.8188	15.4332	14.2729	12.4272	9.8993	7.0568
51	36.9604	31.6613	27.2104	23.8835	21.4703	19.5642	17.9981	16.6796	14.5696	11.6559	8.3483
52	41.1957	35.4819	30.6902	27.0784	24.4234	22.3095	20.5630	19.0863	16.7120	13.4125	9.6398
53	47.8271	41.4886	36.1797	32.1328	29.1066	26.6722	24.6464	22.9239	20.1373	16.2320	11.7226
54	54.4584	47.4952	41.6692	37.1872	33.7897	31.0349	28.7297	26.7615	23.5626	19.0515	13.8054
55	65.8345	57.8388	51.1522	45.9418	41.9199	38.6238	35.8448	33.4586	29.5555	24.0031	17.4799
56	77.2105	68.1824	60.6352	54.6964	50.0500	46.2126	42.9599	40.1557	35.5483	28.9546	21.1544
57	99.8914	88.8782	79.6655	72.3092	66.4419	61.5420	57.3560	53.7257	47.7213	39.0491	28.6793
58	122.5723	109.5740	98.6957	89.9220	82.8338	76.8713	71.7521	67.2957	59.8942	49.1435	36.2041
59	188.1689	169.6339	154.0808	141.3076	130.7586	121.7720	113.9878	107.1650	95.7462	78.9823	58.5478
60	253.7655	229.6937	209.4658	192.6931	178.6833	166.6727	156.2235	147.0343	131.5981	108.8210	80.8914

Table 2.3 Values of Time Integral for Sucrose Gradient Centrifugation

Temperature = 5°C Particle Density 1.50

Adapted from O.M. Griffith, (1986) Techniques of Preparative, Zonal, and Continuous Flow Ultracentrifugation. *Beckman Instruments, Inc.*

Temperature 5.0°C Particle Density 1.50

WT. PCT. SUCROSE	ZD=5	ZD=0	ZD=-5	ZD=-10	ZD=-15	ZD=-20	ZD=-25	ZD=-30	ZD=-40	ZD=-60	ZD=-100
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	0.0000	0.0000	0.2618	0.1420	0.0975	0.0743	0.0600	0.0503	0.0380	0.0256	0.0155
2	0.0000	0.0000	0.5236	0.2840	0.1950	0.1485	0.1199	0.1006	0.0760	0.0511	0.0309
3	0.0000	0.5800	0.7341	0.4132	0.2883	0.2215	0.1799	0.1515	0.1151	0.0778	0.0472
4	0.0000	1.1600	0.9446	0.5424	0.3815	0.2944	0.2398	0.2023	0.1541	0.1044	0.0635
5	0.0000	1.5267	1.1263	0.6634	0.4722	0.3670	0.3003	0.2541	0.1944	0.1323	0.0808
6	0.0000	1.8933	1.3080	0.7843	0.5628	0.4395	0.3607	0.3059	0.2347	0.1602	0.0980
7	1.0838	2.1757	1.4721	0.9000	0.6523	0.5124	0.4222	0.3591	0.2765	0.1896	0.1164
8	2.1675	2.4581	1.6362	1.0157	0.7417	0.5852	0.4836	0.4122	0.3183	0.2189	0.1347
9	2.7132	2.6969	1.7894	1.1286	0.8310	0.6591	0.5466	0.4672	0.3621	0.2500	0.1544
10	3.2589	2.9356	1.9426	1.2414	0.9203	0.7330	0.6096	0.5221	0.4058	0.2810	0.1741
11	3.6527	3.1492	2.0893	1.3532	1.0106	0.8087	0.6748	0.5793	0.4518	0.3141	0.1952
12	4.0464	3.3628	2.2360	1.4649	1.1008	0.8843	0.7400	0.6365	0.4978	0.3471	0.2163
13	4.3701	3.5615	2.3794	1.5771	1.1930	0.9625	0.8079	0.6965	0.5465	0.3825	0.2392
14	4.6937	3.7601	2.5228	1.6893	1.2851	1.0407	0.8758	0.7565	0.5952	0.4178	0.2620
15	4.9795	3.9504	2.6655	1.8034	1.3802	1.1222	0.9471	0.8199	0.6471	0.4558	0.2868
16	5.2652	4.1406	2.8081	1.9175	1.4752	1.2037	1.0184	0.8833	0.6989	0.4938	0.3116
17	5.5296	4.3272	2.9522	2.0349	1.5743	1.2894	1.0939	0.9508	0.7545	0.5350	0.3387
18	5.7940	4.5137	3.0962	2.1522	1.6733	1.3750	1.1693	1.0182	0.8101	0.5762	0.3658
19	6.0472	4.7002	3.2438	2.2744	1.7775	1.4659	1.2498	1.0905	0.8702	0.6210	0.3956
20	6.3003	4.8866	3.3914	2.3965	1.8817	1.5567	1.3303	1.1627	0.9302	0.6658	0.4253
21	6.5493	5.0763	3.5446	2.5250	1.9923	1.6538	1.4169	1.2408	0.9955	0.7150	0.4583
22	6.7982	5.2659	3.6978	2.6534	2.1029	1.7509	1.5035	1.3189	1.0608	0.7642	0.4912
23	7.0486	5.4618	3.8587	2.7900	2.2215	1.8557	1.5974	1.4039	1.1323	0.8185	0.5278
24	7.2990	5.6577	4.0196	2.9265	2.3400	1.9605	1.6912	1.4889	1.2038	0.8728	0.5644
25	7.5556	5.8629	4.1907	3.0731	2.4683	2.0745	1.7938	1.5822	1.2828	0.9332	0.6055
26	7.8121	6.0681	4.3617	3.2197	2.5966	2.1885	1.8964	1.6755	1.3617	0.9935	0.6466
27	8.0796	6.2861	4.5456	3.3788	2.7367	2.3137	2.0096	1.7788	1.4496	1.0612	0.6929
28	8.3470	6.5040	4.7294	3.5378	2.8768	2.4389	2.1228	1.8820	1.5374	1.1288	0.7392
29	8.6303	6.7385	4.9294	3.7121	3.0314	2.5777	2.2487	1.9973	1.6360	1.2052	0.7920
30	8.9136	6.9729	5.1293	3.8864	3.1859	2.7165	2.3746	2.1125	1.7345	1.2816	0.8447
31	9.2183	7.2284	5.3494	4.0796	3.3581	2.8718	2.5161	2.2424	1.8461	1.3687	0.9052
32	9.5229	7.4839	5.5694	4.2728	3.5303	3.0271	2.6575	2.3723	1.9576	1.4557	0.9657
33	9.8553	7.7660	5.8144	4.4893	3.7242	3.2028	2.8181	2.5201	2.0852	1.5559	1.0357
34	10.1877	8.0480	6.0593	4.7058	3.9181	3.3784	2.9786	2.6678	2.2127	1.6560	1.1057
35	10.5563	8.3639	6.3357	4.9515	4.1393	3.5795	3.1629	2.8380	2.3602	1.7724	1.1876
36	10.9248	8.6798	6.6121	5.1972	4.3604	3.7805	3.3472	3.0081	2.5076	1.8888	1.2695
37	11.3396	9.0386	6.9282	5.4797	4.6157	4.0134	3.5613	3.2063	2.6801	2.0257	1.3665
38	11.7543	9.3973	7.2442	5.7621	4.8710	4.2463	3.7754	3.4044	2.8525	2.1626	1.4634
39	12.2290	9.8111	7.6111	6.0915	5.1700	4.5199	4.0277	3.6384	3.0569	2.3257	1.5796
40	12.7036	10.2249	7.9779	6.4209	5.4689	4.7935	4.2799	3.8723	3.2612	2.4887	1.6957
41	13.2570	10.7109	8.4111	6.8116	5.8247	5.1202	4.5819	4.1530	3.5073	2.6861	1.8346
42	13.8104	11.1968	8.8442	7.2023	6.1805	5.4469	4.8838	4.4337	3.7533	2.8834	1.9734
43	14.4675	11.7776	9.3645	7.6735	6.6111	5.8434	5.2511	4.7759	4.0542	3.1259	2.1506
44	15.1246	12.3583	9.8847	8.1447	7.0417	6.2398	5.6184	5.1180	4.3551	3.3684	2.3278
45	15.9185	13.0640	10.5199	8.7222	7.5711	6.7285	6.0722	5.5416	4.7289	3.6710	2.5470
46	16.7124	13.7697	11.1551	9.2997	8.1005	7.2171	6.5259	5.9651	5.1026	3.9736	2.7661
47	17.6902	14.6436	11.9450	10.0204	8.7631	7.8303	7.0965	6.4987	5.5749	4.3576	3.0591
48	18.6680	15.5175	12.7349	10.7410	9.4256	8.4434	7.6671	7.0322	6.0471	4.7416	3.3520
49	19.8979	16.6220	13.7372	11.6584	10.2714	9.2279	8.3987	7.7175	6.6554	5.2384	3.7020
50	21.1278	17.7265	14.7395	12.5758	11.1171	10.0124	9.1302	8.4027	7.2637	5.7351	4.0520
51	22.7103	19.1539	16.0395	13.7694	12.2203	11.0380	10.0883	9.3017	8.0640	6.3912	4.5344
52	24.2927	20.5813	17.3395	14.9629	13.3235	12.0635	11.0464	10.2007	8.8642	7.0473	5.0167
53	26.3790	22.4709	19.0664	16.5528	14.7966	13.4358	12.3307	11.4077	9.9415	7.9340	5.6717
54	28.4653	24.3605	20.7932	18.1426	16.2696	14.8080	13.6150	12.6147	11.0188	8.8207	6.3266
55	31.2944	26.9326	23.1512	20.3193	18.2910	16.6947	15.3839	14.2795	12.5085	10.0514	7.2399
56	34.1234	29.5047	25.5091	22.4960	20.3123	18.5813	17.1527	15.9443	13.9981	11.2821	8.1531
57	38.0795	33.1141	28.8277	25.5671	23.1703	21.2539	19.6624	18.3099	16.1200	13.0415	9.4644
58	42.0356	36.7235	32.1462	28.6382	26.0283	23.9264	22.1721	20.6754	18.2418	14.8008	10.7756
59	47.7696	41.9721	36.9851	33.1268	30.2139	27.8473	25.8597	24.1561	21.3711	17.4044	12.7245
60	53.5036	47.2206	41.8240	37.6154	34.3994	31.7681	29.5473	27.6367	24.5003	20.0080	14.6734

CHAPTER 3

CHARACTERIZATION OF HIV REPLICATION COMPLEXES EARLY AFTER CELL-TO-CELL INFECTION

3.1 INTRODUCTION AND AIMS

Shortly after HIV has entered the cytoplasm of a host cell, the viral RNA genome is found in the cytoplasm of the host cell in the form of a nucleoprotein complex (Ellison *et al.*, 1990; Farnet and Haseltine, 1990; Farnet and Haseltine, 1991; Bukrinsky *et al.*, 1993). It is within this nucleoprotein complex, it is assumed, where the single-stranded RNA genome is converted into a linear, double-stranded DNA molecule by the viral enzyme reverse transcriptase. It is believed that the nucleoprotein complex containing the double-stranded DNA molecule then migrates into the nucleus where integration into the host genome takes place. HIV nucleoprotein complexes isolated from the cytoplasm and nucleus of infected cells are both capable of mediating the integration of the viral DNA into heterologous DNA targets *in vitro* (Ellison *et al.*, 1990; Farnet and Haseltine, 1990). For murine leukaemia virus, it was demonstrated the viral capsid protein was associated with a cytoplasmic nucleoprotein complex of ~160S in size (Brown *et al.*, 1987; Fujiwara and Mizuuchi, 1988; Bowerman *et al.*, 1989)

At the commencement of this study, knowledge of HIV replication complexes was limited to the above demonstration of *in vitro* integration. The aim of this section was to use a one-step cell-to-cell infection model (Li and Burrell, 1992; Li *et al.*, 1992) to investigate the structure and protein composition of the HIV replication complexes in the cytoplasm and in the nucleus of the infected cells. In this section, the use of DNA PCR to identify such complexes meant that, by definition, only those structures that were associated with products of *de novo* reverse transcription would be detected.

3.2 RESULTS

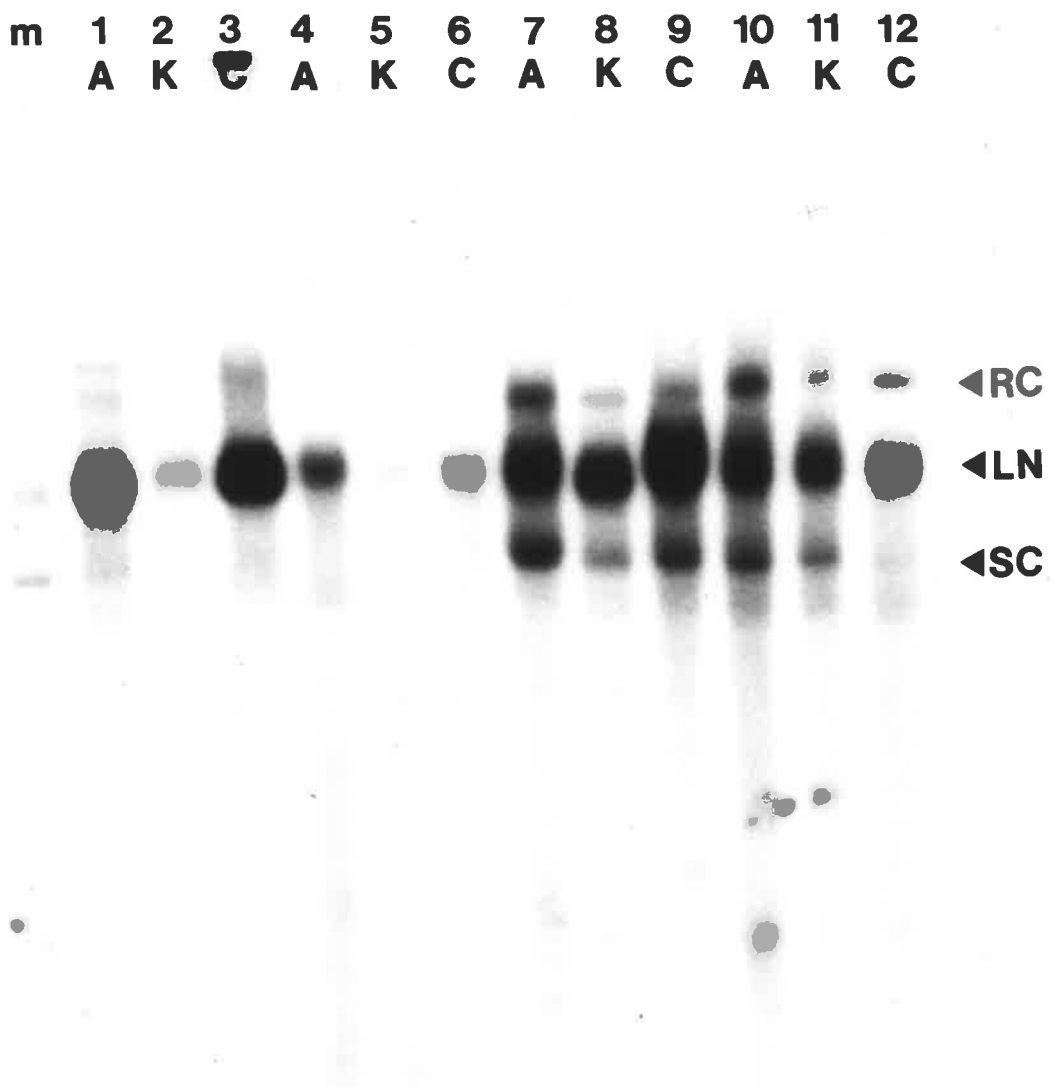
3.2.1 Cell-to-Cell Infection

In our cell-to-cell infection model, HIV persistently-infected cells (H3B) are mixed with uninfected cells (HUT-78) in a ratio of 1:4. After 3-4 hours giant cell formation and ballooning of cells is seen and unintegrated linear DNA can be detected by Southern analysis The level of unintegrated linear DNA reaches a peak at about 6 hours after mixing, with the appearance of the circular forms of DNA at about 8 hours. For this study, cells were harvested at 6 hours after mixing to ensure that only the linear form was present.

3.2.2 Extraction of HIV Replication Complexes

An appropriate extraction procedure, for extracting DNA-containing replication complexes from the separate cytoplasmic and nuclear compartments, needed to be developed. Three buffers previously used by others to isolate cytoplasmic and nuclear extracts were compared. Buffer A (Brown *et al.,* 1987) contained 10mM Tris-HCl (7.4), 225mM KCl, 5mM $MgCl_2$, 1mM DTT, 20µg/ml aprotinin. Buffer K (Farnet and Haseltine, 1990) consisted of 20mM Hepes (pH 7.4), 150mM KCl, 5mM $MgCl_2$, 1mM DTT, 20µg/ml aprotinin. Thirdly, cell wash buffer (CWB) (Vaughn *et al.,* 1990) was composed of 5mM Tris-HCl (7.4), 50mM KCl, 0.5mM EDTA, 0.05mM Spermine, 0.125mM spermidine, 2mM DTT. Cells ($2.5x10^6$) were harvested at 6 and 18 hours after infection, by centrifuging the cells at 930rpm for 3 mins, washing the cells in one of the three extraction buffers, centrifuging again and resuspending the cells in 250µl of the corresponding extraction buffer. The cells were finally lysed using 0.05% Triton X-100 for 10 minutes at room temperature. Overall, the recovery of unintegrated DNA from the cytoplasm and nucleus using buffer A or CWB were similar (Figure 3.1). Buffer K was the least efficient buffer for the recovery of unintegrated DNA from both the cytoplasm and the nucleus. Buffer A and CWB were compared further a number of times and buffer A was finally chosen for the extraction of the replication complexes.

Figure 3.1 Comparison of extraction buffers for efficient DNA recovery. Buffer A **(A)**, buffer K **(K)** and cell wash buffer CWB **(C)** were prepared as described in the text. Cells were harvested at 6 and 18 hours after cell-to-cell infection, and lysed with 0.05% Triton X-100 for 10 minutes at room temperature in buffers A, K, or C as indicated. Extracts were treated with proteinase K and extracted with phenol prior to detection by Southern hybridization as described in Materials and Methods. Lanes 1, 2, 3 = cytoplasmic extracts 6 hours after infection. Lanes 4, 5, 6 = nuclear extracts 6 hours after infection. Lanes 7, 8, 9 = cytoplasmic extracts 18 hours after infection. Lanes 10, 11, 12 = nuclear extracts 18 hours after infection. RC: relaxed circular form of unintegrated DNA. LN: linear DNA. SC= supercoiled form of unintegrated DNA. m: HIV unintegrated DNA marker containing RC, LN and SC forms of DNA.


m
1 A
2 K
3 C
4 A
5 K
6 C
7 A
8 K
9 C
10 A
11 K
12 C
◀RC
◀LN
◀SC

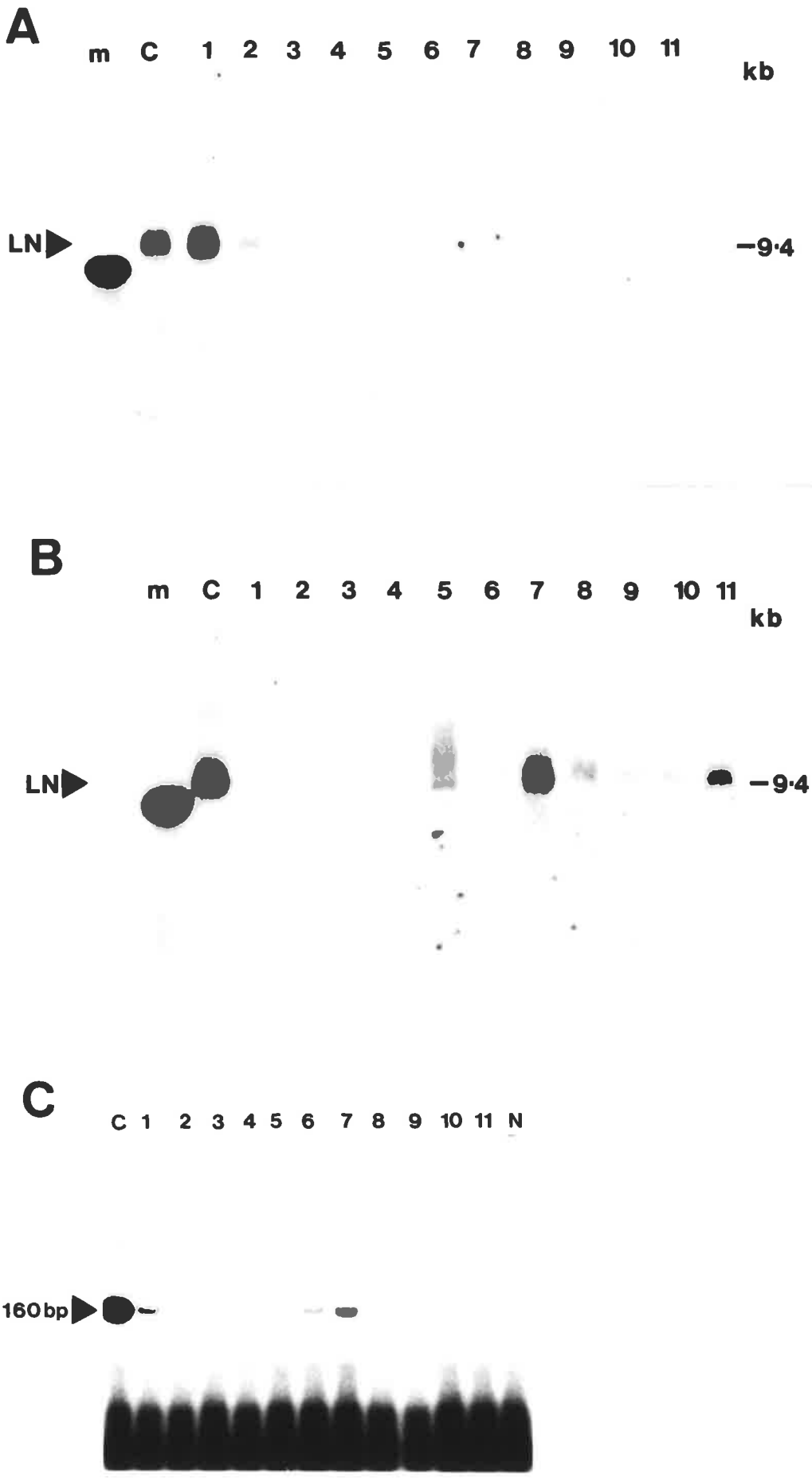
The aim of this extraction was to lyse the cell membrane and to release the cytoplasm, but to retain the integrity of the viral replication complexes in the cytoplasm and the nuclei intact. The nuclei could then be disrupted and the nuclear fraction collected separately. Hence the concentration of detergent, used to lyse the cells, was also examined in terms of damage caused to the nuclei. Two mild detergents, Triton X-100 and digitonin were tested. Various concentrations of Triton X-100 and digitonin (0.025% , 0.05% , 0.1% , 0.05% , 1%) at both room temperature and 37°C for 10, 15, and 30 minutes were compared. The lysed cells were mixed with an equal volume of 0.1% Trypan blue and examined under a light microscope. Conditions in which the cell membranes were disrupted but the nuclei were still intact and undamaged were sought. Best results were obtained with 0.05% Triton X-100 in buffer A for 10 minutes at room temperature (data not shown).

3.2.3 Cytoplasmic HIV Replication Complexes Following Cell-to-Cell Infection

Cytoplasmic extracts harvested 6 hrs after cell-to-cell infection were examined for the presence of HIV DNA-containing nucleoprotein complexes. The cytoplasmic extracts were subjected to velocity sedimentation through sucrose gradients (15%-30%) at 35000 rpm at 4°C, and the viral DNA was detected by Southern hybridization. After 3hrs centrifugation, all unintegrated HIV DNA sedimented to the bottom of the gradient (Figure 3.2A), suggesting that it was associated with proteins in a nucleoprotein complex. After reducing the centrifugation time to 50 mins at 35000 rpm at 4°C, the cytoplasmic nucleoprotein complex was found in fraction 7 (21% sucrose) (Figure 3.2B). Both Southern blotting (Figure 3.2B) and PCR (Figure 3.2C) detected the DNA in the same position in the sucrose gradient. From these results, the sedimentation coefficient of this HIV structure was calculated as ~320S as shown in section 3.2.4. Unintegrated HIV DNA deproteinated by treatment with proteinase K and phenol

Figure 3.2 Sucrose gradient sedimentation of HIV replication complex in cytoplasmic extracts 6hrs after cell-to-cell virus infection.

Six hours after cell mixing, cytoplasmic extracts were prepared as described in section 2.2.2 and a 1ml aliquot was loaded onto 15%-30% sucrose gradients, which were then centrifuged at 35000 rpm for **(A)** 3hrs at 4°C and **(B)** 50 mins at 4°C, in a Beckman SW41 rotor. The gradients were fractionated into 1ml fractions and viral DNA was extracted and identified by Southern analysis as described in Materials and Methods. **(C)** As for **(B)** except that HIV DNA detection was by PCR, using primers gag1 and gag2, as described in Materials and Methods. 1-11 refer to fractions from bottom to top of the gradient. C: unfractionated cytoplasmic extract. m in **(A)** and **(B)**: 9.4 Kb Sac I HIV-1 DNA marker 100pg. N in **(C)** PCR negative control. LN: linear form of unintegrated DNA.


A
m C 1 2 3 4 5 6 7 8 9 10 11
kb
LN
9·4
B
m C 1 2 3 4 5 6 7 8 9 10 11
kb
LN
9·4
C
C 1 2 3 4 5 6 7 8 9 10 11 N
160bp

extraction prior to sedimentation, did not enter the sucrose gradient, remaining at the top (Figure 3.3).

Cytoplasmic extracts were treated with RNAse A (as described in section 2.2.2) prior to velocity sedimentation through sucrose gradient, in order to clarify whether RNA was involved in the ~320S nucleoprotein complex. RNAse A treatment did not affect the size of the complex. Similarly , when sucrose fractions containing the complex were isolated and treated with RNAse A, and then subjected to velocity sedimentation for a second time on a sucrose gradient, the complex sedimented to the same position.

The possibility that the virus-producing H3B cells might contain maturing replication complexes with viral DNA already present was answered by analysing a zero hour cell extract (Figure 3.4A). No DNA-containing complexes were detected at zero hours, indicating the complexes detected at 6 hrs (Figure 3.4B) were due to a new round of viral replication after cell mixing.

In order to investigate whether the 320S nucleoprotein complex could be dissociated into smaller subunits by detergent, cytoplasmic extracts were treated with various concentration of SDS (0.1% , 0.5% , 1%) , NP-40 (0.5% , 1%) and triton X-100 (0.5% , 1% , 2%), for 10 mins at room temperature, prior to loading onto a 15%-30% sucrose gradient. Such detergent treatment had either no effect on the complex size , or led to a gradient profile resembling that seen with deproteinated DNA. These results suggest the 320S complex could not be dissociated into smaller complexes by using relatively mild detergent conditions and hence was probably not an aggregate of smaller subunits loosely linked by weak hydrophobic interactions.

The presence of lipid membrane within the complex could lead to the complex having such a large sedimentation coefficient. Caliguiri and Tamm (1970) showed that

Figure 3.3 **Linear viral DNA**, prepared from cell-to-cell (6hrs) infection and deproteinated by treatment with proteinase K and phenol extraction (section 2.2.1, Hirt Extraction) prior to sedimentation. Deproteinated DNA was layered onto 15%-30% sucrose gradient, and centrifuged at 35000 rpm for 3hrs at 4°C. The gradients were fractionated into 1ml fractions and viral DNA was extracted and identified by Southern analysis as described in section 2.2.11. 1-11 refer to fractions from bottom to top of the gradient. C: unfractionated deproteinated DNA. M: Sac I HIV-1 DNA marker 100pg. LN: linear unintegrated DNA.

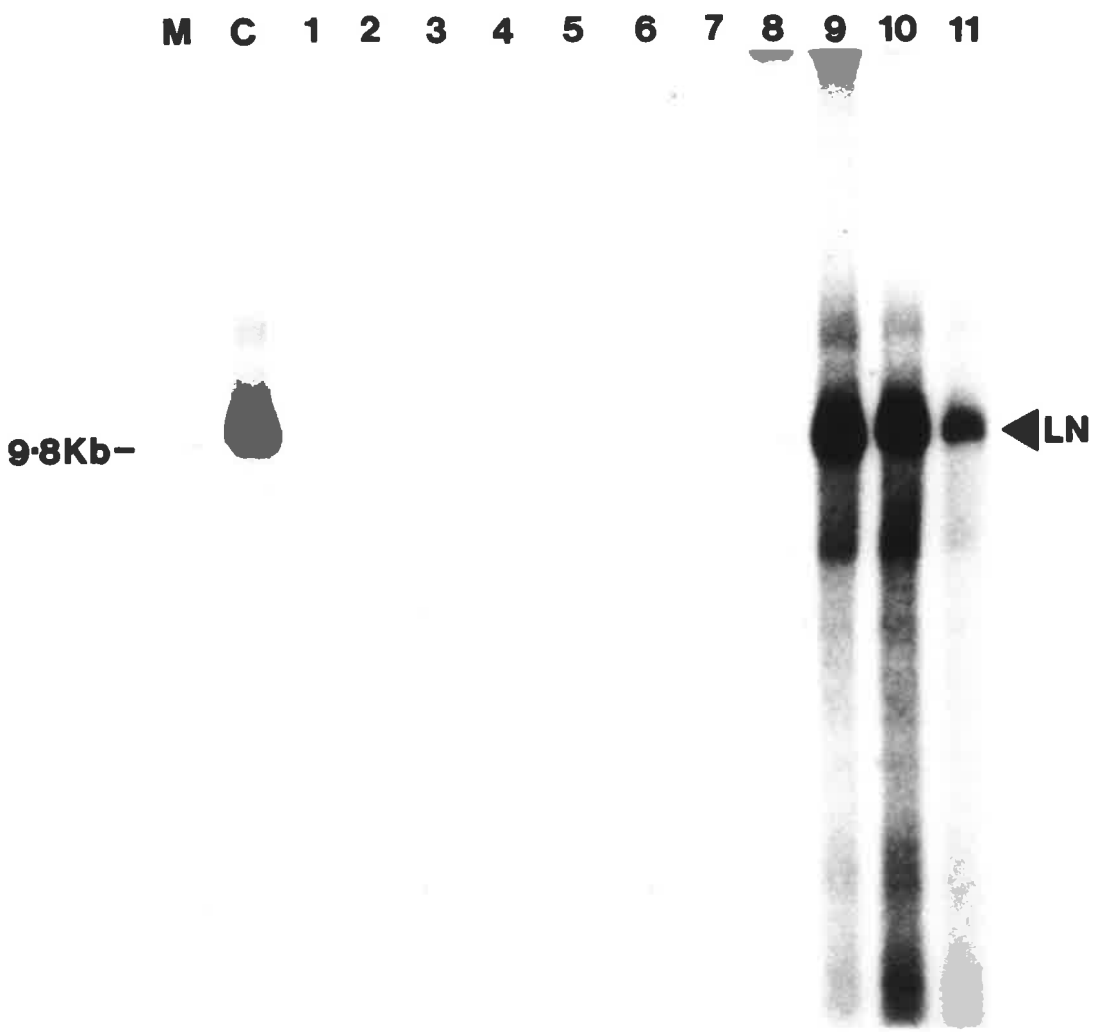

M C 1 2 3 4 5 6 7 8 9 10 11
9·8Kb–
LN

Figure 3.4 Sucrose gradient sedimentation of HIV replication complex in cytoplasmic extract from a cell-to-cell virus infection.

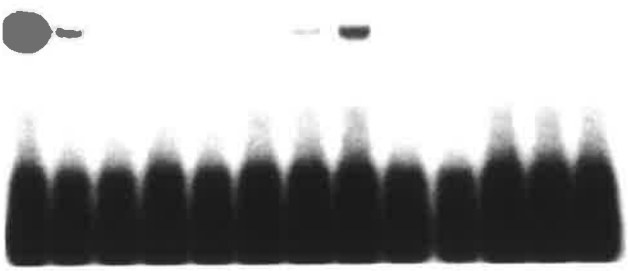
(A) Zero and **(B)** six hours after cell mixing, cytoplasmic extracts were prepared and RNAse A treated as described in Materials and Methods and a 1ml aliquot was loaded onto 15%-30% sucrose gradients, which were then centrifuged at 35000 rpm for 50 mins, at 4°C in a Beckman SW41 rotor. 1ml fractions (1-11) were collected from the bottom of the gradient. DNA was prepared and detected by PCR as described in section 2.2.8. N: PCR negative control. C: unfractionated extracts.

A 0 Hr fractions

C 1 2 3 4 5 6 7 8 9 10 11

B 6 Hr fractions

C 1 2 3 4 5 6 7 8 9 10 11 N



isopycnic centrifugation in discontinuous sucrose density gradients allowed the separation of smooth and rough membranes in cytoplasmic extracts (Caliguiri and Tamm, 1970). Cytoplasmic extracts containing the HIV nucleoprotein complex were subjected to isopycnic centrifugation in discontinuous sucrose gradients and the unintegrated HIV DNA was detected by PCR , using *gag* -specific primers (Figure 3.5). Unintegrated HIV DNA was found in the lower portion of the gradient (density, 1.27-1.3 g/ml). This gradient position corresponds to the area where ribosomes and other nucleoproteins band , and was distinct from the location of cellular membranes (1.16-1.25 g/ml)(Caliguiri and Tamm, 1970). Hence the 320S cell-to-cell cytoplasmic nucleoprotein complex was not significantly associated with any cellular membranes.

3.2.4 Calculation of Sedimentation Coefficient

The sedimentation coefficient of the nucleoprotein complex was estimated by the method of McEwen (1967), as described in section 2.3.4. In the case of DNA nucleoprotein complexes, we used the table for densities of 1.3 (see Table 2.2), since after isopycnic centrifugation in discontinuous sucrose gradients the complex banded at a density of approximately 1.3 g/ml (section 3.2.3). Temperature = 4°C. Using these tables we can obtain the Time Integral (I) value for sucrose at the meniscus of the gradient and at the separated zone for the particle.

$$\Delta I = I \text{ (sucrose \% of banding position)} - I(15\%)$$

$$= I\,(21\%) - I(15\%) = 5.4366 - 4.1502$$

$$= 1.2864$$

then the Sedimentation Coefficient $(S) = \Delta I / \omega^2 t$

where $\omega^2 = (0.10472 \times RPM)^2$

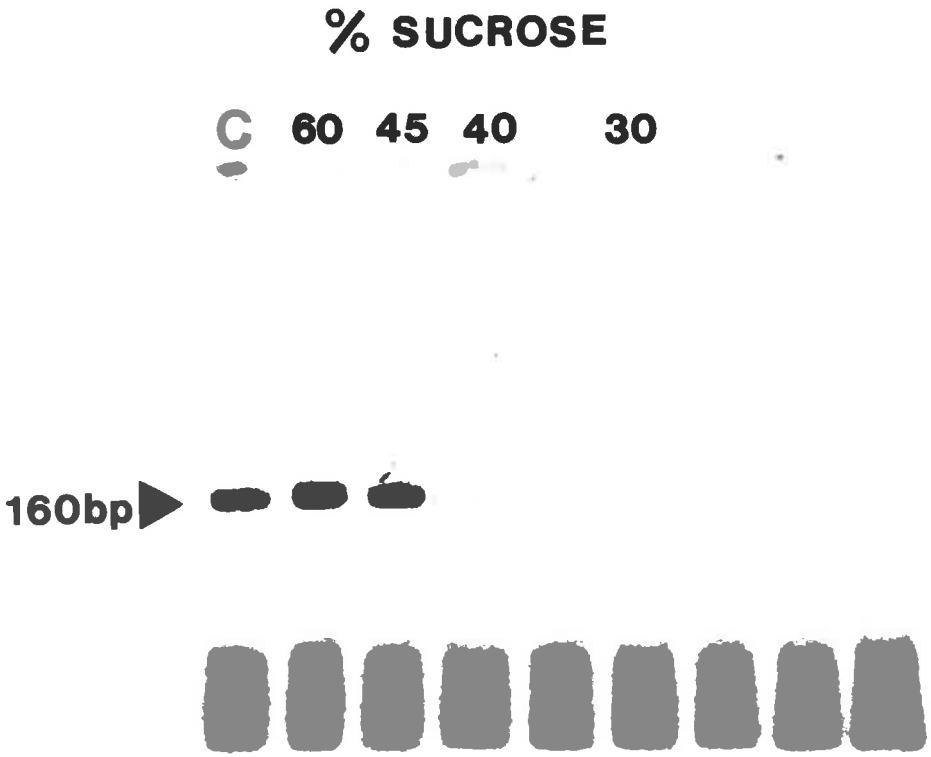
$RPM = 35\,000$

$\omega^2 = (0.10472 \times 35\,000)^2$

$\omega^2 = 1.343 \times 10^7 \text{ sec}^{-2}$

Figure 3.5 Isopycnic centrifugation of HIV cytoplasmic replication complex 6 hrs after a cell-to-cell virus infection.

Discontinuous sucrose-density gradients were prepared as described in section 2.3.3 and infected cytoplasmic extracts were centrifuged at 26000 rpm for 16hrs at 4°C in a Beckman SW41 rotor. Fractions were collected from the bottom of the gradient. Viral DNA was prepared and detected by PCR as described in section 2.2.8. Unintegrated HIV DNA was detected in the lower portion of the gradient where the density of sucrose was between 1.27-1.3 g/ml. C: unfractionated cytoplasmic extract.


% SUCROSE
C
60
45
40
30
160bp

and $t = 50$ minutes

$= 3000$ sec

Therefore Sedimentation Coefficient $(S) = \Delta I / \omega^2 t$

$= 1.2864 / 1.343 \times 10^7 \times 3000$

$= 1.2864 / 4.03 \times 10^{10}$

$= 3.192 \times 10^{-11}$ sec

$= 320$ S

The sedimentation coefficient calculations were further verified using ^{3}H-uridine labelled poliovirus as an internal marker (160S) in the sucrose gradient. After a 3 hour centrifugation at 35000 rpm at 4°C on a 15% - 30% sucrose gradient the ^{3}H-uridine labelled poliovirus was found in fraction 5 (25.5% sucrose). This correctly corresponded to a sedimentation coefficient of 160S.

3.2.5 Characterization of HIV DNA-containing Nuclear Complexes

Nuclear extracts of cells at 6hrs following cell-to-cell infection were treated with RNAseA and analysed on 15%-30% sucrose gradients in a similar way to the cytoplasmic extracts(Figure 3.6). After 3 hrs sedimentation at 35000 rpm , HIV DNA was found in fractions 8 and 9 (Figure 3.6A), indicating that the nuclear HIV nucleoprotein complex was much smaller than the cytoplasmic complex, with a sedimentation coefficient of 80S (Figure 3.6B). These results suggested that the replication complexes underwent structural changes associated with transport from the cytoplasm to the nucleus.

3.2.6 Calculation of Nuclear Complex Sedimentation Coefficient

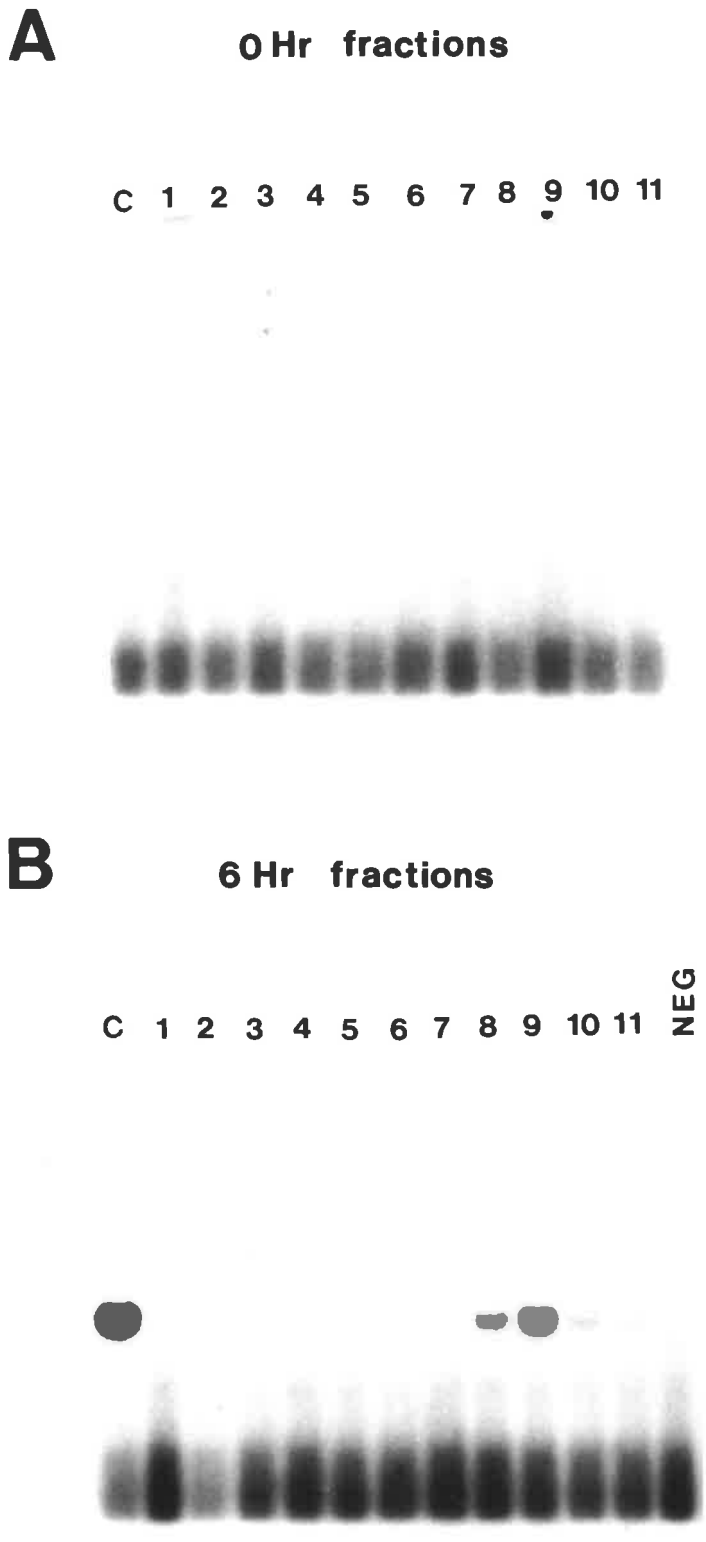
$\Delta I = I$ (sucrose % of banding position) - I(15%)

$= I(20.5\%) - I(15\%) = 5.32505 - 4.1502$

$= 1.17485$

Figure 3.6 Sucrose gradient sedimentation of HIV replication complex in nuclear extracts from a cell-to-cell virus infection.

(A) Zero and **(B)** six hours after cell mixing, nuclear extracts were prepared and RNAse A treated as described in Materials and Methods and a 1ml aliquot was loaded onto 15%-30% sucrose gradients, which were then centrifuged at 35000 rpm for 3hrs, at 4°C in a Beckman SW41 rotor. 1ml fractions (1-11) were collected from the bottom of the gradient. DNA was prepared and detected by PCR as described in section 2.2.8. N: PCR negative control. C: unfractionated extracts


A
0 Hr fractions
C 1 2 3 4 5 6 7 8 9 10 11
B
6 Hr fractions
C 1 2 3 4 5 6 7 8 9 10 11 NEG

$\omega^2 = 1.343 \times 10^7$ sec^{-2} (as in section 3.2.4)

and $t = 3$ hours $= 10800$ sec

Therefore Sedimentation Coefficient (S) $= \Delta I / \omega^2 t$

$= 1.17485 / 1.343 \times 10^7 \times 10800$

$= 8.1 \times 10^{-12}$ sec

$= 80$ S

3.2.7 Protein Composition of HIV Replication Complexes

Sucrose gradient fractions containing the peak of viral DNA from both cytoplasmic and nuclear extracts were analysed by immunoprecipitation using antisera to the viral proteins integrase, reverse transcriptase, matrix protein (p17), capsid protein (p24), Vpu, Vpr, Vif, protease, monoclonal antibody to histones (H1 and core histones H2a, H2b, H3, H4) and also monoclonal antibody to RNA polymerase II (175 kD subunit). All antisera used detected the appropriate protein in Western-blot analysis.

Initially, immunoprecipitation followed by Western-blot analysis was attempted, however no bands corresponding to viral proteins were osbserved. Hence, Western-blot detection was not sufficiently sensitive to detect the low level of protein associated with the complexes isolated on sucrose gradients. Hence PCR detection of viral DNA component of the HIV replication complex in an immunoprecipitate was used to demonstrate that viral replication complexes had been precipitated by the particular antibody used.

HIV DNA was detected in the pellet after precipitation of cytoplasmic extracts with antisera to integrase, reverse transcriptase, p17, protease, Vpr, and histones (Figure 3.7A). In contrast, with nuclear extracts, only antisera to integrase, protease and histones precipitated HIV DNA-containing complexes (Figure 3.7B). These findings (Table 3.1) suggested that the complexes had lost their association with detectable

Figure 3.7 Immunoprecipitation of HIV replication complexes.

Sucrose gradient fractions containing **(A)** cytoplasmic replication complex and **(B)** nuclear replication complex were immunoprecipitated with antiserum to histones, integrase, reverse transcriptase (RT), matrix protein p17, protease, capsid protein p24, RNA polymerase II, vpu, vpr and vif. pA-Seph: consists of sucrose fraction containing protein A-sepharose and no antibody. Neg: is the PCR negative control. DNA was detected by PCR as described in section 2.2.8.

A
histone
integrase
RT
p17
protease
p24
pA-Seph
Neg
ANTISERUM TO
histones
integrase
p24
pol
vpu
vpr
vif
pA-Seph
Neg
B
histone
integrase
RT
p17
protease
p24
pA-Seph
Neg
ANTISERUM TO

Table 3.1 Proteins detected in the cytoplasmic and nuclear HIV replication complexes. N/A= not tested.

PROTEINS ASSOCIATED	CYTOPLASMIC COMPLEX	NUCLEAR COMPLEX
capsid / p24	-	-
matrix / p17	+	-
RT	+	-
Vpr	+	N/A
integrase	+	+
protease	+	+
histone	+	+
Vpu	-	N/A
Vif	-	N/A
RNA pol	-	N/A

matrix and RT proteins as part of the process of transport from the cytoplasm to the nucleus, while integrase, protease and histone proteins remained in association with the complexes. The major capsid protein p24 was not detected, with any of three independent p24 antisera, in complexes from either cellular fraction, indicating p24 was not associated with HIV replication complexes. This indicates that the capsid protein, despite being a major structural component of the virus, is not associated with the structure involved in viral DNA replication, while all the viral-coded enzymes, the p17 matrix protein, Vpr, and cellular histones are all associated with the structures engaged in viral DNA replication.

3.2.8 Micrococcal Nuclease Analysis of the Replication Complexes

In section 3.2.7, both cytoplasmic and nuclear replication complexes were shown to have histones associated with them. Histones are conserved DNA-binding proteins of eukaryotes that are associated with DNA in nucleosome structures. Micrococcal nuclease is an endonuclease that cleaves DNA preferentially between nucleosomes, resulting in a repeat length ladder. DNA not bound to histones is completely digested by micrococcal nuclease. In this study, cytoplasmic and nuclear replication complexes were digested with micrococcal nuclease to investigate whether the association of the HIV unintegrated DNA with histones was organized in a nucleosomal structure.

Micrococcal nuclease digestion conditions were established using chicken red blood cell nuclei. DNA from these nuclei was used as a positive control for generating nucleosome repeat patterns by digestion with micrococcal nuclease in each study. The repeat length of chicken DNA nucleosomes was found to be 200bp-210bp as previously described (Horz and Zachau, 1980). Purified HIV DNA (1ng) was also digested with 60 units of micrococcal nuclease for 5 minutes at 37°C, as a control, as micrococcal nuclease completely digests DNA not associated with proteins.

Cytoplasmic and nuclear extracts containing the replication complexes were adjusted to 1mM $CaCl_2$ and digested with micrococcal nuclease for various times at 37°C. The reaction was stopped with the addition of EDTA to a final concentration of 20mM and the cleaved DNA was deproteinated with SDS and proteinase K. The DNA samples were then extracted with phenol-chloroform and ethanol precipitated. The DNA samples were run on a 1.5% agarose gel and analysed by Southern hybridization.

The purified HIV DNA (1ng) was totally digested by the micrococcal nuclease as expected. Full-length HIV DNA was detected in extracts not digested with micrococcal nuclease. Both cytoplasmic and nuclear extracts digested with micrococcal nuclease resulted in a smear (Figure 3.8). No uniform ladder was observed but on the other hand, the DNA was not totally digested even after 20 minutes of digestion, while the equivalent amount of purified DNA was degraded only after 5 minutes of incubation with micrococcal nuclease (lane 1 in Figure 3.8). These results suggest that the association between histones and other proteins, and the HIV DNA in the replication complex is most likely in a form different from the repeating nucleosomal structure found with eukaryotic chromosomes.

3.2.9 Cytoplasmic HIV Replication Complexes Following Infection With Cell-free Virus

HIV nucleoprotein complexes formed during an infection with cell-free virus were also examined. Cells were harvested 8hrs after infection with HIV, when linear viral DNA was the predominant form. The cytoplasmic extracts were subjected to velocity sedimentation through a 15%-30% sucrose gradient for 3hrs at 35000 rpm at 4°C and the gradient fractions were examined by Southern blotting. Under these conditions unintegrated HIV DNA was found at the bottom of the gradient (Figure 3.9A), suggesting that the unintegrated HIV DNA was associated in a discrete nucleoprotein complex of a large size (estimated at > 320S). However, when these cytoplasmic extracts were treated with RNAse A prior to sucrose gradient sedimentation under

Figure 3.8 Micrococcal nuclease analysis of cytoplasmic and nuclear replication complexes.

M: 100bp DNA ladder (Gibco-BRL). m: radioactively labelled pUC19 digested with HpaII DNA size markers. HIV:SacI HIV-1 DNA marker 100pg. Lane 1 = 1000pg of SacI HIV-1 DNA marker digested with micrococcal nuclease. C: untreated cytoplasmic extract. Lanes 2, 3, 4 = cytoplasmic extract treated with 60U of micrococcal nuclease for 5, 10, and 20 mins at 37°C, respectively. N: untreated nuclear extract. Lane 5 = nuclear extract digested with 60 U of micrococcal nuclease for 5 mins at 37°C.

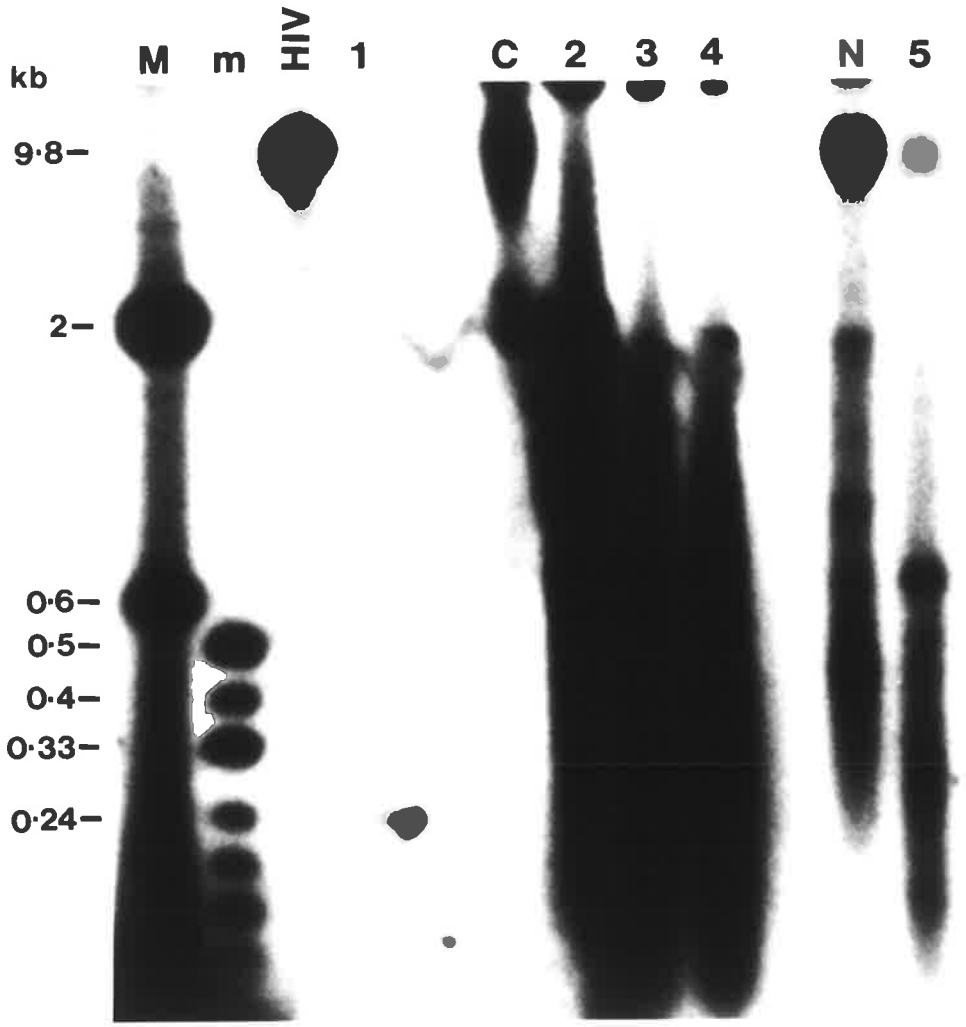
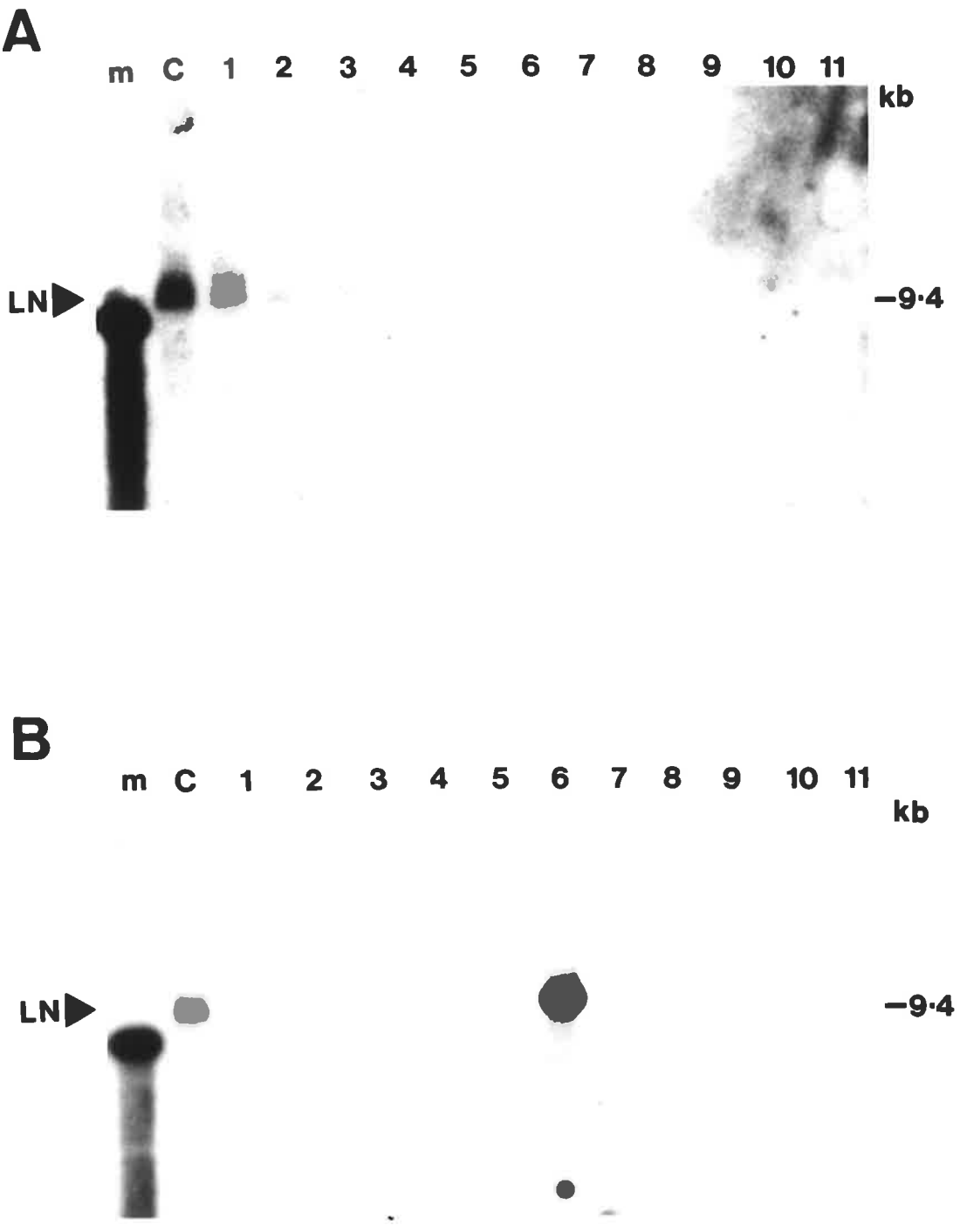

kb
M
m
HIV
1
C
2
3
4
N
5
9·8
2
0·6
0·5
0·4
0·33
0·24

Figure 3.9 Sucrose gradient sedimentation of HIV replication complex in cytoplasmic extracts from a cell-free virus infection.

Cytoplasmic extracts were prepared as described in section 2.2.2 and loaded onto a 10ml 15%-30% sucrose gradient. Gradients were centrifuged at 35000 rpm for 3hrs at 4°C in a Beckman SW41 rotor. Fractions (1ml) were deproteinized and assayed for DNA using agarose gel electrophoresis and Southern blot hybridization. **(A)** Sedimentation of cytoplasmic extract without RNAse A treatment prior to loading. **(B)** Sedimentation of cytoplasmic extract treated with RNAse A (20µg/ml) for 30 mins at room temperature prior to loading onto sucrose gradient. 1-11 refer to fractions from bottom to top of the gradient. C: unfractionated cytoplasmic extract analysed on the same gel. m: SacI HIV-1 DNA marker 100pg.


A
m C 1 2 3 4 5 6 7 8 9 10 11
kb
LN
9·4
B
m C 1 2 3 4 5 6 7 8 9 10 11
kb
LN
9·4

identical conditions, the complex was found in the middle of the sucrose gradient corresponding to ~160S (Figure 3.9B). Farnet and Haseltine (1990) used similar conditions including RNAse A digestion and identified a similar structure of ~160S. However, the present study suggested that the native nucleoprotein complex present 8hrs post infection was in fact considerably larger due to the presence of RNA, which was removed by RNAse A digestion. In contrast, Farnet and Haseltine have reported that at 4 hours post infection the sedimentation value of the HIV cytoplasmic replication complex was not affected by omission of RNAse digestion (Farnet and Haseltine, 1991).

3.3 DISCUSSION

Unintegrated HIV DNA, isolated from the cytoplasm of cells 6 hrs after cell-to-cell transmission of infection, was observed to sediment as a discrete complex of approximately 320S. Nuclear replication complexes, however, were found to be much smaller with a sedimentation coefficient of 80S.

The term "pre-integration complexes" has been used in the past to refer to similar HIV DNA-containing nucleoprotein structures (Ellison *et al.*, 1990; Farnet and Haseltine, 1990; Farnet and Haseltine, 1991; Bukrinsky *et al.*, 1993). However, this study showed that the HIV nucleoprotein complex isolated from the cytoplasm was not identical to the intranuclear structure that is likely to represent the immediate precursor to chromosomal integration *in vivo*. Secondly, although the cytoplasmic nucleoprotein complex is capable of integrating into artificial target DNA *in vitro* (Ellison *et al.*, 1990; Farnet and Haseltine, 1990), integration may not be the only fate for such complexes, as expression of retroviral RNA and proteins has been reported to occur in the absence of integration (Panganiban and Temin, 1983; Stevenson *et al.*, 1990). Hence the term "replication complex" was used, in preference to "preintegration complex".

Immunoprecipitation-PCR studies indicated that the viral proteins integrase, reverse transcriptase, protease, matrix p17, Vpr and also histones were associated with the unintegrated DNA in the 320S cytoplasmic replication complex. The core protein p24 was not detected. As p24 is one of the most abundant viral proteins in infected cells, this finding also demonstrated that the HIV DNA detected using the other antisera had not been co-precipitated non-specifically, but instead was incorporated in a specific structure containing the proteins identified by the corresponding antisera.

The replication complex found in the nucleus was smaller (80S) than the cytoplasmic structure (320S). This reduction in size could involve the replication complex shedding proteins not required for any nuclear functions or alternatively, a minor species of smaller structures may have been preferentially transported. Immunoprecipitation-PCR studies indicate that the nuclear replication complex was associated only with the viral enzymes integrase and protease as well as with histones. While the viral protein integrase is essential for integration, the role of protease in the nuclear complex is not clear. Histones are conserved DNA-binding proteins of eukaryotes that are associated with DNA in nucleosome structures. The association of histones with newly synthesized HIV DNA in both cytoplasmic and nuclear replication complexes was unexpected and has not been previously reported. This association of histones with the replication complexes may act to stablize the complexes and, in addition, may act to position the unintegrated DNA during the process of integration.

Micrococcal nuclease is an endonuclease that cleaves DNA preferentially between nucleosomes resulting in a uniform ladder when the digested DNA is analysed by gel electrophoresis. The DNA content of nucleosomes varies from one organism to the next , ranging from 150 to 240 base pairs per unit. DNA not protected by histones is completely digested by micrococcal nuclease. Digestion of the cytoplasmic and nuclear replication complexes with micrococcal nuclease resulted in a smear rather than a uniform repeat length ladder, suggesting the HIV DNA of the replication complexes

was associated with histones in a structure different from nucleosomes. Equivalent amounts of purified HIV DNA were completely digested by micrococcal nuclease. This indicates that the HIV DNA in the replication complexes was, to a certain extent, resistant to nucleases. There are five major classes of histones : H1, H2A, H2B, H3 and H4. The antibody against histones used in the immunoprecipitation studies consisted of a mixture of antibodies to all species of histones and not to specific individual histones, and therefore it is not known whether all histones were present in the replication complexes. Without all histones present, nucleosomal organisation is unlikely. In any case, the histones found in the replication complexes were probably associated with the unintegrated viral DNA in a structure different from nucleosomes.

The viral enzyme reverse transcriptase was found associated with the cytoplasmic replication complex but not with the nuclear replication complex. This finding is compatible with progressive release of reverse transcriptase from the complex, after the completion of reverse transcription, although details about the rate and mechanism of such a process are not known. The viral protein R (Vpr) was also shown to be present in the cytoplasmic replication complex. The function of Vpr is unclear, although, it is believed that it may play a role in *trans* activation of viral gene expression or RNA processing (Cohen *et al.*, 1990). Vpr is the only regulatory product of HIV packaged within virion particles (Cohen *et al.*, 1990; Yuan *et al.*, 1990). Vpr has been shown to be localized in the nucleus in infected cells, in particular with the chromatin and nuclear matrix fraction (Lu, *et al.*, 1993). The significance of Vpr being associated with the cytoplasmic replication complex is at present unclear (see below).

The matrix protein p17 was also shown to be present in cytoplasmic but not nuclear replication complexes. Proteins require an active nuclear localization sequence (NLS) to be transported into the nucleus through pores in the nuclear envelope (Garcia-Bustos *et al.*, 1991; Silver, 1991). Proteins that do not possess their own NLS may enter the nucleus via co-transport with another protein containing an NLS. This

translocation of proteins through the nuclear pores into the nucleus requires ATP. Once in the nucleus, proteins may remain associated with the NLS-binding protein or may dissociate, allowing the NLS-binding protein to recycle back into the cytoplasm. A nuclear localization signal was found at the N-terminus of the matrix protein p17 of simian immunodeficiency virus (Delchambre *et al.*, 1989) and recently in the HIV p17 matrix protein. It has been shown that the HIV p17 matrix protein, by virtue of an NLS at its N terminus, contributes to the karyophilic properties of the viral preintegration complex and influences the ability of the virus to replicate within non-proliferating cells (Bukrinsky *et al.*, 1993). The matrix protein in HIV may therefore be important for transport of the viral replication complex from the cytoplasm into the nucleus. Once the replication complex has migrated into the nucleus, the HIV p17 matrix protein is no longer required and as a result might be shed from the complex. Though Vpr lacks a classical NLS, the carboxyl-terminal portion of the protein is rich in basic amino acids. A truncation mutation which removes the carboxyl-terminal 19 amino acids was found to impair Vpr localization in the nucleus (Lu *et al.*, 1993). Therefore Vpr may also play a role in the targeting of the replication complex to the nucleus.

The loss of proteins (reverse transcriptase and p17) that are not required for intra-nuclear functions may explain, at least in part, the reduction in sedimentation coefficients of nuclear (80S) compared to cytoplasmic (320S) replication complexes.

In 1991, Farnet and Haseltine reported that integrase was the only viral protein that was associated with the viral DNA in the cytoplasmic nucleoprotein complex found in infection with cell-free virus. However in 1993, at the time our results were published, Bukrinsky *et al.*, reported that the viral proteins integrase, matrix (p17), and reverse transcriptase were associated with viral nucleic acids following infection with cell-free virus, in agreement with our results. In this study (Bukrinsky *et al.*, 1993), the presence of protease was not investigated. Capsid (p24) protein was not detected , in

agreement with our findings. In the study by Farnet and Haseltine, infected cells were lysed in the presence of detergent (0.5% Triton X-100) (Farnet and Haseltine 1991), conditions that according to Bukrinsky *et al* ., disrupted the association of the matrix protein (p17) from the viral nucleic acids (Bukrinsky *et al.,* 1993). The presence of detergent and hence the dissociation of reverse transcriptase and p17, was suggested by Bukrinsky *et al.* as a possible explanation for the discrepancy between these two results. However, mild detergent conditions (0.5% Triton X-100) similar to those used by Farnet and Haseltine were also employed in this study to lyse infected cells. Despite the presence of detergent, integrase was not the only viral protein detected in our study. The greater sensitivity of immunoprecipitation followed by the detection of the precipitated DNA by PCR, could be an alternative explanation for the discrepancy between reports. Both in this study and in the study reported by Bukrinsky *et al.*, immunoprecipitation followed by PCR was used. Farnet and Haseltine immunoprecipitated radiolabelled viral proteins and detected these proteins by fluorography. Hence the detection method employed by Farnet and Haseltine may not be as sensitive.

In infection with cell-free virus, the replication complexes were reduced in size from >320S to ~160S after RNAse treatment. In contrast, complexes found after cell-to-cell transmission sedimented at 320S with or without RNAse treatment. Detergent treatment did not lead to disaggregation of the 320S cytoplasmic nucleoprotein complex into discrete subunits, and isopycnic centrifugation in discontinuous sucrose gradients showed that it was not membrane / lipid associated. These results, taken together, suggest a genuine difference, in size and probably composition, between cytoplasmic HIV replication complexes in a cell-to-cell infection and those in a cell-free virus infection. In similar studies on murine leukemia virus (MLV), full-length unintegrated viral DNA was organized, together with viral capsid protein, in a cytoplasmic 160S nucleoprotein complex that was similar to and probably derived from the core of extracellular virions(Bowerman *et al.,* 1989). It has also been shown in a cell-free virus

infection system that the HIV-1 cytoplasmic nucleoprotein complex had a similar sedimentation value (Farnet and Haseltine, 1990)(and also shown in Figure 3.9B). Ultra-structural studies suggest that morphological maturation of HIV virions occurs after the virion has been released from the cell via the process of "budding" (Gelderblom, 1991). However, in cell-to-cell transmission of HIV infection, cell-free virus is not necessarily involved and mature POL products are already present within these cells prior to budding. Cell-associated reverse transcriptase and other viral gene products present within the donor cells are probably responsible for transmission of infection (Li and Burrell, 1992; Sato *et al.*, 1992; Li *et al.*, 1994). Hence, it is not surprising that the structure and protein composition of the HIV replication complex in a cell-to-cell infection differed from that found in a cell-free HIV infection. However, the precise form of the precursor structure in the donor H3B cells and the mechanism of activation of reverse transcription have not been identified.

CHAPTER 4

KINETICS OF REVERSE TRANSCRIPTION - SYNTHESIS OF UNINTEGRATED HIV DNA

4.1 INTRODUCTION

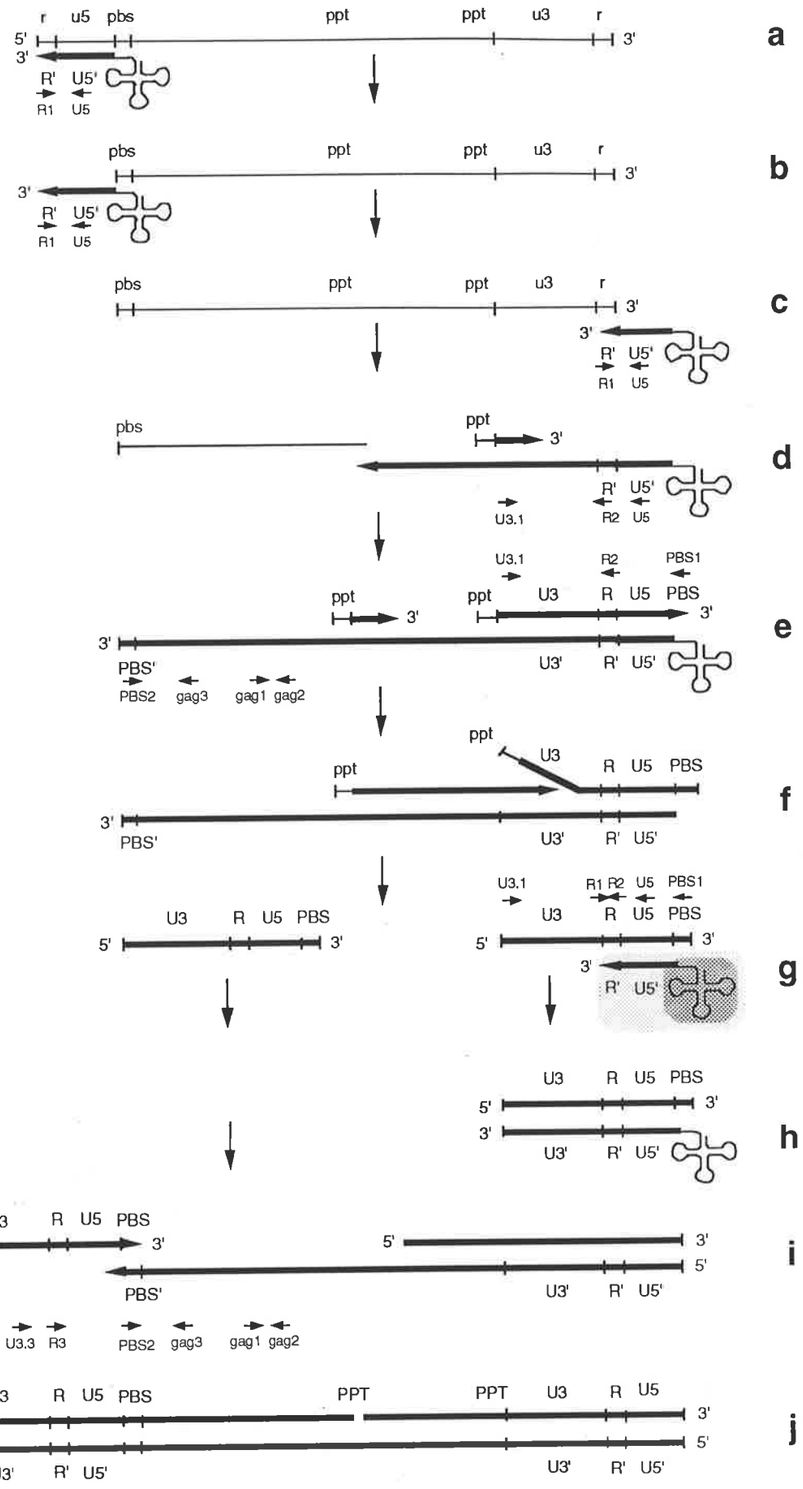
The process of reverse transcription can be divided into several discrete steps (Figure 4.1): (i) Synthesis of a short stretch of DNA (the "minus-strand strong-stop DNA") from the primer binding site (PBS) near the 5' end of one genomic viral RNA molecule, using a tRNA as primer (Figure 4.1A). (ii) Transfer of the minus-strand strong-stop DNA from the 5' end of the viral RNA to the 3' end of the same, or a second molecule of viral RNA (Figure 4.1C). (iii) Continued synthesis of the minus-strand DNA, accompanied by degradation of the RNA template by RNAse H activity of the viral reverse transcriptase; this process leaves a polypurine tract (PPT) at specific sites in the 3' end of the viral RNA to serve as the primer for the synthesis of the plus-strand viral DNA using the minus-strand DNA as template (Figure 4.1 D & E). (iv) This newly synthesized short stretch of plus-strand DNA ("plus-strand strong-stop DNA") is then transferred from the 5' end to the 3' end of newly made, partially completed minus-strand DNA (Figure 4.1 I). (v) After the second template transfer, the remainder of the plus-strand DNA as well as the 3' end of the minus-strand DNA are synthesized, to yield a linear double stranded DNA. Although the basic steps of reverse transcription have been known for some time from studies of type C retroviruses and some aspects of these have recently been confirmed for HIV, a detailed kinetic study delineate the multiple events in this process, has been lacking and the current data are highly controversial.

The aim of this study was to investigate the time course of various components of the reverse transcription process using our synchronized, one-step cell-to-cell infection system. Reverse transcription kinetics were followed by measuring the rate of

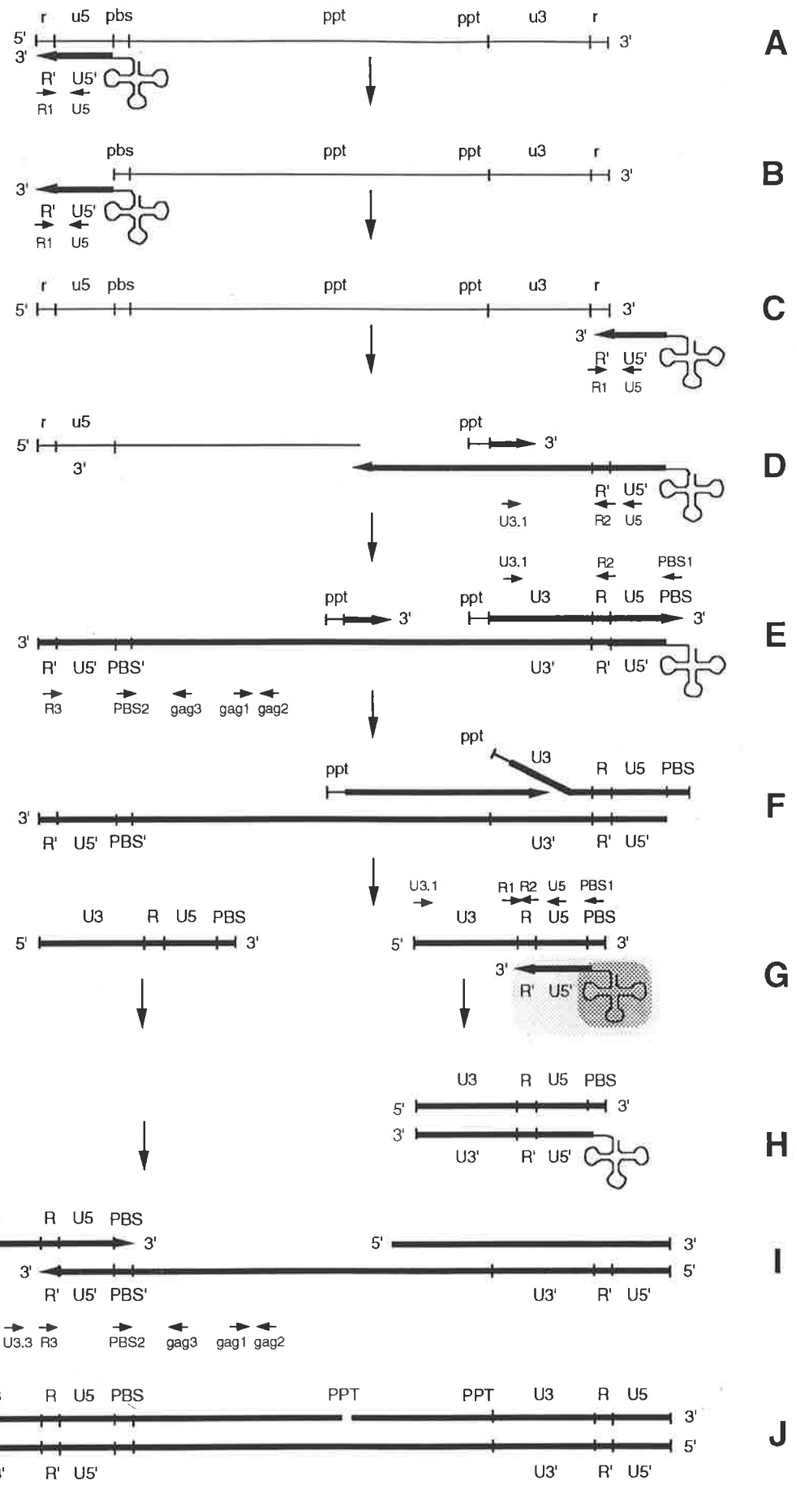
Figure 4.1 **HIV reverse transcription**: intramolecular (left column, **a-j**) and intermolecular (right column, **A-J**) models. The thin lines represent RNA, thick lines represent DNA and  represents tRNA. Lower case letters (eg. u3, r, u5, pbs, ppt) denote RNA while upper case letters (eg. U5, R, U3, PBS, PPT) denote DNA. **(a/A)**: Synthesis of minus strand strong-stop DNA. **(b/B)** & **(c/C)**: First template transfer. **(d/D)** & **(e/E)**: Extension of minus strand DNA and synthesis of plus strand strong-stop DNA. **(f/F)**, **(g/G)** & **(h/H)**: Synthesis of double-stranded (ds) strong-stop DNA. **(i/I)**: Second template transfer. **(j/J)**: Full length ds DNA. Horizontal small arrows indicate deoxyoligonucleotide primers used in this study. The sequences of these primers are listed in the text and also Materials and Methods.

This diagram is adapted from Li *et al*, (1993) *Virology*, **194**:82.

INTRA-molecular



INTER-molecular



appearance of unintegrated cDNA species using a quantitative polymerase chain reaction (PCR) analysis. In addition to its high sensitivity as a nucleic acid detection technique, PCR was employed in this study, essentially for its ability to quantitate DNA structures heterogenous in length but representing completion of each of the ordered sequence of specific steps during reverse transcription (Varmus and Brown, 1989; Zack *et al.*, 1990; Li *et al.*, 1993). Apart from the strong-stop DNA populations the intermediate reverse transcripts are heterogenous in length thus unsuitable for Southern blot analysis, where only populations of similar length molecules can be analysed. The PCR analysis in this study was restricted to relatively low amplification (20-22 cycles) and applied to relatively large numbers of cells (10^5) in each sample to achieve more reproducible results.

4.2 RESULTS

4.2.1 Isolation of Unintegrated HIV DNA from Cell-to-Cell Infection

The one-step cell-to-cell HIV infection model, as described in Chapter 3, was slightly modified in this study. HIV persistently-infected cells (H3B) were mixed with uninfected cells (HUT-78) at a ratio of 2:1, in an attempt to ensure a tightly synchronous infection in the early hours after mixing. At appropriate time points (0, 1, 1.5, 2, 2.5, 2.75, 3, 3.25, 3.5, 3.75, 4, 8 hours) after cell mixing, cells were harvested by low-speed centrifugation and extrachromosomal DNA was prepared by the Hirt procedure in the presence of 0.5 mg/ml proteinase K (Hirt 1967), as described in Chapter 2. Time 0 was taken as the point at which cells were first mixed. Extrachromosomal DNA was also isolated from HUT-78 cells and H3B cells. The Hirt extracts were treated with 100 µg/ml RNAseA (Boehringer Mannheim) for 1 hour at 37°C , extracted with phenol-chloroform-isoamylalcohol and precipitated by ethanol. The DNA samples were further treated with RNAse H (Boehringer) at 20U/ml for 20 mins at 37° C, extracted again with phenol-chloroform-isoamylalcohol and precipitated by ethanol. Digestion with RNAse A and RNAse H insured any RNA associated with the newly

synthesized DNA in DNA/RNA hybrids was eliminated. The purified DNA samples were then analysed and quantitated by PCR (as in Chapter 2, section 2.2.8).

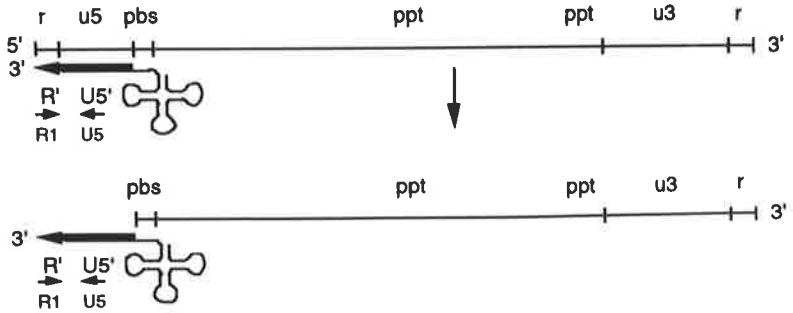
4.2.2 Time Course of Viral DNA Synthesis

The process of reverse transcription was followed by PCR using radioactively labelled primers (see section 2.2.7) to the different regions of the genome (see Figures 4.1 - 4.6). Oligonucleotide primers were 5'-labelled with γ-^{32}P-ATP (Bresatec) using T4 polynucleotide kinase (Bresatec) in a standard kinase labelling reaction (see Chapter 2). Unintegrated DNA from $1x10^5$ cells was analysed at each time point by 20 cycles of PCR (see Chapter 2). The PCR products were run on 8% PAGE gels. These gels were dried and the PCR products visualized and analysed by both autoradiography and the phospho-imager (see Chapter 2). Quantitation of the PCR was performed by comparison of experimental samples with standards containing known amounts of HXB2 viral DNA, isolated from a plasmid clone of λHXB2, originally obtained from B.Hahn and G.Shaw through the AIDS Research and Reference Program NIAID, NIH, Bethesda. The viral DNA was excised with an XbaI digest. Copy number standards (10K, 50K, 100K copies) were amplified simultaneously with the time course products,in separate tubes, enabling the number of pixels to be converted into copy numbers. The observed signals of the U5/R, U5/U3 and U3/R standards were divided by two to determine genome copy numbers, to account for the presence of two LTRs in each molecule of HXB2 DNA.

Each PCR time course was repeated in triplicate and two independent readings taken from each individual PCR gel by the phospho-imager. The average was recorded and a standard deviation determined. In most cases less than 10% variation was observed.

Primers U5 and R1, allowed detection of the first step of reverse transcription, the synthesis of the minus strong-stop DNA (Figure 4.2A). This pair of primers would

Figure 4.2 Analysis of HIV reverse transcription using primers R1/U5. **(A)** Location of primers and the first viral DNA species detected by these primers during reverse transcription (see Figure 4.1). **(B)** Detection of viral DNAs by PCR using primer pair R1/U5. Extrachromosomal DNA samples from ~10^5 mixed cells were amplified by PCR and viral specific bands (arrows) detected as described in section 2.2.8. Numbers on top of tracks indicate the time point (hours post infection) at which extrachromosomal DNA was prepared. Track N: PCR negative control. Tracks 10K, 50K and 100K: copy number standards at 10,000, 50,000 and 100,000 respectively. Track H3B: ~10^5 viral donor cells. Track HUT78: ~10^5 recipient cells. **(C)** Kinetic analysis: Three independent gels of each of **(B)** were exposed twice on a PhosphorImager and their pixel readings were converted to copy number using copy number standards run on the same gel. Copy numbers (average +/- S.D.) were plotted against time (hours post infection).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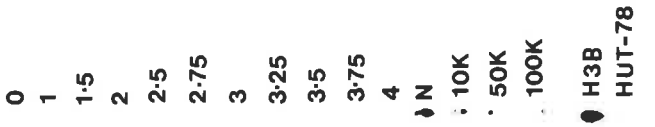
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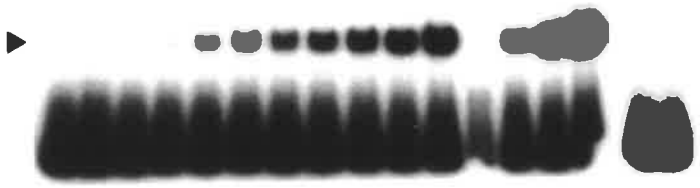

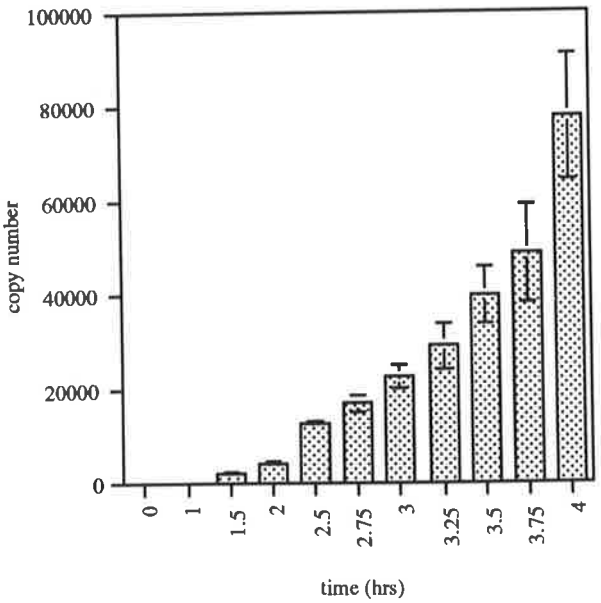
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2.75
3
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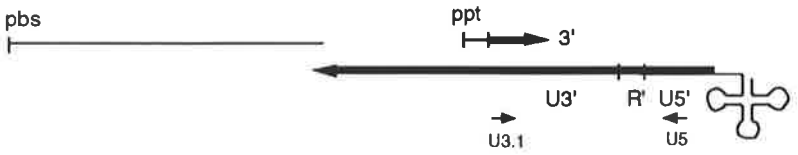
3.5
3.75
4
time (hrs)

C

Figure 4.3 Analysis of HIV reverse transcription using primers U3.1/U5. **(A)** Location of primers and the first viral DNA species detected by these primers during reverse transcription (see Figure 4.1). **(B)** Detection of viral DNAs by PCR using primer pair U3.1/U5. Extrachromosomal DNA samples from ~10^5 mixed cells were amplified by PCR and viral specific bands (arrows) detected as described in section 2.2.8. Numbers on top of tracks indicate the time point (hours post infection) at which extrachromosomal DNA was prepared. Track N: PCR negative control. Tracks 10K, 50K and 100K: copy number standards at 10,000, 50,000 and 100,000 respectively. Track H3B: ~10^5 viral donor cells. Track HUT78: ~10^5 recipient cells. **(C)** Kinetic analysis: Three independent gels of each of **(B)** were exposed twice on a PhosphorImager and their pixel readings were converted to copy number using copy number standards run on the same gel. Copy numbers (average +/- S.D.) were plotted against time (hours post infection).


pbs
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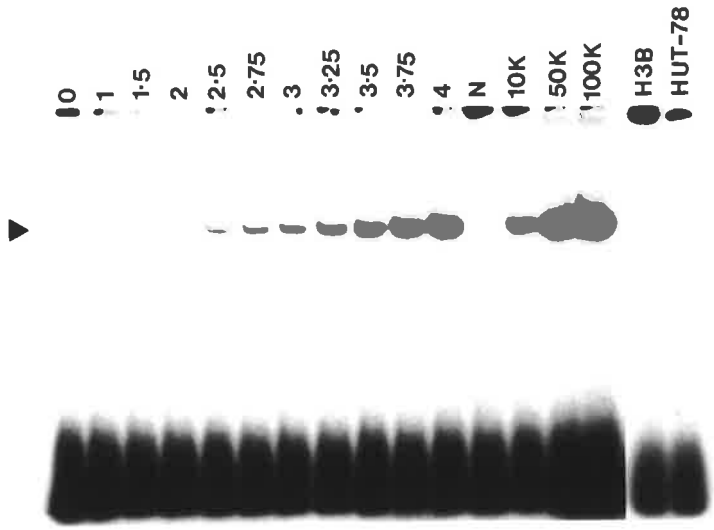
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50K
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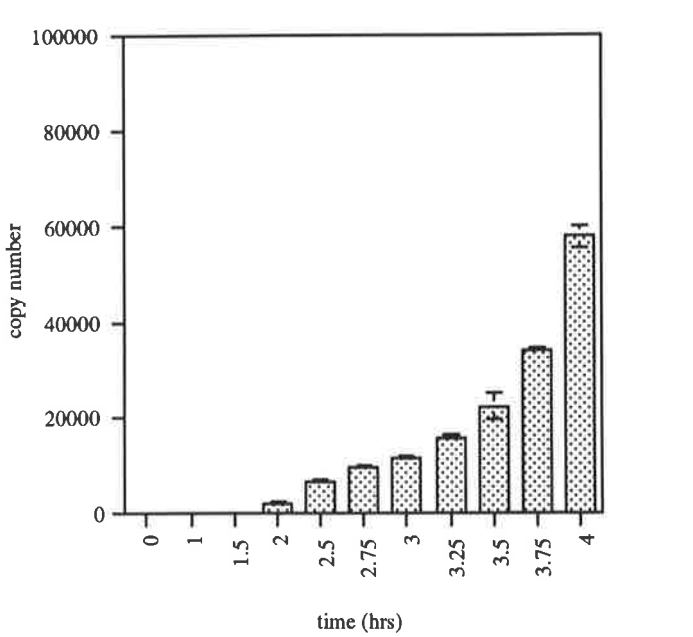
H3B
HUT-78

B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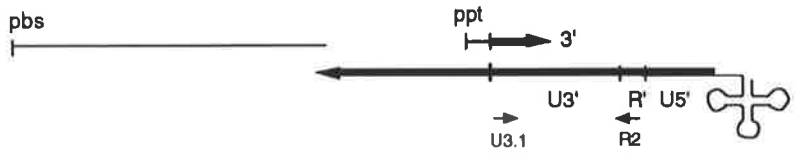
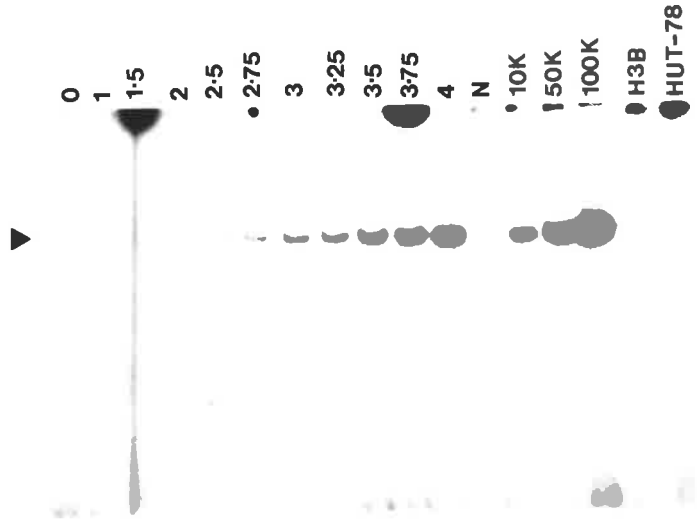
4
time (hrs)

C


pbs
ppt
3'
U3'
R'
U5'
U3.1
R2

A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1·5
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3
3·25
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3·75
4
N
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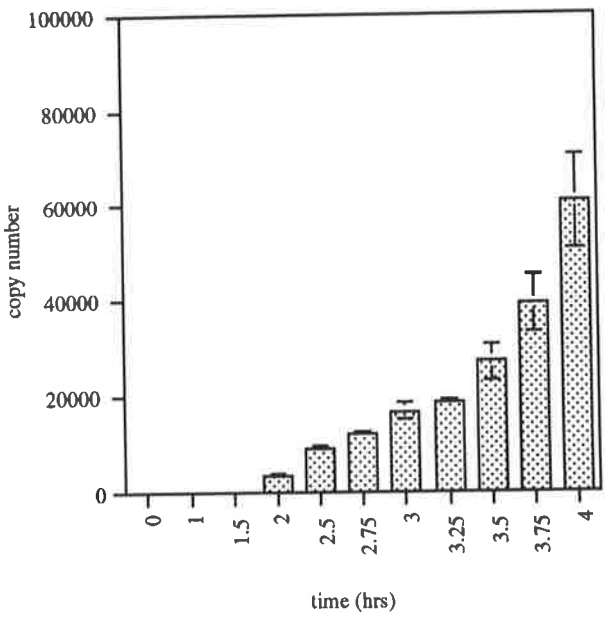
50K
100K
H3B
HUT-78

B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copy number
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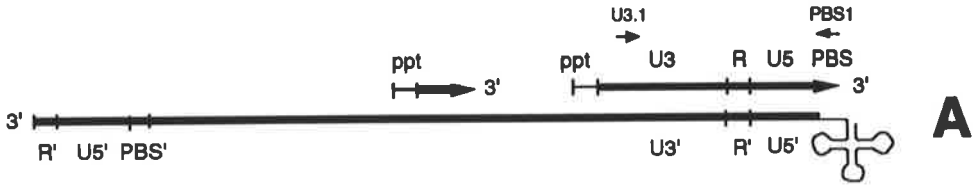
3.5
3.75
4
time (hrs)

C

Figure 4.4 Analysis of HIV reverse transcription using primers U3.1/R2. **(A)** Location of primers and the first viral DNA species detected by these primers during reverse transcription (see Figure 4.1). **(B)** Detection of viral DNAs by PCR using primer pair U3.1/R2. Extrachromosomal DNA samples from ~10^5 mixed cells were amplified by PCR and viral specific bands (arrows) detected as described in section 2.2.8. Numbers on top of tracks indicate the time point (hours post infection) at which extrachromosomal DNA was prepared. Track N: PCR negative control. Tracks 10K, 50K and 100K: copy number standards at 10,000, 50,000 and 100,000 respectively. Track H3B: ~10^5 viral donor cells. Track HUT78: ~10^5 recipient cells. **(C)** Kinetic analysis: Three independent gels of each of **(B)** were exposed twice on a PhosphorImager and their pixel readings were converted to copy number using copy number standards run on the same gel. Copy numbers (average +/- S.D.) were plotted against time (hours post infection).

Figure 4.5 Analysis of HIV reverse transcription using primers U3.1/PBS1. **(A)** Location of primers and the first viral DNA species detected by these primers during reverse transcription (see Figure 4.1). **(B)** Detection of viral DNAs by PCR using primer pair U3.1/PBS1. Extrachromosomal DNA samples from ~10^5 mixed cells were amplified by PCR and viral specific bands (arrows) detected as described in section 2.2.8. Numbers on top of tracks indicate the time point (hours post infection) at which extrachromosomal DNA was prepared. Track N: PCR negative control. Tracks 10K, 50K and 100K: copy number standards at 10,000, 50,000 and 100,000 respectively. Track H3B: ~10^5 viral donor cells. Track HUT78: ~10^5 recipient cells. **(C)** Kinetic analysis: Three independent gels of each of **(B)** were exposed twice on a PhosphorImager and their pixel readings were converted to copy number using copy number standards run on the same gel. Copy numbers (average +/- S.D.) were plotted against time (hours post infection).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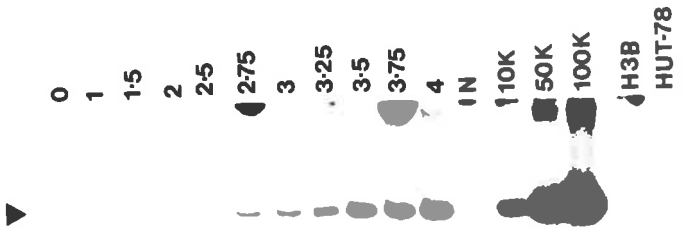
ppt
3'
ppt
U3
R
U5
PBS
3'
3'
R'
U5'
PBS'
U3'
R'
U5'

A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10K
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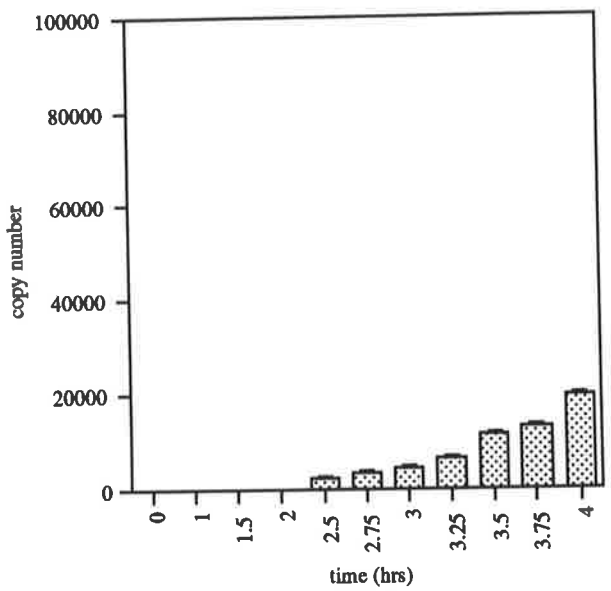
H3B
HUT-78

B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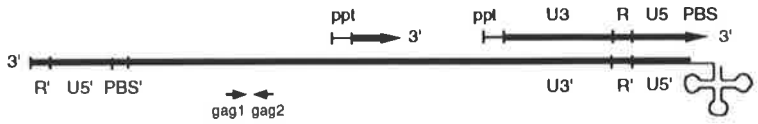
3.5
3.75
4
time (hrs)

C

Figure 4.6 Analysis of HIV reverse transcription using primers gag1/gag2. **(A)** Location of primers and the first viral DNA species detected by these primers during reverse transcription (see Figure 4.1). **(B)** Detection of viral DNAs by PCR using primer pair gag1/gag2. Extrachromosomal DNA samples from ~10^5 mixed cells were amplified by PCR and viral specific bands (arrows) detected as described in section 2.2.8. Numbers on top of tracks indicate the time point (hours post infection) at which extrachromosomal DNA was prepared. Track N: PCR negative control. Tracks 10K, 50K and 100K: copy number standards at 10,000, 50,000 and 100,000 respectively. Track H3B: ~10^5 viral donor cells. Track HUT78: ~10^5 recipient cells. **(C)** Kinetic analysis: Three independent gels of each of **(B)** were exposed twice on a PhosphorImager and their pixel readings were converted to copy number using copy number standards run on the same gel. Copy numbers (average +/- S.D.) were plotted against time (hours post infection).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ppt
U3
R
U5
PBS
3'
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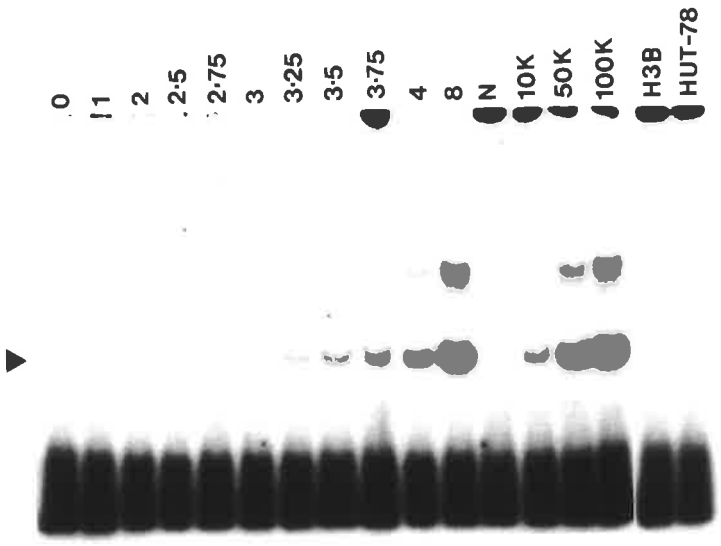
PBS'
gag1
gag2
U3'
R'
U5'

A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50K
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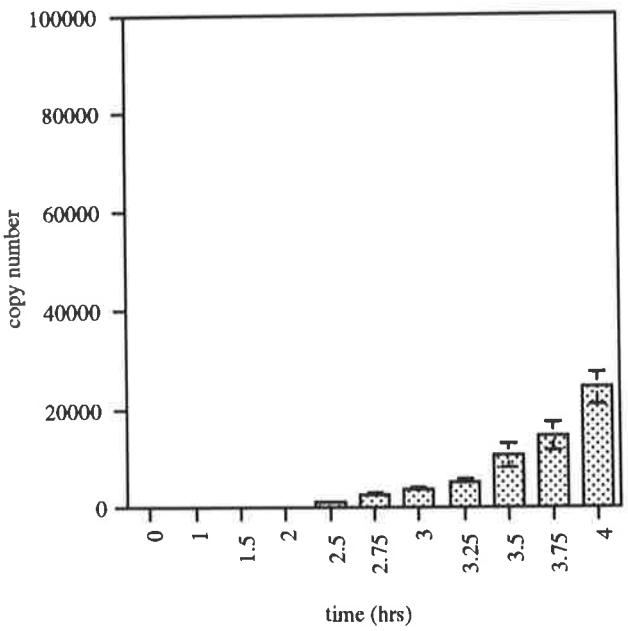
H3B
HUT-78

B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copy number
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2
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2.75
3
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3.5
3.75
4
time (hrs)

C

also detect all the subsequently elongated HIV DNA. As shown by figures 4.2B and 4.2C, the first appearance of the minus strong-stop DNA, as detected by the primer pair R1/U5, occurred 1.5 hours after initial infection.

Primers U5 / U3.1 (see Figure 4.3A) and R2 /U3.1 (see Figure 4.4A) allowed detection of the DNA product after the first template transfer and the extension of the minus-strand DNA after the first switch. Results from these PCR gels (Figures 4.3B and 4.4B) show that this product was first detected around 2 hrs after infection. These results suggested that there seemed to be a temporary time delay associated with the first template transfer.

Primer pair U3.1 / PBS1 allowed the complete plus-strand strong-stop DNA to be detected (Figure 4.5A). Figure 4.5B shows that the first appearance of the complete plus-strand strong-stop DNA occurs at approximately 2.5 hours after infection. This result suggests another time delay in association with the initiation and synthesis of the plus-strand DNA. The extension of the minus-strand DNA through to the gag region was detected by the gag1/gag2 primers at 2.5-2.75 hours (Figure 4.6B).

When analysing the copy number results, approximately 2000 copies of the minus strong-stop DNA was detected per 1 x 10^5 cells after 1.5 - 2 hours of cell mixing. After this time there was a steady increase in the number of minus strong-stop copy number to 80,000 copies by the end of 4 hours. Therefore in our cell-to-cell infection system, reverse transcription was not initiated simultaneously by all structures capable of this, but progressively through the time course within the single cycle of infection.

By 4hr after cell-to-cell HIV infection, when every species of viral DNA was still increasing (without evidence of reaching a plateau) and sufficient numbers of full length DNA had been made, there were ~80,000 copies of minus-strand strong-stop

DNA in 100,000 mixed cells (66,600 virus donor cells and 33,400 recipient cells). At the same time point, the copy number of the newly extended minus-strand DNA, after the first transfer, was estimated at ~60,000 by PCR quantitation using both the U3.1/U5 and the U3.1/R2 primer pairs. These results suggest at least a 70-80% success rate for the first template transfer. Considering that the lower copy number of the latter DNA species may also be due to the delayed appearance of this species of DNA following the temporary arrest associated with the first template transfer, the actual success rate of the first template switch is expected to be higher than 70-80%. Similar arguments also apply to relevant analyses that follow. At 4 hours, the copy number of the complete plus-strand strong-stop DNA, as detected by U3.1/PBS1, was estimated at 20,000. Comparison with the estimate of ~60,000 for the post transfer and extended minus-strand DNA suggested a success rate of at least 30-35% for plus-strand DNA initiation and synthesis on the DNA template at the 3' end PPT site. However, unlike the DNA species detected by previous three pairs of primers, which are present twice in the full length HIV proviral genome and once in the double stranded strong-stop DNA population (step G in Figure 4.1), the species of DNA detected by U3.1/PBS1 primers, appeared only once in the full length HIV DNA and once in the double stranded strong-stop DNA. Hence the actual success rate for the initiation of plus-strand DNA synthesis is expected to be higher. The copy number of the near-fully extended minus-strand DNA detected by the gag1/gag2 primer, was estimated at ~25,000. Nominally, this is just over ~40% of those DNA molecules (60,000) that had completed the first template transfer and extended to U3 (detected by primer pairs U3.1/U5 and U3.1/R2). However, this gag sequence appears only once in the full-length HIV DNA, while sequences detected by either of the latter primers appear twice in full-length HIV DNA and also in double-stranded strong-stop DNA species; thus the actual success rate of minus-strand DNA extension ~7,500 bases from U3.1 position to gag1 position should be more than doubled the figure of 40%. This is an indication that in activated T-cells, at least in our HUT78/H3B experimental system, premature termination or drop-off of reverse transcripts are rare events.

The above data also indicated that from the first appearance of the extended minus-strand DNA following the first transfer (at 2hr post infection) to the first appearance of the near full length minus-strand DNA 7,500 bases away (at 2.5hr post infection), there was a time lapse of ~30 minutes. Thus, disregarding time allowance for the first template transfer and for the initiation events of the minus and plus-strand DNA synthesis, the rate of HIV reverse transcription of the minus-strand DNA using viral RNA as template, in virus infected cells, was estimated at ~250 bases per minute, or ~4 bases per second. As we will see in the following experiments, this rate of reverse transcription *in vivo*, was further estimated at being ~4-5 bases per second.

4.2.3 Inter or Intra-molecular Events During the First Template Switch

Based on experiments using spleen necrosis virus-based vectors, but with different constructions and different selection procedures in helper cell lines, the first template transfer in retroviral reverse transcription (the transfer of the minus strand strong-stop DNA) was proposed to be exclusively intermolecular (Panganiban and Fiore, 1988), either intermolecular or intramolecular (Hu and Temin, 1990) or completely intramolecular (Jones *et al.,* 1994). No detailed study is available, to the best of our knowledge, regarding the molecular mechanisms of the first template transfer in HIV reverse transcription in virus-infected cells.

Like all retroviruses, HIV has two copies of its genome packaged in its core. The two plus-strands of single-strand RNA are present as a dimer, joined in a noncovalent linkage. Reverse transcription is initiated with the binding of the host tRNA primer to the PBS on the 5' end of one of the RNA molecules. As the minus-strand strong-stop DNA is being synthesized from the 5' end of the RNA, the RNAse H activity of the reverse transcriptase molecule degrades the U5 and R regions of the RNA template. This frees the minus strong-stop DNA , allowing it to bind to the 3' end of either the same molecule or the second RNA molecule. However RNAse H

digestion of the first RNA template results in this first RNA molecule lacking the U5 and R regions at the 5' end, while the second RNA molecule will still retain its 5'end. Therefore if the first strand transfer were intra-molecular (jumps to the 3' end of the same RNA molecule), the extension of the minus DNA strand would stop at the PBS and further completion of the 3' end of the minus-strand DNA (through U5-R-U3) would be delayed since most of these regions of its template are missing until the second template switch has occurred. Alternatively, if the first jump were intermolecular (to the other molecule of RNA), minus-strand DNA synthesis would extend to the border of U3/R and only the synthesis of the U3 region at the 3' end of the minus-strand DNA might be delayed, due to a possible temporal arrest associated with the second template switch.

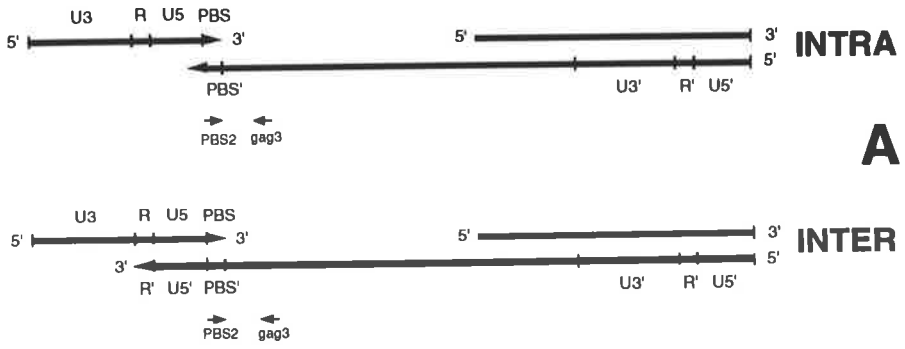
By isolating reverse transcription products (ie. unintegrated DNA) from our cell-to-cell infection system at various times after infection and designing PCR primers directed to the 5' end of the genome it seemed possible to determine whether the first switch was inter- or intra-molecular if there was a time delay being associated with the second template transfer, as seen with the first template transfer. Figure 4.1 shows the different consequences from the first template transfer being inter- or intramolecular. If the first template transfer was intramolecular (left model of Figure 4.1 A-H) the synthesis of the U5 & R regions in the 3' end of the minus-strand DNA, as detected by PCR using primer pair R3/gag3, would happen only after the second template switch when the transferred plus-strand strong-stop DNA could act as template (steps H & I in Figure 4.1, left model). However, if the first template transfer was intermolecular, then the synthesis of the U5 and R regions, as detected by PCR using primer pair R3/gag3, would occur as a continuation of the minus-strand DNA extension using RNA as template; this would not require the second template transfer (steps d & e in Figure 4.1, right model). In either intra- or intermolecular models of the first template transfer, the synthesis of the U3 regions at the 3' end of the minus-strand DNA, as detected by PCR using primer pairs U3.3/gag3 and U3.1/gag3, must use the transferred plus-strand

strong-stop DNA as template. Detection of the DNA between the PBS2 and the gag3 primers would be similar in either case since the PBS region is not degraded as it is part of the tRNA, and RNAseH only degrades RNA in a DNA:RNA hybrid and not RNA in a RNA:RNA hybrid. Therefore, the first appearance and accumulation kinetics should be similar to those observed using gag1/gag2 primers. In summary, (i) completed minus-strand viral DNA detected by primer pair PBS2/gag3 should appear with the similar kinetics to that detected by primer pair gag1/gag2; (ii) if the first switch were intermolecular, completely extended minus-strand DNA detected by primer pair R3/gag3 should also appear with kinetics similar to (i); (iii) if the first switch were intramolecular, and the second switch imposes a time lag (as seen with the first switch above), then the product detected by R3/gag3 should appear significantly later than (i); (iv) if the second switch imposes a time lag the product detected by U3.3/gag3 should appear later, than the above, regardless of whether the first switch were inter- or intramolecular. Given the rate of HIV reverse transcription estimated in the previous section, primer pairs U3.3/gag3 and U3.1/gag3 would probably detect HIV DNA with similar kinetic characteristics.

In this set of PCR reactions, primer pairs PBS2/gag3 (Figure 4.7A), R3/gag3 (Figure 4.8A), U3.3/gag3 (Figure 4.9A) and U3.1/gag3 (Figure 4.10A) were used with reaction conditions as described in Chapter 2, section 2.2.8, except the thermocycling temperatures used were 94°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes. PCR products obtained with primers PBS2 / gag3 were first detected at about 2.5 - 2.75 hours after infection (Figure 4.7). The primer pair PBS2/gag3 would be expected to detect completely extended minus-strand viral DNA with kinetic characteristics similar to the primer pair gag1/gag2. The time course of appearance of both these PCR products were in fact very similar (Figure 4.6B and 4.7B).

Each PCR with the aforementioned primer pairs was repeated three times and in each PCR reaction the gag3 primer was labelled. Each individual PCR gel was

Figure 4.7 Kinetic analysis of HIV reverse transcription using primer pair PBS2/gag3. **(A)** Location of primers and the first viral DNA species detected by these primers during reverse transcription (see Figure 4.1). **(B)** Detection of viral DNAs by PCR using primer pair PBS2/gag3. Extrachromosomal DNA samples from ~10^5 mixed cells were amplified by PCR and viral specific bands (arrows) detected as described in section 2.2.8. Numbers on top of tracks indicate the time point (hours post infection) at which extrachromosomal DNA was prepared. Track N: PCR negative control. Tracks 10K, 50K and 100K: copy number standards at 10,000, 50,000 and 100,000 respectively. Track H3B: ~10^5 viral donor cells. Track HUT78: ~10^5 recipient cells. **(C)** Kinetic analysis: Three independent gels of each of **(B)** were exposed twice on a PhosphorImager and their pixel readings were converted to copy number using copy number standards run on the same gel. Copy numbers (average +/- S.D.) were plotted against time (hours post infection).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gag3
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INTER
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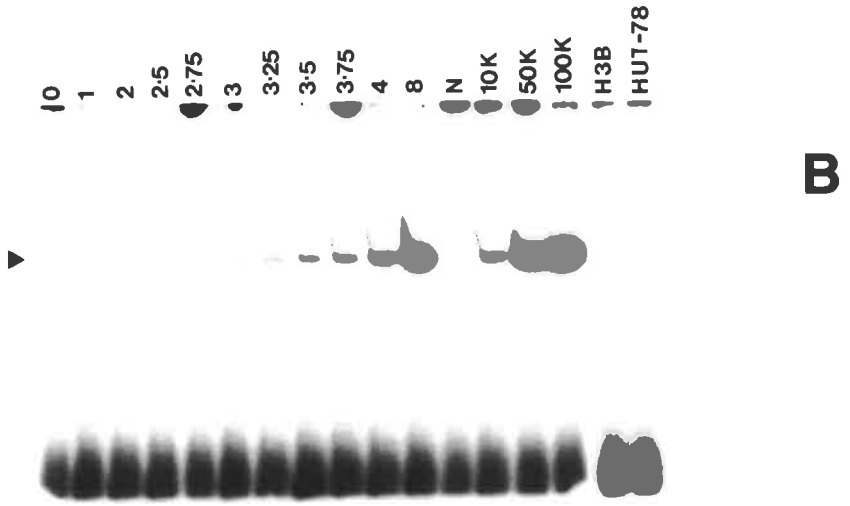
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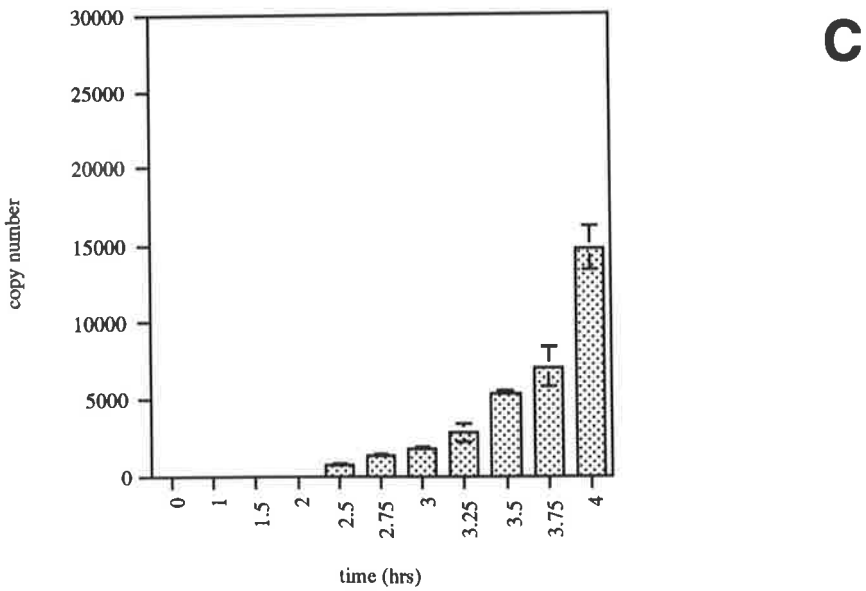
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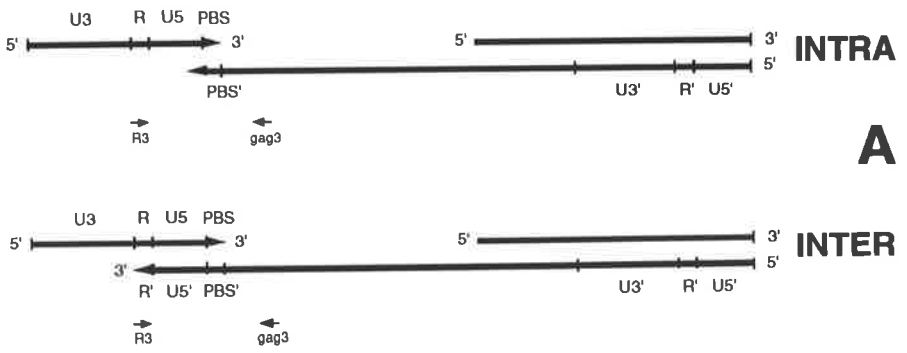
3.5
3.75
4
time (hrs)
C

Figure 4.8 Analysis of HIV reverse transcription using primer pair R3/gag3. **(A)** Location of primers and the first viral DNA species detected by these primers during reverse transcription (see Figure 4.1). **(B)** Detection of viral DNAs by PCR using primer pair R3/gag3. Extrachromosomal DNA samples from ~10^5 mixed cells were amplified by PCR and viral specific bands (arrows) detected as described in section 2.2.8. Numbers on top of tracks indicate the time point (hours post infection) at which extrachromosomal DNA was prepared. Track N: PCR negative control. Tracks 10K, 50K and 100K: copy number standards at 10,000, 50,000 and 100,000 respectively. Track H3B: ~10^5 viral donor cells. Track HUT78: ~10^5 recipient cells. **(C)** Kinetic analysis: Three independent gels of each of **(B)** were exposed twice on a PhosphorImager and their pixel readings were converted to copy number using copy number standards run on the same gel. Copy numbers (average +/- S.D.) were plotted against time (hours post infection).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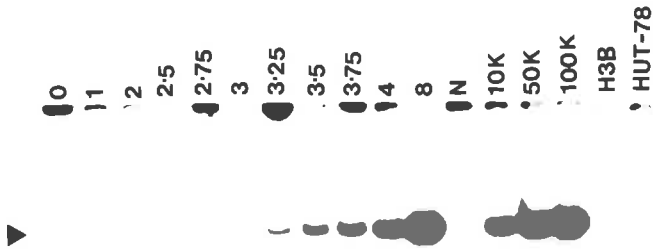
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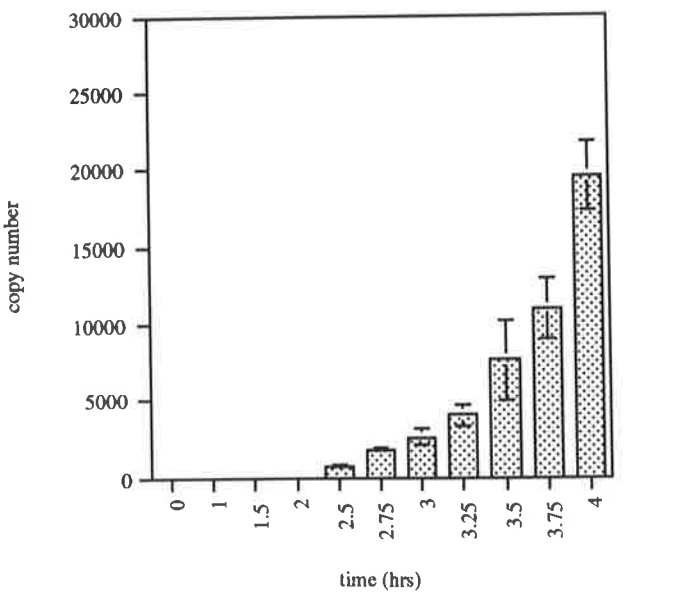
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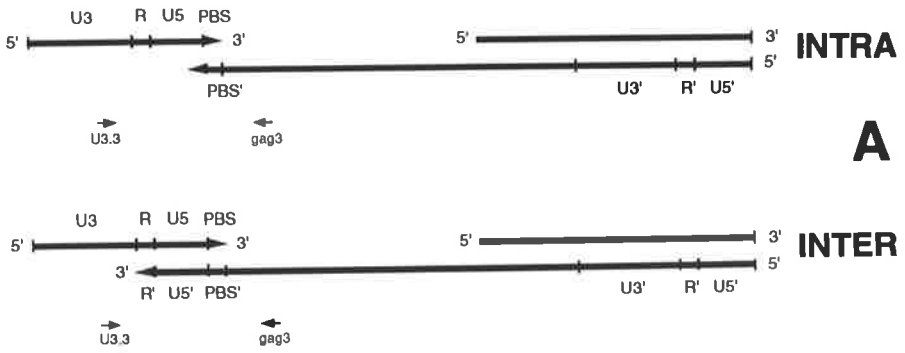
3.5
3.75
4
time (hrs)
C

Figure 4.9 Analysis of HIV reverse transcription using primer pair U3.3/gag3. **(A)** Location of primers and the first viral DNA species detected by these primers during reverse transcription (see Figure 4.1). **(B)** Detection of viral DNAs by PCR using primer pair U3.3/gag3. Extrachromosomal DNA samples from ~10^5 mixed cells were amplified by PCR and viral specific bands (arrows) detected as described in section 2.2.8. Numbers on top of tracks indicate the time point (hours post infection) at which extrachromosomal DNA was prepared. Track N: PCR negative control. Tracks 10K, 50K and 100K: copy number standards at 10,000, 50,000 and 100,000 respectively. Track H3B: ~10^5 viral donor cells. Track HUT78: ~10^5 recipient cells. **(C)** Kinetic analysis: Three independent gels of each of **(B)** were exposed twice on a PhosphorImager and their pixel readings were converted to copy number using copy number standards run on the same gel. Copy numbers (average +/- S.D.) were plotted against time (hours post infection).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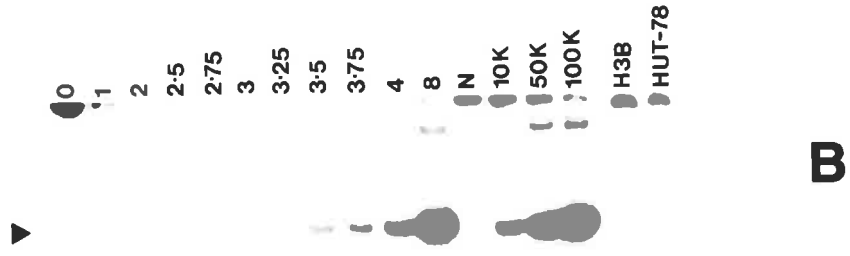
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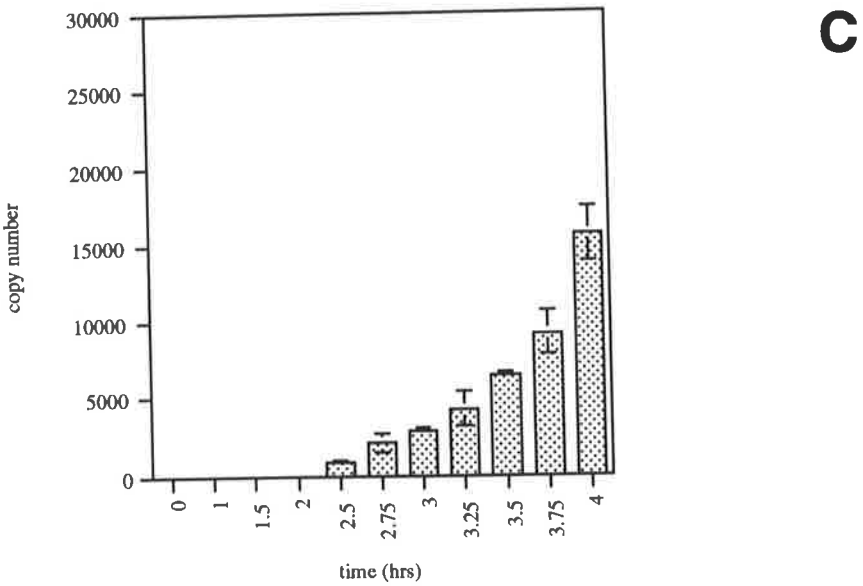
HUT-78

B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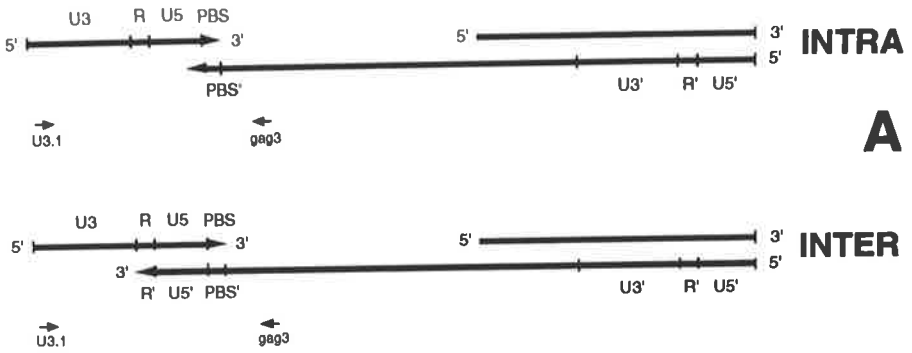
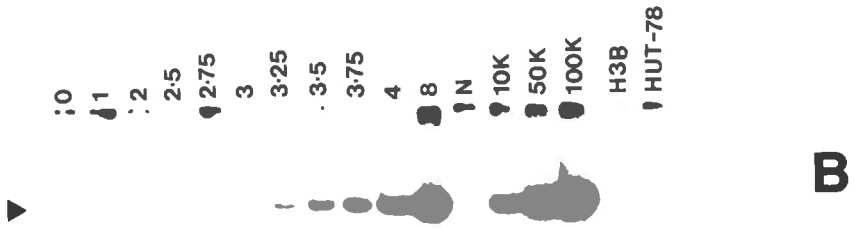
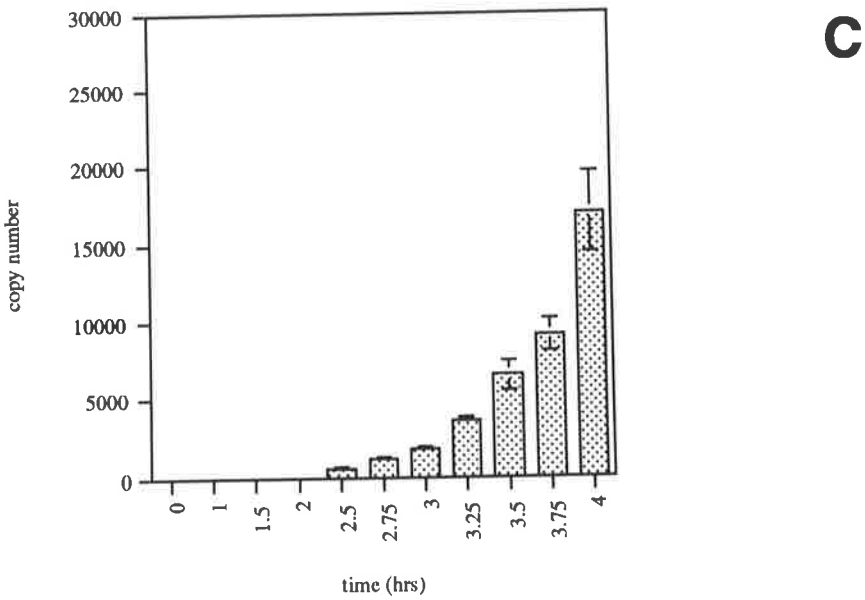
3.5
3.75
4
time (hrs)

C

Figure 4.10 Analysis of HIV reverse transcription using primer pair U3.1/gag3. **(A)** Location of primers and the first viral DNA species detected by these primers during reverse transcription (see Figure 4.1). **(B)** Detection of viral DNAs by PCR using primer pair U3.1/gag3. Extrachromosomal DNA samples from ~10^5 mixed cells were amplified by PCR and viral specific bands (arrows) detected as described in section 2.2.8. Numbers on top of tracks indicate the time point (hours post infection) at which extrachromosomal DNA was prepared. Track N: PCR negative control. Tracks 10K, 50K and 100K: copy number standards at 10,000, 50,000 and 100,000 respectively. Track H3B: ~10^5 viral donor cells. Track HUT78: ~10^5 recipient cells. **(C)** Kinetic analysis: Three independent gels of each of **(B)** were exposed twice on a PhosphorImager and their pixel readings were converted to copy number using copy number standards run on the same gel. Copy numbers (average +/- S.D.) were plotted against time (hours post infection).


U3
R
U5
PBS
5'
3'
PBS'
U3'
R'
U5'
INTRA
U3.1
gag3
A
U3
R
U5
PBS
5'
3'
R'
U5'
PBS'
U3'
R'
U5'
INTER
U3.1
gag3


0
1
2
2·5
2·75
3
3·25
3·5
3·75
4
8
N
10K
50K
100K
H3B
HUT-78
B


30000
25000
20000
15000
10000
5000
0
copy number
0
1
1.5
2
2.5
2.75
3
3.25
3.5
3.75
4
time (hrs)
C

exposed twice on a PhosphorImager and the copy numbers of DNA species detected by each of these pairs of primers were quantitated by comparison with copy number standards run on the same gel. It was found that with all four primer pairs (PBS2/gag3, R3/gag3, U3.3/gag3, U3.1/gag3) the kinetics of reverse transcription looked very similar, with the initial detection of PCR product at ~2.5 hours post infection. The detection of all four transcripts at the same time suggested that the second template switch was not associated with any time delay, as is seen with the first template transfer; this implied that the second transfer may employ different and more rapid mechanism in contrast to the first template transfer which imposed a significant delay (see above). It also meant that the above strategy did not allow differentiation between intra- versus intermolecular mechanism of the first template transfer. The copy number of viral DNA that completed the second template transfer, as estimated by using the U3.3/gag3 and U3.1/gag3 primer pairs, was 16,000-17,000 at 4hr post infection, suggesting a nominal 80-85% success rate for the second template switch compared to the estimated 20,000 copies of the completed plus-strand strong-stop DNA at 4hrs post infection by using U3.1/PBS1 primers (Figure 4.5). As the U3.3/gag3 and U3.1/gag3 sequences are present only once, in full-length DNA, while sequences prior to the switch (U3.1/PBS1) are also present in the double-stranded strong-stop DNA which accumulates in addition to the full-length product (Li *et al.*, 1993), the actual success rate for the second template switch is expected to be higher than 80-85%.

Artificially facilitated extension of the second template transfer *in vitro*, may be envisaged, as a result of the denaturing and re-annealing processes involved in the PCR reaction. For example, the plus-strand strong-stop DNA in step E (Figure 4.1) might be denatured from its native structure during PCR and then annealed to the 3' end of the minus-strand DNA and elongated by Taq polymerase and dNTP, despite the fact that the second template switch may not have taken place *in vivo* in the sample being examined. To test this possibility, we performed a control experiment in which DNA samples from different time points post infection were denatured and annealed for 20

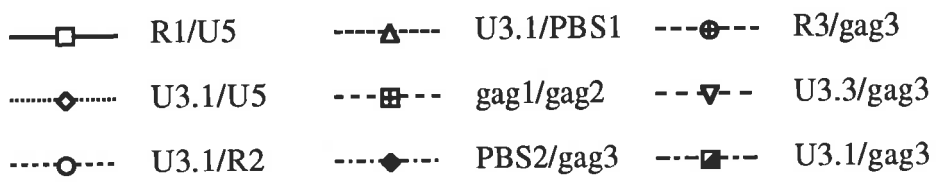
cycles using thermocycling conditions identical to a typical PCR reaction in the presence of Taq polymerase, dNTPs, etc., but without primers (so there is no amplification of discrete length DNA product). Primers were then added and amplification was allowed for another 20 cycles. No increased signals using R3/gag3 or U3.1/gag3 primers were detectable as compared to a normal PCR. Actually, in each case a slightly decreased signal was observed probably due to a slight loss of activity of Taq polymerase during the first 20 cycles of mock PCR (data not shown). We thus concluded that the possible "PCR facilitated transfer" did not significantly influence our analysis.

4.2.4 Overview of Reverse Transcription Kinetics Following Cell-to-Cell HIV Infection

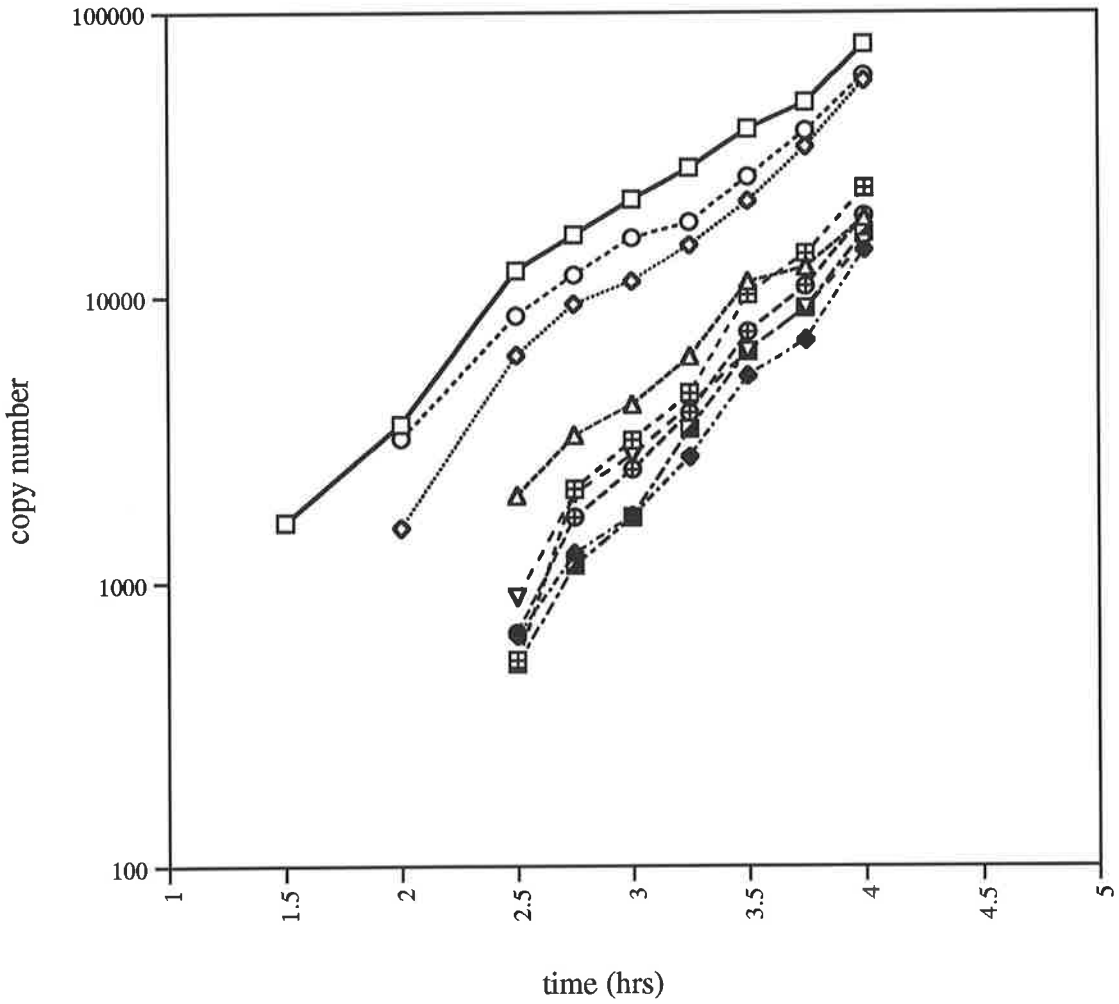
The above data demonstrated a time delay of ~30 minutes between the first appearance of the minus strand strong-stop DNA (as detected by R1/U5) at 1.5hr post infection and the first appearance of the post transfer, newly extended minus-strand viral DNA (as detected by U3.1/U5 and U3.1/R2) at 2hr post infection (Figure 4.3 and 4.4). This estimate was relatively crude because of the limited number of time points observed. On the other hand, when the overall kinetics were analysed (Figure 4.11), the positions of curves representing accumulation of each species of DNA indicated that the average time interval between the accumulation of equal copy numbers of the minus-strand strong-stop DNA (R1/U5) and the post transfer, newly extended minus-strand viral DNA (average of U3.1/U5 and U3.1/R2) could be estimated more accurately as 15-30 minutes. Similarly, Figure 4.11 also clearly demonstrated that the time delay associated with the initiation and synthesis of the plus-strand strong-stop DNA (detected by U3.1/PBS1) was more pronounced and could be estimated as 40-50 minutes in contrast to the crude estimate of 30 minutes based on the first appearance of each species (Figures 4.2C - 4.6C). Thirdly, based on the first appearance of the newly extended minus strand viral DNA following the first template transfer (as detected by U3.1/U5 and U3.1/R2 at 2hr post infection, Figures 4.3C and 4.4C), compared with either the near full-length minus-strand viral DNA ~7,500 bases apart (as detected by gag1/gag2 at ~2.5hr

Figure 4.11 Overview of the kinetics of viral DNA synthesis following cell-to-cell transmission of HIV infection.

Copy numbers (in log scale) of viral DNA as detected by each of the nine pairs of primers (see Figures 4.2 - 4.10) are plotted against time (hours post infection):



For locations of primers, see Figure 4.1.


100000
10000
1000
100
copy number
1
1.5
2
2.5
3
3.5
4
4.5
5
time (hrs)

post infection, Figure 4.6C) or full-length minus-strand viral DNA ~9,000 bases apart (as detected by U3.1/gag3, also at ~2.5hr post infection, Figure 4.10C), the rate of HIV reverse transcription was estimated at 250-300 bases per minute. In contrast, based on the analysis shown in Figure 4.11, the time interval between accumulation of equal copy numbers of post transfer, newly extended minus-strand DNA and the completed minus-strand viral DNA was estimated as ~50-60 minutes. This translates into a rate of HIV reverse transcription in infected T cells of ~150-180 bases per minute. The above analysis could not distinguish whether or not this rate varied at different stages in the replication cycle.

Taking both lines of evidence into account, we suggest that (1) in virus infected T cells the temporary arrest associated with the first template switch in the process of HIV reverse transcription was 15-30 minutes and the time delay specifically associated with the initiation and synthesis of the plus-strand strong-stop DNA was 40-50 minutes; (2) the rate of HIV reverse transcription of the minus-strand DNA using viral RNA as template in virus infected T cells was 150-180 bases per minute.

4.3 DISCUSSION AND CONCLUSIONS

Using a one-step cell-to-cell transmission infection model and quantitative PCR with primers to different regions of the genome, we were able to monitor the progression of HIV reverse transcription. Oligonucleotide primers were specifically designed for detecting the initial appearance of the minus-strand strong-stop viral DNA, the post-transfer and newly extended minus-strand viral DNA, the plus-strand strong-stop viral DNA and the fully extended minus-strand viral DNA.. In addition to its high sensitivity as a nucleic acid detection technique, PCR is able to quantitate DNA molecules heterogenous in length. Quantitative PCR using 5'-labelled primers was thus employed to identify molecules that had completed each of the different stages of

reverse transcription. Figure 4.11 shows an overview of the kinetics of viral DNA synthesis as detected by each of the nine pairs of primers used in this study.

The presence of the minus strong-stop DNA was first detected 1.5 hours after cell mixing. Primers to the U5 and U3 regions and also to the U3 and R regions first detected the post transfer extension of the minus strong-stop DNA 2 hours after cell mixing. First detection of the gag region were seen at 2.5 hours. These results suggest there is a significant time delay in the initiation of reverse transcription, and in the first template switch. However once the minus strong-stop has jumped to the 3' end of the other RNA, the synthesis of the minus DNA is efficient , with minus-strand synthesis being near complete within 2.5 hours (Figures 4.7-4.10). Synthesis of the plus strong-stop DNA was first detected around 2.5 hours. Hence the synthesis of the minus DNA and the synthesis of the plus strong-stop are occurring simultaneously (Figures 4.5C and 4.6C), indicating that two molecules of reverse transcriptase must be attached and synthesizing DNA at the same time. The plus strong-stop DNA can then jump to the 3' end of the newly synthesized minus-strand of DNA and complete the synthesis of the plus-strand of DNA and also act as template for the completion of the 3' of the minus-strand of DNA.

These results demonstrate that even following cell-to-cell transmission of HIV infection, where production of mature virions and the early steps of virus attachment and penetration into the susceptible cells may be bypassed (Li and Burrell, 1992; Li *et al.*, 1992; Sato *et al.*, 1992; Li *et al.*, 1994), there still is a considerable time delay of 1-1.5 hrs, before the initiation of minus-strand HIV DNA synthesis. This initial time delay may be required for cell fusion and activation of reverse transcription, which may involve conformational or structural changes to the viral nucleocapsid, a correctly orientated RNA secondary structure, and the interactions of this structure with viral reverse transcriptase and the tRNA primer and possible other viral and cellular proteins (Aiyar *et al.*, 1994; Li *et al.*, 1994). A time delay was also observed with the first

template transfer and the initiation of the plus-strand HIV DNA synthesis. Of the two template transfers, the first template transfer has been better studied. This template transfer is thought to require RNase H activity to generate the single strand overhang in the R region of the minus-strand DNA. There is also evidence that the R region may or may not be complete, suggesting premature strand transfer may occur (Klaver and Berkhout, 1994). However, the actual transfer reaction was reported not to require the RNase H domain, and an unidentified function of the viral reverse transcriptase has been suggested as being responsible for the strand transfer synthesis (Buiser *et al.*, 1991). Other viral proteins such as the nucleocapsid protein, have been reported to activate the first template transfer in Moloney murine leukaemia virus reverse transcription (Allain *et al.*, 1994). For HIV, it has been reported that purified HIV reverse transcriptase alone could catalyse the template transfer reaction *in vitro* (Peliska and Benkovic, 1992). Time delay was also shown to be associated with the initiation and synthesis of the plus-strand DNA (Figure 4.5). These time delays constitute a large part of the overall time required for the completion of the full length double stranded DNA.

Generally in retroviruses, minus-strand synthesis is thought to occur in a continuous fashion at the relatively slow rate of approximately 2000 nucleotides per hour, until at least part of the PBS has been copied (Varmus and Brown, 1989). Such rates have been based on studies with type C retroviruses, in which the first complete linear DNA molecules were detected approximately 4hr after infection (Varmus and Swanstrom, 1982; Brown *et al.*, 1989). Similarly, studies on HIV, using one-step virus growth conditions and either cell-free virus or cell-to-cell transmission routes, showed that the complete linear HIV DNA molecules were also first detected by Southern blot at approximately 4hrs post infection (Kim *et al.*, 1989a; Li and Burrell, 1992; Sato *et al.*, 1992; Barbosa *et al.*, 1994). However, *in vitro* kinetic studies of HIV reverse transcription, using bacterially expressed purified HIV reverse transcriptase and poly(A)$dT_{(20)}$ as template, have yielded an estimated rate of DNA synthesis of 10-15

nucleotides per second or 36,000-45,000 nucleotides per hour (Huber *et al.*, 1989). It was further shown that DNA synthesis was less rapid with RNA and DNA templates of random sequence (Huber *et al.*, 1989). A similar *in vitro* primer extension experiment, using HIV reverse transcriptase but globin mRNA as template and $(dT)_{16}$ as primer and dNTP concentrations mimicking those in activated peripheral blood lymphocytes, reported an ~100 fold lower rate of DNA synthesis of 70-100 nucleotides in 15 minutes or 280-400 nucleotides per hour (Gao *et al.*, 1993). With the use of PCR, Stevenson *et al.* (1990) reported that near full-length HIV DNA (POL region) could be detected in infected MT2 cells within 30 minutes of the initial exposure to cell-free virus. Zack and co-workers used PCR and primers specific for different regions of HIV DNA to show that the minus-strand strong-stop DNA could be detected in both resting and activated lymphocytes at 2.5hr post infection, while full-length HIV DNA could be detected only in activated peripheral blood lymphocytes 6hr after HIV infection (Zack *et al.*, 1990; Zack *et al.*, 1992). A recent PCR-based study of HIV reverse transcription kinetics reported that viral DNA synthesis was completed 12hr to 16hr after HIV infection of a T-cell line, but required 36hr in primary human macrophages (Collin and Gordon, 1994). All the PCR-based kinetic studies mentioned above used low multiplicity of infection, believing that the high sensitivity of PCR would allow a single cycle of viral infection in a minority of cells to be effectively analysed in a multi-round infection system.

H3B cells, the HIV infected donor cells in our cell-to-cell infection, contain no episomal DNA, detectable by the PCR conditions used. Therefore each of the intermediate reverse transcript species detected after mixing H3B cells with the non-infected recipient HUT-78 cells, was due to a new round of DNA synthesis. This is important because partially reverse transcribed viral DNA intermediates have been found in the HIV virions as well as in other retrovirus particles (Lori *et al.*, 1992; Trono *et al.*, 1992; Zhu and Cunningham, 1993). The retroviral DNA associated with virions is thought not to be simply a passive contamination on the outside of the virions. This virion DNA is probably responsible for the full amount of minus strong-stop HIV DNA, but

not near full length HIV DNA, detected in the presence of two reverse transcriptase inhibitors at high concentrations, even when a DNase treated virion inoculum was used (Zack *et al.*, 1990). Similarly, this virion DNA may be responsible for the extremely early detection of near full length HIV DNA (gag sequence) at 30 minutes after initial exposure of MT2 cells to HIV (Stevenson *et al.*, 1990). Likewise, the high copy number of the early DNA species at the time of infection (0hr) reported in a recent HIV reverse transcription kinetics study (Collin and Gordon, 1994) might also be due to virion DNA. In the results described in this thesis, pre-existing episomal viral DNA was either not present or below the sensitivity limits of the PCR assay used.

The initiation of plus-strand HIV DNA synthesis may start at either the 3' PPT or the central PPT (Pullen and Champoux, 1990; Charneau and Clavel, 1991; Charneau *et al.*, 1992). *In vitro* studies of processing of the PPT primer have shown that the RNase H activity of the HIV reverse transcriptase cleaved the viral RNA into multiple fragments but only two primers that comprised the near entirety of the PPT could be extended (Huber and Richardson, 1990). This would suggest other RNA fragments were unable to function as successful primers for synthesis of plus-strand strong-stop DNA. In our current experimental design, only the initiation and synthesis of the complete plus-strand strong-stop DNA from the 3' PPT could be detected by the U3.1/PBS1 primers. Plus-strand DNA that had initiated at the central PPT would not include the PBS sequence, as the viral reverse transcriptase would have cleaved the tRNA upon the completion of the plus-strand strong-stop DNA when the newly synthesized minus-strand DNA was used as a template for the first time (Step E in Figure 4.1). It has been reported that the PPT primer could be released intact from the elongated plus-strand DNA via a cleavage at the RNA-DNA junction and could be reused (Huber and Richardson, 1990). This re-processing of PPT primer may slow down the reverse transcription process as a whole, but would have little impact on the first round usage of the PPT primer directly cleaved from the viral RNA.

Our results using PCR primers designed to differentiate DNA species before and after the second template switch showed that DNA species clearly synthesized after the second template switch (detected by U3.1/gag3 & U3.3/gag3 primer pairs) appeared at the same time and increased in a similar kinetics to DNA species clearly synthesized before the second template switch (detected by PBS2/gag3), regardless of the intra- or the intermolecular model for the first template switch (as seen in Figure 4.11). This clearly implicates a more rapid second template transfer as compared to the first one. Thus inter- and intra-molecular mechanisms for this switch could not be distinguished in the experimental system used.

In summary, we have measured the actual *in vivo* kinetics of a number of different discrete steps that take place during HIV reverse transcription. The initial lag period before the commencement of reverse transcription (1.5hrs) and time delays associated with the first template switch (15-30 mins) and initiation and synthesis of the plus-strand strong-stop DNA (40-50 mins) constituted a large part of the time taken for the first appearance of full length linear DNA. The second template transfer was found to be more rapid than the first one and apparently did not impose temporal arrest on the reverse transcription process. The actual rate of HIV minus-strand DNA synthesis in T-cell lines was estimated at 150-180 bases per minute or ~50-60 mins for the synthesis of 9,000 bases. If we allow another 50-60 mins for the synthesis of the major part of the plus-strand DNA, the first appearance of double stranded full-length DNA might be expected at ~3.5 - 4hrs post infection, in agreement with the Southern-based kinetic studies on type C oncoviruses, visna virus and HIV. It should be emphasized that this study is based on a one-step viral infection model and that PCR was employed not for its high sensitivity but rather its ability to quantitate DNA structures heterogenous in length but belonging to a population of molecules specific for each of the steps during retroviral reverse transcription. The rate of reverse transcription in different cells may vary, depending on cell types and activation status of the cell, and is linked to the levels of deoxynucleotides in the cell (Zack *et al.*, 1990; Gao *et al.*, 1993; Collin and

Gordon, 1994; Krogstad *et al.*, 1994). In this context, it is worth noting that in two recent similar studies (both using human primary macrophages and the same strain of HIV, both using PCR techniques), a one-step infection model detected transferred and extended HIV ENV DNA at 2hr post infection (Munis *et al.*, 1992), while a low multiplicity infection model first detected transferred and newly extended U3/U5 HIV DNA ~10hr post infection (Collin and Gordon, 1994). We believe a one-step model is more suited to kinetic analyses of replication events at the cellular level. Further detailed studies, using optimal experimental model systems on viral reverse transcription and other early events in the HIV replication cycle may hold promise for better understanding and control of HIV infection.

CHAPTER 5

RNA-CONTAINING COMPLEXES AND THE EARLY MOLECULAR EVENTS OF HIV CELL-TO-CELL INFECTION

5.1 INTRODUCTION

In retroviral infection with cell-free virus, the intracellular structure that gives rise to the DNA-containing replication complex is thought to consist of the two genomic RNA molecules and possibly some viral proteins derived from the viral core (Bowerman *et al.,* 1989). It is generally believed that maturation of retrovirus reverse transcriptase occurs at or after budding of the virus from infected cells (Witte and Baltimore, 1978; Eisenman *et al.,* 1980). For HIV, structural studies have suggested that virion maturation may occur after virion assembly and budding (Arnold and Arnold, 1991; Gelderblom, 1991). In a cell-to-cell HIV infection, this budding process is by-passed and therefore the requirement of viral assembly, release and maturation of the virions is also by-passed (Li and Burrell, 1992; Sato *et al.,* 1992; Li *et al.,* 1994). As a result, the viral RNA-containing, early cytoplasmic complex that takes part in viral DNA synthesis may be different in the cell-to-cell transmission setting from that found in an infection with cell-free virus.

In this chapter, these 'very early' molecular structures involved in reverse transcription machinery in a cell-to-cell infection were investigated. Firstly , the virus donor cells (H3B) used in our cell-to-cell infection were investigated to determine the origin of potential reverse transcription complexes (ie. replication complexes). The presence of replication complexes was then investigated at different times after infection.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of Cytoplasmic Lysate from H3B Donor Cells

H3B cells were vortexed at 80% of the full force of the vortex machine (Maxi Mix II, Thermolyne Corporation, subsidiary of Sybron, Iowa U.S.A.) for 3 x 10 seconds (to remove budded virus particles from the cell surface), followed by a more vigorous vortex at 100% force of the vortex machine for a further 3 x 1 minute, resulting in the forced-release of 'budding-associated' virus particles. After removal of budding-associated particles, the cells were immediately freeze-thawed in fresh cell culture medium three times and the cell lysate was spun at 1000 x g for 3 minutes, followed by 8000 x g for 10 minutes (Li *et al.*, 1994). The clear supernatant (H3B cytoplasmic lysate) was then analysed on sucrose gradients.

5.2.2 Analysis of viral RNA-containing complexes in H3B Cytoplasmic Lysate by Sucrose Gradient Sedimentation and RNA Detection.

H3B cytoplasmic lysate were adjusted to 0.5% Triton X-100 and 6% sucrose then layered onto a 10ml gradient of 15%-30% sucrose in buffer A [10mM Tris pH7.4, 150mM KCl, 1mM dithiothreitol, 20mg/ml aprotinin] containing 0.5% Triton X-100 and centrifuged for 90 minutes at 35,000 rpm at 4°C using a Beckman SW41 rotor. Fractions (1ml) were collected and 200µl of each fraction was individually processed to extract RNA using the urea-SDS method as described in Chapter 2. The resulting RNA samples were then treated with RNase-free DNase I (1 unit per 100µl reaction) at 37°C for 1hr and further extracted with phenol-chloroform and precipitated by ethanol. Purified RNA samples were reverse transcribed using Rous-Associated Virus-2 reverse transcriptase (Amersham) then amplified by PCR, using primers gag1 and gag2, for 25 cycles (see section 2.10). PCR products were resolved on an 8% polyacrylamide gel , transferred to nylon membrane and detected by Southern hybridization as described in section 2.10.2, using internal oligonucleotide primers.

5.2.3 RT Assays

Routine exogenous RT assays as measured by [^{3}H]-thymidine incorporation using a synthetic template (poly(A).(dT)$_{10}$) were carried out as described by Hoffman *et al.*, (1985). Endogenous RT assays were performed using a method essentially as described by Yong *et al.*, (1985), except that the final reaction mix contained 0.0125% Triton X-100 and 15µg/ml melittin (Sigma). The RT assays were performed by Alice Stevenson.

5.3 RESULTS

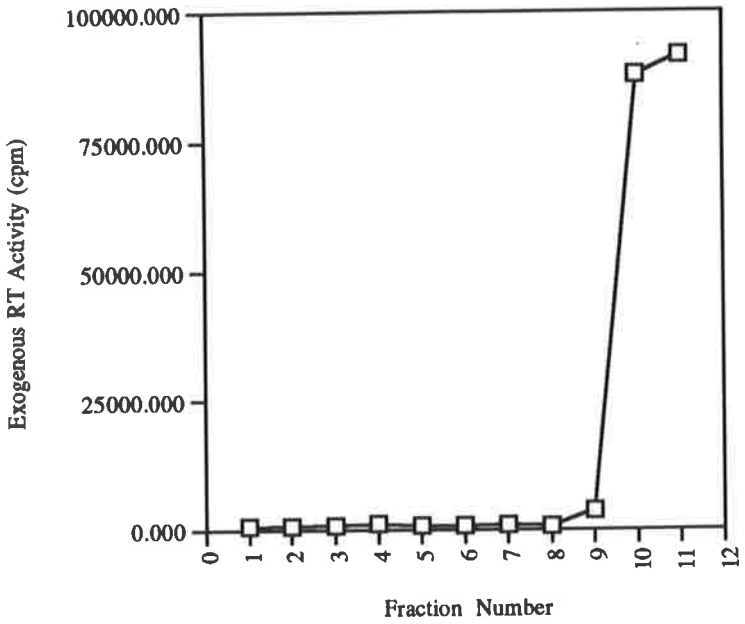
5.3.1 Cell-Associated Reverse Transcriptase Activities in HIV-Persistently Infected Cells

In an effort to identify the origin of the replication complexes isolated after cell-to-cell infection, the virus donor cells (H3B) used in our cell-to-cell infection were first investigated. Virus donor cells were vortexed under controlled conditions of increasing severity to release virus that was budded but trapped, and partially budded virus. This ensured any newly released virus particles associated with the cell surface were removed. After removal of budding-associated virus particles, the cells were immediately freeze-thawed in fresh cell culture medium three times and the nuclear and cell debris removed by centrifugation. This clean cytoplasmic lysate was subjected to sucrose gradient sedimentation. The gradient was fractionated (1ml fractions) and each individual fraction was assayed for exogenous RT activity, endogenous RT activity and for the presence of viral RNA. Triton X-100 (0.5%) was included in the sucrose gradient to eliminate non-specific protein-RNA associations.

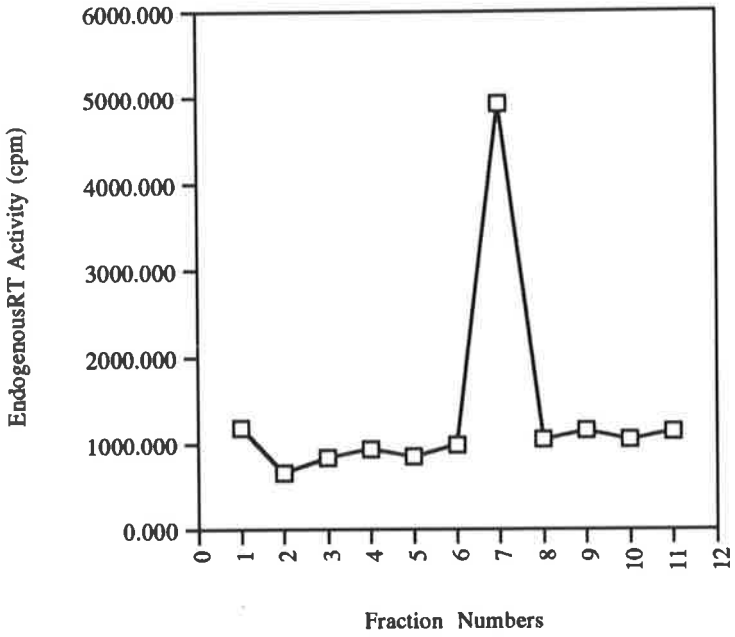
Figure 5.1A shows the majority of the cell-associated exogenous RT activity was found in the top two fractions (fractions 10 and 11) of the sucrose gradient, while endogenous RT activity (Figure 5.1B) was mainly present in one distinct peak, in fraction 7 (22% sucrose). Using HIV *gag* specific primers and RT-PCR, it was demonstrated that the genomic length, HIV RNA was also found in fraction 7 (Figure

Figure 5.1 Physical form of cell-associated HIV RT in H3B cells. Cell-associated RT preparation equivalent to 4×10^7 H3B cells was analysed by sucrose gradient centrifugation (35K/90mins/4°C) as described in the text. Eleven 1ml fractions (fraction 1, bottom; fraction 11, top) were collected. **(A)** 20µl of each gradient fraction was used for exogenous RT assay and **(B)** 10µl for endogenous RT assay. **(C)** 200µl of each fraction was processed for genomic length HIV RNA detection by RT-PCR as described in Material and Methods (Chapter 2). Track N: RT-PCR negative control. Arrow indicates the 160bp amplification product.

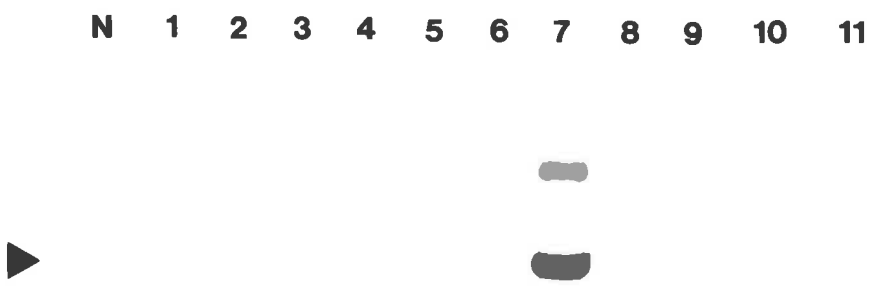
A


100000.000
75000.000
50000.000
25000.000
0.000
Exogenous RT Activity (cpm)
0 1 2 3 4 5 6 7 8 9 10 11 12
Fraction Number

B


6000.000
5000.000
4000.000
3000.000
2000.000
1000.000
0.000
EndogenousRT Activity (cpm)
0 1 2 3 4 5 6 7 8 9 10 11 12
Fraction Numbers

C


N 1 2 3 4 5 6 7 8 9 10 11

5.1C). These results indicated that while the majority of cell-associated exogenous RT was in a soluble (non-sedimentable) form, the endogenous activity was found in a particulate structure containing genomic HIV RNA. This structure sedimented more slowly than the viral DNA-containing 320S cytoplasmic replication complex found early after cell-to-cell transmission in HIV infection, as described in Chapter 3. Taking into account that the complex was located at the 22% sucrose level of the gradient, the sedimentation coefficient of the RNA complex was estimated by the method of McEwen, (1967) as described in section 2.3.4. However, neither the density nor the ratio of RNA to protein is known for the RNA-containing complexes isolated in this study. For RNA and ribosomes a density of 1.5 is recommended (see Table 2.2). Most proteins and some plant and bacterial viruses generally have densities of approximately 1.3 (Table 2.2). If the density of the complex was assumed to be 1.3, then the sedimentation coefficient would be calculated to be 180S. However if the density was 1.5, the sedimentation coeffient would be 210S. Thus within these limits a sedimentation coefficient of approximately 180S-200S was estimated.

5.3.2 Viral DNA and RNA-containing Replication Complexes at Different Stages of Reverse Transcription.

In Chapter 4, various primer pairs were used to follow the different stages of reverse transcription. Oligonucleotide primers directed to the U5 and R regions allowed the detection of the minus strong-stop DNA and hence the initial stages of reverse transcription to be detected (see section 4.2.2). The minus strong-stop DNA was detected as early as 1.5 hours after infection, with the near full-length viral DNA detectable after 3 hours by PCR.

To relate their products to the cytoplasmic structure involed, cytoplasmic extracts (section 2.2.2) isolated 2 and 6 hours after a cell-to-cell infection (1×10^8 cells) were subjected to sucrose gradient sedimentation and fractionated (1ml). DNA was extracted from 100µl samples and RNA from 200µl samples of each sucrose fraction. Viral DNA

in gradient fractions was detected by PCR using radioactively labelled primers U5 and R1 for the 2 hours fractions and primers gag1 and gag2 for the 6 hours fractions. In both the 2 hrs and 6 hrs gradient fractions (Figure 5.2A and 5.3A), the DNA peak corresponded to a sedimentation coefficient of 320S. These results show that by the time minus strong-stop DNA synthesis is completed, the nucleoprotein complex involved is of 320S size. This further implies that further steps in reverse transcription proceed within this 320S cytoplasmic replication complex.

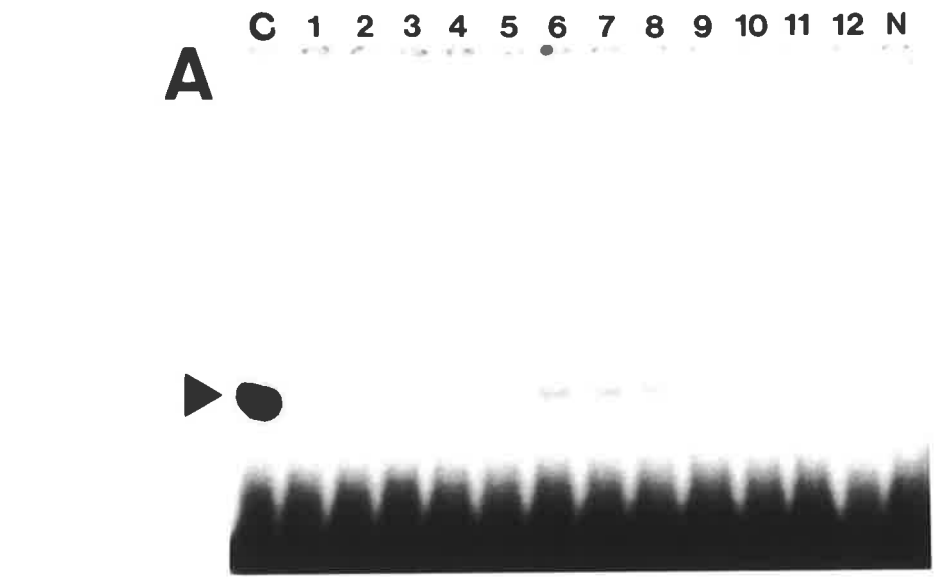
Using RT-PCR and HIV *gag* specific primers, it was demonstrated that the genomic length , HIV RNA peak was found in fraction 8 and 9 (22% sucrose), for both 2 and 6 hour extracts (Figure 5.2B and 5.3B). This position corresponded to a value of 180S-200S, equivalent in size to the RNA complex isolated from the H3B donor cells.

Individual gradient fractions were assayed for exogenous RT activity and endogenous RT activity. The majority of the cell-associated exogenous RT activity was again found in the top of the sucrose gradient, while endogenous RT activity was not detected in any of the fractions, in contrast to results obtained with the lysate of H3B cells.

Immunoprecipitation-PCR studies were carried out with the 180S-200S RNA complexes, in an attempt to characterize the proteins associated with the RNA. The detection of HIV RNA in an immunoprecipitate, was taken as evidence that viral RNA complexes had been precipitated by the particular antibody used. As with the DNA-containing replication complexes in Chapter 3, antisera to the viral proteins reverse transcriptase, integrase, protease, p17, p24 and also histones were used in these immunoprecipitation studies. However, viral RNA was not detected in the pellets after immunoprecipitation with any of the antisera used, most likely due to RNA degradation during the incubation involved. A number of attempts were made to prevent this degradation: (i) Firstly shorter incubation times at 4°C were used, eg.4 or 2 hours

Figure 5.2 Sucrose gradient sedimentation of HIV DNA- and RNA-containing replication complexes isolated from the cytoplasm of mixed cells 2 hours after a cell-to-cell infection.

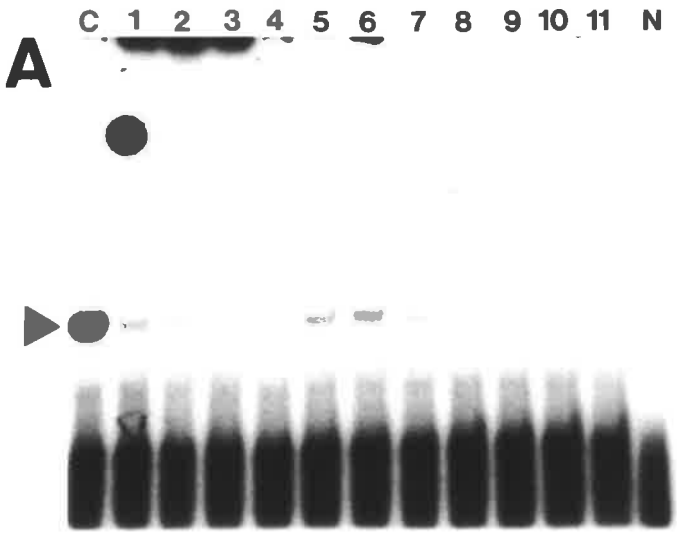
Cytoplasmic extracts were prepared as described in section 2.2.2 and a 1ml aliquot was loaded onto 15%-30% sucrose gradients, which were then centrifuged at 35000 rpm for 1hrs at 4°C, in a Beckman SW41 rotor. The gradients were fractionated into 1ml fractions. **(A)** Viral DNA was extracted from 100µl of each gradient fraction with HIV DNA detected by PCR, as described in section 2.2.8, using the primer pair R1/U5 for the detection of the minus-strand strong-stop (see Figure 4.1 and 4.2). **(B)** RNA was extracted from 200µl of each gradient fraction and genomic length HIV RNA was detected by RT-PCR as described in section 2.2.9. 1-11 refer to fractions from bottom to top of the gradient. C in **(A)**: unfractionated cytoplasmic extract. N in **(B)**: PCR negative control.

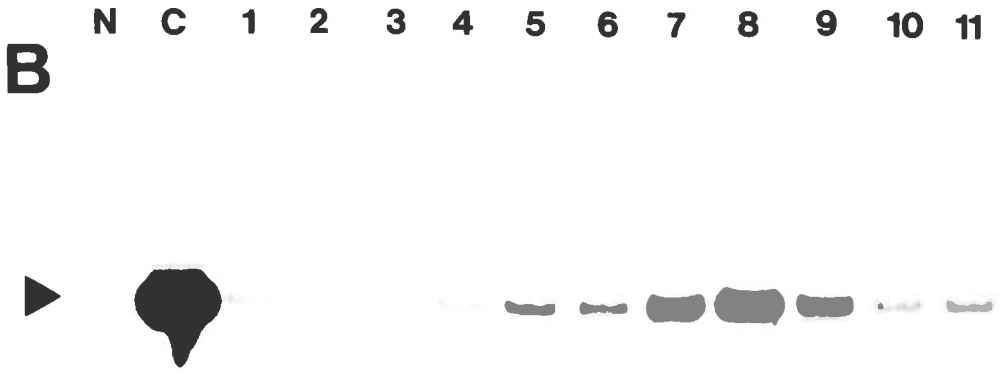

A
C 1 2 3 4 5 6 7 8 9 10 11 12 N


B
N C 1 2 3 4 5 6 7 8 9 10 11 12

Figure 5.3 Sucrose gradient sedimentation of HIV DNA- and RNA-containing replication complexes isolated from the cytoplasm of mixed cells 6 hours after a cell-to-cell infection.

Cytoplasmic extracts were prepared as described in section 2.2.2 and a 1ml aliquot was loaded onto 15%-30% sucrose gradients, which were then centrifuged at 35000 rpm for 1hr at 4°C, in a Beckman SW41 rotor. The gradients were fractionated into 1ml fractions. **(A)** Viral DNA was extracted from 100µl of each gradient fraction with HIV DNA detected by PCR, as described in section 2.2.8, using the primer pair gag1/gag2 for the detection of near full-length DNA (see Figure 4.1). **(B)** RNA was extracted from 200µl of each gradient fraction and genomic length HIV RNA was detected by RT-PCR as described in section 2.2.9. 1-11 refer to fractions from bottom to top of the gradient. C in **(A)**: unfractionated cytoplasmic extract. N in **(B)**: PCR negative control.


A
C 1 2 3 4 5 6 7 8 9 10 11 N


B
N C 1 2 3 4 5 6 7 8 9 10 11

incubation with the antiserum instead of an overnight incubation as previously used; (ii) RNAsin, an RNAse inhibitor was included throughout the immunoprecipitation procedure; and (iii) antisera were incubated at 55°C for 45 mins in the presence of 0.1M iodoacetic acid, 0.15M sodium acetate(pH 5.2) and then snap cooled on ice and $CaCl_2$ added to a final concentration of 5mM, in an attempt to inactivate any RNAses present (Maniatis *et al.*, 1982). Despite these added precautions to prevent degradation, an RNA signal was not detected in either the pellet or supernatant of the immunoprecipitation reaction after incubation, indicating that the RNA was being degraded, rather than not being immunoprecipitated. Future attempts at identifying proteins associated with this RNA complex will require the removal of the possible contaminating RNAase activity present in the antisera. Two possible procedures for removing this RNAse activity exist: (i) Antiserum can be mixed with Macaloid, a clay that absorbs RNAase (Maniatis *et al.*, 1982) or (ii) RNAase can be removed by affinity chromatography on agarose 5'-(4-aminophenylphosphoryl uridine-2'(3')-phosphate (Maxwell *et al.*, 1977; Maniatis *et al.*, 1982).

5.3.3 Detection of the Double-Stranded Strong-Stop DNA in the Replication Complex

A discrete double-stranded strong-stop HIV DNA with a length of ~650 base pairs was isolated from cells following cell-to-cell transmission of infection (Li *et al.*, 1993). This species of DNA corresponded to the region representing the right hand long terminal repeat of the proviral DNA. It was proposed that a transient free plus-strand strong-stop DNA is released from its template by displacement synthesis and subsequently used as a template for the synthesis of its complementary minus-strand during the process of reverse transcription in our cell-to-cell infection. The proposed transient free plus-strand strong-stop DNA may also mediate the second template switch (see Figure 4.1 F-I).

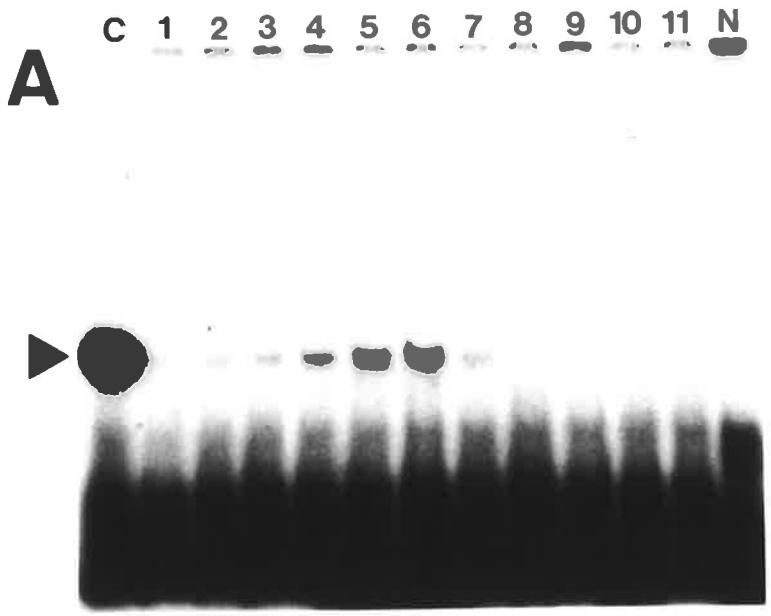
The association of this double-stranded strong-stop DNA with the cytoplasmic replication complex was investigated. A cytoplasmic extract from a 6 hour cell-to-cell infection was analysed on a sucrose gradient, fractionated and DNA extracted from each fraction. Double-stranded strong-stop DNA and the proposed transient plus-strand strong stop DNA were detected with PCR using radioactively labelled primers U3.1 and PBS1, while the near full-length DNA and hence the replication complex was detected with primers gag1 and gag2. The position of these primers is indicated in Figure 4.1. As deproteinated DNA sedimented at the top of the sucrose gradient using our centrifugation conditions (see Chapter 3, section 3.2.3), detection of DNA with the U3.1 / PBS1 primers and not with the *gag* primers in one of the top fractions would indicate that the double-stranded strong-stop product is released from the replication complex. However if the double-stranded strong-stop product remains associated with the complex the DNA profiles will look identical. Figure 5.4A shows the PCR detection of the replication complex using the gag primers. As expected the replication complex was located in fractions 5 and 6 (320S). Detection of the double-stranded strong-stop DNA is shown in figure 5.4B. Again the DNA peak was found in fractions 5 and 6, with the DNA profiles of the two PCRs looking identical. No DNA signal was detected outside the replication complex by either pair of primers. These results suggest that the double-stranded strong-stop DNA had not separated from the replication complex as a discrete product, but most likely remained associated with the functional HIV replication complex.

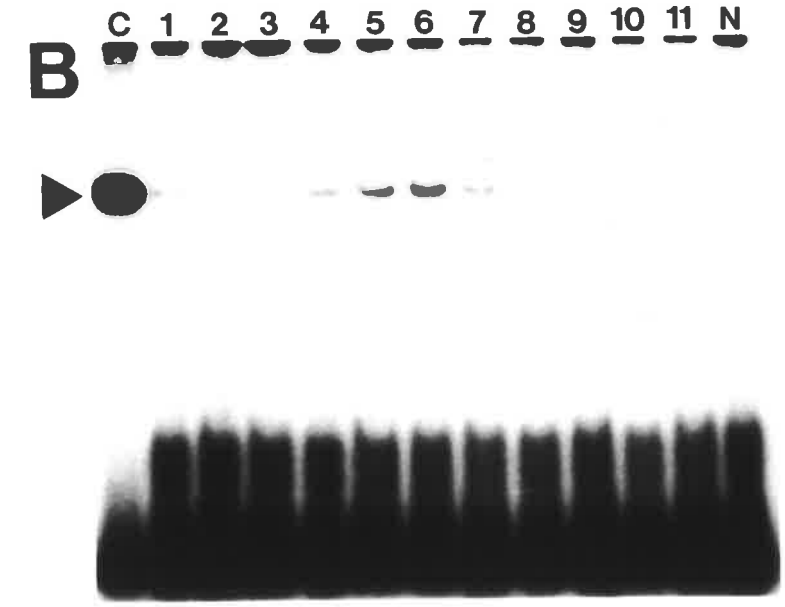
5.4 Discussion

Throughout Chapters 3 and 4 a one-step, synchronous cell-to-cell transmission of HIV infection, using HIV-persistently infected cells as virus donor cells (H3B cells) and HUT-78 cells as recipient cells, has been used to investigate the process of reverse transcription, and the structure and protein composition of the replication complex defined by the presence of a partial or final product of reverse transcription. In this

Figure 5.4 Detection of the double-stranded strong-stop DNA in replication complexes after sucrose gradient sedimentation.

Cytoplasmic extracts were prepared as described in section 2.2.2 and a 1ml aliquot was loaded onto 15%-30% sucrose gradients, which were then centrifuged at 35000 rpm for 1hr at 4°C, in a Beckman SW41 rotor. The gradients were fractionated into 1ml fractions. Viral DNA was extracted from 100µl of each gradient fraction with HIV DNA detected by PCR, as described in section 2.2.8, using the primer pair **(A)** gag1/gag2 for the detection of near full-length DNA, and **(B)** U3.1/PBS1 for the detection of full-length DNA as well as the double-stranded strong-stop DNA (see Figure 4.1). 1-11 refer to fractions from bottom to top of the gradient. C: unfractionated cytoplasmic extract. N: PCR negative control.


A
C 1 2 3 4 5 6 7 8 9 10 11 N


B
C 1 2 3 4 5 6 7 8 9 10 11 N

chapter, the origin of the replication complex and hence the origin of the RNA template utilized in the reverse transcription process in our cell-to-cell infection was investigated.

Firstly, the association of reverse transcriptase with viral genomic length RNA in the virus donor cells (H3B cells) was investigated, as only in specific association with viral RNA (not as free enzyme) can we expect the reverse transcriptase to start synthesizing viral DNA *in vivo*. Cytoplasmic lysates from virus donor cells (H3B cells) from which budding-associated virus particles had been removed, were analysed on sucrose gradients. Genomic RNA was found to sediment as a discrete structure with a sedimentation coefficient of 180S-200S. The fraction containing this genomic HIV RNA also corresponded with the peak fraction of endogenous RT activity. Therefore genomic HIV RNA in our H3B virus donor cells exists in association with reverse transcriptase in a complex of 180S-200S. The presence of other proteins in this structure could not be explored due to RNA degradation.

Next, the relationship between the 180S-200S RNA-containing complex in H3B cells and the 320S DNA-containing cytoplasmic replication complex during the process of reverse transcription was investigated. Cytoplasmic extracts isolated from a cell-to-cell infection 2 hours after cell mixing were subjected to sucrose gradient velocity sedimentation. The DNA and RNA content of the sucrose gradient fractions were analysed. RT-PCR using HIV *gag* specific primers demonstrated that the genomic length, HIV RNA again sedimented as a discrete complex with a sedimentation coefficient of 180S-200S. As shown by the reverse transcription kinetics in Chapter 4, only the initial stages of reverse transcription were detectable by 2 hours. Oligonucleotide primers directed to the U5 and R regions of the genome, detect the minus-strand strong-stop DNA and hence the earliest product of reverse transcription. PCR with these primers showed that the minus-strand strong-stop DNA was found in a complex of approximately 320S. However, the DNA peak observed in the sucrose gradient of the cytoplasmic extract at 2hrs was less defined than at 6hrs, with the

DNA peak being broader (spanning fractions 6 through to fraction 8) and not as sharp. This result could represent the gradual transition of the 180S-200S RNA complex to the 320S DNA containing replication complex with the initiation of reverse transcription, although pulse chase or inhibitor studies would be required to explore this point more definitively. At 6 hours after infection, the DNA-containing replication complex (identified with gag primers) sedimented sharply at the 320S position, while genomic HIV RNA was again found in a complex 180S-200S.

These results are compatible with a model whereby the incoming infectious structure from the H3B virus donor cells consists of a 180S-200S RNA complex containing genomic length HIV RNA, the viral enzyme reverse transcriptase and probably other viral and cellular proteins. Despite the presence of mature reverse trasncriptase and an appropriate RNA template that functions *in vitro* in exogenous and endogenous assays, the H3B donor cells do not contain any episomal DNA detectable by Southern hybridization, indicating that *in vivo* reverse transcription does not occur to any significant extent in these cells. Once co-cultivated with CD4+ HUT-78 cells, however , reverse transcription is triggered by an unknown mechanism (Li *et al.,* 1994), with the initial stages of reverse transcription being detected 1.5 hours after cell mixing. The earliest product of reverse transcription and subsequent DNA synthesis are found within a 320S complex. It is likely that the 180S-200S structure is a precursor to the 320S structure, with the conversion occurring either as a cause or as a result of the initiation of reverse transcription. This conversion may involve conformational changes, permeability changes, addition of further viral or cellular proteins and/or other mechanisms.

In the reverse transcription model proposed by Li *et al.*, a transient plus-strand strong-stop DNA mediated the second template transfer and a double-stranded strong-stop by-product of ~650 base pairs is produced (Li *et al.,* 1993). In an attempt to determine whether this double-stranded strong-stop DNA was released from the

replication complex in which the whole process of reverse transcription takes place, or whether it remained associated with the replication complex, its sedimentation properties were characterized on a sucrose gradient. Both near full-length DNA and the plus-strand strong-stop DNA were detected only in the same fractions as the 320S replication complex, indicating that the double-stranded strong-stop by-product most likely remains associated with the replication complex and is not released into the cytoplasm.

In a cell-free virus infection, mature virus particles are involved in the infection of susceptible host cells. Structural studies with HIV have suggested that the final maturation of virion morphology occurs after virion assembly and budding (Arnold and Arnold, 1991; Gelderblom, 1991). In our cell-to-cell infection, this budding process is by-passed, as direct cell membrane fusion occurs between infected and uninfected cells. Theoretically then, H3B cells would be capable of donating HIV-specific intermediates representing all the stages of replication taking place in these cells. In particular, immature core containing unprocessed viral proteins such as precursor reverse transcriptase along with the genomic RNA are likely candidates for precursors of replication. However, our results in this study demonstrate that H3B cells contained significant cell-associated reverse transcriptase in a form that was active in both exogenous and endogenous assays *in vitro* but inactive *in vivo*. In addition, it was also recently demonstrated that following cell-to-cell transmission of HIV infection to susceptible cells, *de novo* reverse transcription was initiated without detectable evidence for further synthesis or proteolytic processing of HIV reverse transcriptase (Li *et al.*, 1994). Thus we can assume that, within the H3B donor cells, the two copies of the genomic RNA are associated with fully-cleaved, but inactive, reverse transcriptase and possibly also with the precursor or mature forms of the viral proteins integrase, p17 , protease and Vpr and in a 180S-200S complex. When the H3B cells are mixed with HUT-78 cells, attachment involving the CD4+ receptor takes place, followed by cell fusion. Presumably the 180S-200S RNA complex is released into the cytoplasm of the HUT-78 cells and the reverse transcriptase enzyme is activated by an unknown process,

contributed by the HUT-78 cells, leading to the initiation of reverse transcription. With this initiation of reverse transcription, the 180S-200S RNA complex undergoes a structural change resulting in the 320S DNA containing replication complex. The above proposal is answerable to further detailed investigations.

CHAPTER 6

CONCLUDING DISCUSSION AND OVERVIEW

6.1 CONCLUDING DISCUSSION

In this study, an established one-step, single cycle model for synchronous cell-to-cell transmission of HIV infection, using HIV-persistently infected cells as virus donor cells and HIV-susceptible cells as recipient cells (Li and Burrell, 1992), was employed to investigate the early events of HIV infection. It has been previously demonstrated, with this model, that rapid *de novo* reverse transcription is mandatory for the new round of HIV replication that follows cell-to-cell virus transmission. It has also been demonstrated that possible contributions made by small amounts of cell-free virus in the system are insignificant and that the donor cells, rather than free virions, are responsible for initiation of infection (Li *et al.,* 1992; Li *et al.,* 1994).

Past studies using synchronized one-step growth conditions in a fully permissive infection, have first detected newly synthesized full length retroviral DNA, 3-5hr after infection. Examples include type C oncoviruses (for review see Varmus and Swanstrom, 1982; Varmus and Brown, 1989), visna virus (Harris *et al.*, 1981) and HIV (Kim *et al.,* 1989a; Farnet and Haseltine, 1990; Farnet and Haseltine, 1991; Li and Burrell, 1992; Li *et al.,* 1992; Sato *et al.,* 1992; Barbosa *et al.,* 1994). These one-step viral infection studies were based on Southern blot analysis of the unintegrated viral DNA. This means that only discrete lengths, in most cases the full length, DNA was analysed. In this study, in addition to full-length DNA molecules, the intermediate transcripts from reverse transcription were also analysed, in particular for the kinetic studies. Apart from the strong-stop DNA species, these intermediate reverse transcripts, by definition, are heterogenous in length thus unsuitable for Southern blot analysis. Hence PCR was employed as the detection method because in addition to its high sensitivity as a nucleic acid detection technique, it is able to quantitate DNA molecules heterogenous in length

but representing the completion of each of the ordered sequence of specific steps during reverse transcription (Varmus and Brown, 1989; Zack *et al.*, 1990; Li *et al.*, 1993). We thus combined a classical one-step viral infection model with this versatile technique to study the kinetics of the different steps of HIV reverse transcription following cell-to-cell infection. Results from these studies are presented in Chapter 4. The final product of retroviral reverse transcription, the full-length unintegrated viral DNA in infected cells, is associated with viral and cellular proteins in the viral replication complex. The one-step cell-to-cell HIV infection model was employed in conjunction with PCR and classical virological methods: eg. Southern blotting, sucrose gradient centrifugation, immunoprecipitation and enzyme activity assays, to characterize the cytoplasmic and nuclear HIV replication complexes and to study the transition from viral RNA-containing complexes to viral DNA-containing complexes early after infection. These results are presented in Chapters 3 and 5.

In our cell-to-cell infection, the HIV infected donor cells (H3B cells) contain no episomal DNA, therefore any unintegrated DNA detected after mixing H3B cells with the non-infected recipient HUT-78 cells is due to a new round of DNA synthesis, that is, reverse transcription. Our results have shown that each of the intermediate reverse transcript species we analysed, from the minus strong-stop DNA to the near full length DNA post second template switch, were synthesized *de novo* in our experimental system as the virus donor cells, recipient cells and infected mixed cells in the first 1hr post infection never gave any specific signal (Chapter 4). When the virus donor cells (H3B cells) were analysed, reverse transcriptase was found to be associated with viral genomic length RNA in a discrete complex with a sedimentation coefficient of 180S-200S (Chapter 5). Presumably this 180S-200S complex is the site of initiation for reverse transcription, as only in specific association with viral RNA (not as free enzyme) can we expect the reverse transcriptase to start synthesizing viral DNA *in vivo*. However, detection of the minus-strand strong-stop and hence the initial step of reverse transcription is associated with a complex of 320S (Chapter 5). This implies that

with initiation of reverse transcription the 180S-200S RNA complex undergoes structural and/or conformational changes to give rise to the 320S DNA-containing replication complex, in which reverse transcription is completed. Unfortunately, due to RNA degradation problems, immunoprecipitation of the 180S-200S RNA complex failed to identify any additional viral proteins that may be associated with this RNA-containing complex. Thus we are unable to determine whether the viral proteins identified with the 320S DNA-containing replication complex are also associated with the 180S-200S RNA-containing complex, or become associated after reverse transcription has been initiated.

Within the H3B virus donor cells the 180S-200S RNA containing complex is reverse transcriptionally inactive. It is not until these donor cells are mixed with CD4+ recipient cells that reverse transcription is activated and viral DNA can be detected. The stimulation signal for the initiation of reverse transcription is unknown. Our PCR kinetic studies on reverse transcription detected the minus-strand strong-stop viral DNA 1.5hr after cell mixing, hence a considerable time delay in the initiation of reverse transcription was observed. Allowing time for the cells in the experimental system to actually meet and for cell fusion to take place may constitute part of the time delay observed before reverse transcription is initiated. CD4/gp120 interaction or one of the events involved in cell fusion, may be the stimulatory signal for reverse transcription, however one would expect a shorter time delay for the initiation of reverse transcription if only receptor interaction or cell fusion was necessary. Stimulation by a cytokine may be involved.

Virion assembly occurs adjacent to the plasma membrane and involves the ordered assembly of the Gag and Gag-Pol polyproteins. During the process of virion assembly, two copies of the HIV RNA genome are also brought to the site of assembly by interacting with the nucleocapsid component of the Gag protein. These polyproteins are believed to remain in this precursor state until the virus is ready to be released from the infected cell. During or shortly after budding, the virion protease is

activated, resulting in the ordered and highly specific cleavage of the Gag and Gag-Pol polyproteins into the mature virion structural proteins and enzymes. This maturation event is essential for virion infectivity. In a cell-to-cell HIV infection, this budding process is by-passed (Li and Burrell, 1992; Sato *et al.,* 1992; Li *et al.,* 1994), hence this cleavage and processing of the Gag and Gag-Pol polyproteins may take place at a different stage or in a different context. Our results show that HIV persistently infected cells (H3B) contained significant amounts of reverse transcriptase, part of which was associated with the genomic RNA in a 180S-200S complex. This reverse transcriptase was in a form that was active in both exogenous and endogenous *in vitro* assays, but inactive *in vivo*. Thus our H3B virus donor cells contain a mature form of reverse transcriptase but this form is not functional in these cells. Hence, if the viral protease can be activated in our H3B cells to cleave the reverse transcriptase enzyme, it may continue the cleaving process of the Gag and Gag-Pol polyproteins and complete the processing of the integrase enzyme, the proteins p17, p24, and nucleocapsid proteins. Thus in our H3B virus donor cells, the 2 copies of genomic RNA may also be associated with other viral and cellular proteins, in addition to reverse transcriptase. Successful techniques to examine the protein composition of the RNA-containing complex would allow examination of this structure.

Unintegrated HIV DNA, isolated from the cytoplasm of acutely infected cells following cell-to-cell transmission infection, was observed to sediment as part of a discrete complex with a sedimentation coefficient of approximately 320S. This replication complex was not associated with cell membranes and could not be dissociated into smaller discrete subunits using detergents. PCR detection of the DNA component of immunoprecipitated HIV early replication complexes showed that the cytoplasmic complexes were associated with the viral enzymes integrase, reverse transcriptase, protease, the matrix protein (p17), Vpr and histones. Nuclear replication complexes, however, were found to be much smaller with a sedimentation coefficient of 80S and were found to be associated only with integrase, protease and histones,

lacking the viral enzyme reverse transcriptase and the matrix protein (p17). Thus transportation of HIV early replication complexes from the cytoplasm to the nucleus is accompanied by a reduction of the sedimentation coefficient from 320S to approximately 80S. These results suggest that HIV DNA within infected cells remains associated with the protein machinery responsible for its replication, transportation into the nucleus and integration into the host genome. If any host components participate in these events, presumably they do so through association with these complexes.

Capsid protein (p24) was not found to be associated with the replication complexes, however, the matrix protein (p17) was present in the cytoplasmic replication complex (Bukrinsky *et al.,* 1993; Karageorgos *et al.,* 1993). Hence the association of p17 and not p24 is a puzzling finding, since p24 surrounds the HIV RNA and the p17 layer is on the outer side of this p24 capsid (see Figure 1.1). How could p17 become associated with the complex and not p24? Could there possibly be two types of p17 or two functions for p17, one as a matrix protein and the other as a nuclear localization protein? This may be possible if one speculates and considers the possibility that the p17 that is going to function as a structural matrix protein is derived from the Gag precursor, and the p17 that becomes the nuclear localization protein is derived from the Gag-Pol precursor. For every one Gag-Pol polyprotein precursor there are 40 Gag precursors, hence sufficient matrix p17 protein molecules can be produced from the Gag precursors, enabling the p17 from the Gag-Pol precursor, along with the other Pol gene products (integrase, reverse transcriptase and protease) to become associated with the HIV RNA, producing the RNA-containing form of the replication complex which transforms into the DNA-containing replication complex with the initiation of reverse transcription.

Investigations into the kinetics of HIV reverse transcription in this cell-to-cell infection, using quantitative PCR, illustrated that HIV reverse transcription is a discontinuous process with an overall high success rate in each of its distinct steps.

Oligonucleotide primers designed for detecting the initial appearance of various reverse transcription intermediates were employed to monitor the progression of viral reverse transcription by quantitative PCR. In our cell-to-cell infection system, the minus-strand strong-stop viral DNA was detected as early as 1.5hrs after the initiation of cell-to-cell HIV infection. The post-transfer and newly extended minus-strand viral DNA was detected 2hrs post infection, whereas both the plus-strand strong-stop DNA and the fully extended minus-strand DNA were detected at 2.5hrs post infection. The initial time delay before the commencement of reverse transcription, in addition to the time delay associated with the first template switch and initiation and synthesis of the plus-strand strong-stop DNA constituted a large part of the time needed before the first appearance of full length double-stranded linear DNA. These kinetics data and the locations of these primers on the HIV proviral genome suggest that once the reverse transcription is initiated the RNA dependent DNA polymerase activity of the HIV reverse transcriptase synthesizes DNA at a rate of 150-180 bases per minute.

Another set of oligonucleotide primers was designed to study the events immediately before and after the second template transfer. In contrast to the time delay associated with the first template switch, data showed that the appearance of viral DNA synthesized after the second template transfer occurred at a time point very close to the time of the appearance of the last piece of DNA synthesized just before the second template switch. This may indicate a more rapid mechanism for the second template transfer as compared to the first transfer. Taken together, our results define a more accurate rate of HIV reverse transcription in T-cell lines than previously suggested and implicate different mechanisms for the two distinct template switches during retrovirus reverse transcription. By specifically looking at the final events in reverse transcription, it was shown that the inter- versus intramolecular mechanisms could not be clearly differentiated using our current experimental system.

Kinetic studies on reverse transcription using the primer pair U5/R1 detected approximately 2000 copies of the minus strong-stop DNA per 1 x 10^5 cells 1.5 hours after cell mixing. After this time there is a steady increase in the minus strong-stop copy number to 80,000 copies by the end of 4 hours. Thus, reverse transcription is not initiated at one time , but gradually through the time course within a single infection cycle. Sucrose gradient analysis of cytoplasmic extracts isolated from a cell-to-cell infection 2 hours after cell mixing (followed by PCR detection with the primers to the U5 and R regions), showed that the minus-strand strong-stop DNA was found in a complex of 320S. However , the DNA peak observed in the sucrose gradient of the 2 hour cytoplasmic extract was less defined , with the DNA peak being broader (spanning fraction 6 through to fraction 8, see figure 5.2) and not sharp. This result could represent the gradual transition of the 180S-200S RNA-containing complex to the 320S DNA-containing replication complex with the initiation of reverse transcription.

6.2 OVERVIEW

By considering all the results obtained during this study of the early events in HIV replication, we can propose a model for the sequence of events that takes place during a cell-to-cell infection of HIV. Our results suggest that within the H3B virus donor cells, most genomic RNA is associated with fully-cleaved, but inactive, reverse transcriptase and possibly also with the precursor or mature forms of other viral and cellular proteins in a 180S-200S complex. One can propose that the activation signal which leads to the viral protease self-cleaving from the Gag-Pol precursor, may continue cleaving and processing the reverse transcriptase and perhaps the remaining proteins. This unknown stimulation signal seems to involve the recipient HUT-78 cells, since the 180S-200S RNA-containing complex, associated with the cleaved but inactive reverse transcriptase, was isolated from H3B cells alone. When the H3B cells are mixed with HUT-78 cells, cell fusion takes place. Presumably the 180S-200S RNA complex is released into the cytoplasm of the HUT-78 cells and the reverse

transcriptase enzyme is activated by an unknown factor, most likely contributed by the HUT-78 cells or the fusion process, leading to the initiation of reverse transcription. With this initiation of reverse transcription, the 180S-200S RNA complex undergoes a structural change resulting in the 320S DNA-containing replication complex. The entire process of reverse transcription is believed to take place within the 320S replication complex, which is associated with the viral integrase, reverse transcriptase, protease, Vpr, p17 and also associated with histones. This process of reverse transcription is a multi-event process involving several distinct steps with different kinetic characteristics that have been discussed in the previous section. With the completion of reverse transcription, the 320S cytoplasmic replication complex migrates into the nucleus. The transportation of the replication complex from the cytoplasm to the nucleus is accompanied by a reduction in size from 320S to 80S, coinciding with the loss of the reverse transcriptase enzyme and the matrix protein p17, which contains a nucleus localization signal. Once inside the nucleus the full-length linear viral DNA may be integrated into the host chromosome. At this stage, the "early events" in the HIV replication cycle is considered complete.

6.3 FUTURE WORK

Further characterization of the RNA-containing complexes will lead to a better understanding of the proteins associated with the RNA template prior to the initiation of reverse transcription. Steps will need to be taken to ensure that any contaminating RNAse is removed from all materials used in these studies, as discussed in Chapter 5. The transition from RNA-containing, to DNA-containing replication complexes is an important and interesting process that needs to be further investigated. The ability to isolate these replication complexes from infected cells, may enable the development of an *in vitro* system, in which the requirements of reverse transcription and the actual transition of RNA-containing to DNA-containing replication complexes can be investigated and monitored *in vitro*. In addition, reverse transcription activation and

factors controlling this process can be investigated. Characterization of complexes present between 0hrs and 1.5hrs after infection may lead to a better understanding of this activation process and transformation of the 180S-200S RNA-containing complex to the 320S DNA-containing complex. The effect of sodium butyrate (an inhibitor of cellular histone deacetylase, Laughlin, *et al.*, 1993)) on the production of histones may allow the role and function of histones in the formation of the replication complex structure to be explored. The presence of protease inhibitors may also influence the formation of the replication complexes.

Future work may also involve construction of full-length HIV clones containing mutations either the integrase, protease, p17 or *vpr* genes. Transfection of these molecular clones into cells may result in provirus defective in these genes. If these cells can then transmit HIV infection, isolation and characterization of the replication complexes from these cell lines may allow determination the functions and roles of these proteins in the early stages of the HIV life-cycle.

Further studies on the inter- or intra-molecular mechanism of template transfers during HIV reverse transcription could lead to a better understanding of the process of reverse transcription. The development of an optimal experimental model system for the study of the inter-/intra-molecular transfers is required. Unintegrated DNA would be required to be isolated at a more frequent time course (eg. every 10 mins), in order to try to detect the template transfers. A double quantitative competitive PCR (QC PCR) (Piatak *et al.*, 1993) can be developed such that two primer pairs (eg. R3/gag3 & U3.3/gag3), spanning the same area of the deletion (or insertion) between PBS and *gag* in the competing template, can be added in the same PCR reaction, such that any short delay in the continued synthesis can be observed. For conventional PCR, lower copy number standards (eg. 5000, 10000, 25000 copies) can be used for a more accurate estimation of copy number. Such stringent experimental conditions may allow the determination of whether inter- or intra-molecular mechanisms are utilized. Hence, such

detailed studies using optimal experimental systems on HIV reverse transcription and other early events in the HIV replication cycle may hold hope for a better understanding and control of HIV infection.

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