THE ROLE OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 NUCLEOCAPSID PROTEIN IN VIRAL REPLICATION

By

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ABSTRACT

Essential macromolecules of reverse transcription of human immunodeficiency virus type 1 include nucleocapsid protein (NCp), reverse transcriptase (RT), $tRNA^{Lys.3}$, and viral RNA. We found NCp7 conferred exquisite specificity to reverse transcription by stabilizing and/or mediating the formation of the $tRNA^{Lys.3}$/RNA complex that is recognized by RT for initiation of (-)ssDNA synthesis, as indicated by suppression of non-specific initiation from self-primed RNAs and the concomitant stimulation of specific initiation of $tRNA^{Lys.3}$. Moreover, the remaining portion of $tRNA^{Lys.3}$, i.e., sequences not directly base-pairing the primer binding site (PBS) of viral RNA, was found to be essential for efficient reverse transcription to occur.

To further study potential roles of the remaining portion of $tRNA^{Lys.3}$ during reverse transcription in the context of an infectious molecular clone, we replaced the original wild-type PBS (complementary to $tRNA^{Lys.3}$) with sequences that are complementary to alternate tRNAs, i.e. $tRNA^{Lys.1,2}$ or $tRNA^{Phe}$. Although the mutant viruses were able to use either $tRNA^{Lys.1.2}$ or $tRNA^{Phe}$ as primers during the early stages of infection, $tRNA^{Lys.3}$ was found to replace these alternate tRNAs as primer during the late stages of infection, accompanying with increasing production of viral progeny. The strict commitment of HIV-1 for choosing $tRNA^{Lys.3}$ as replication primer strongly suggests that the virus has evolved to use this specific tRNA to maintain a growth advantage. Additionally, we found that the A-rich loop and 7 nt-stretch downstream of the PBS were not essential for viral replication, whereas a 54-nt sequence immediately downstream of the PBS was indispensable for viral replication. This sequence was proven to be crucial for both reverse transcription and expression of viral mRNA.

Résumé

La protéine nucléocapside (NCp), la transcriptase inverse (TI), l'ARNt$^{Lys.3}$ et l'ARN viral font parti des macromolécules essentielles à la transcription inverse de l'ARN du virus de l'immunodéficience humaine de type I (VIH-1). Nous avons démontré que NCp7 donne une grande spécificité à la transcription inverse en stabilisant et/ou en permettant la formation du complexe ARNt$^{Lys.3}$/ARN qui est reconnu par la TI pour l'initiation de la synthèse de l'ADN simple brin(-). Ceci est indiqué par la suppression de l'initiation non-spécifique de la synthèse de l'ADN par l'auto-amorçage de l'ARN, ainsi que par la stimulation de l'initiation spécifique par l'ARNt$^{Lys.3}$. De plus, il a été démontré que la partie de l'ARNt$^{Lys.3}$ qui ne s'associe pas directement au site de liaison de l'amorce (Primer Binding Site: PBS) de l'ARN viral, est essentielle pour la transcription inverse efficace.

Afin d'étudier plus en détail les rôles potentiels de cette partie de l'ARNt$^{Lys.3}$ durant la transcription inverse dans le contexte d'un clone moléculaire infectieux, nous avons remplacé le PBS original de type sauvage (complémentaire à l'ARNt$^{Lys.3}$) par des séquences complémentaires à d'autres ARNt, i.e. ARNt$^{Lys.1,2}$ ou ARNt^{Phe}. Bien que les virus mutés étaient capables d'utiliser soit l'ARNt$^{Lys.1,2}$ ou l'ARNt^{Phe} comme amorce durant les premiers stades de l'infection, l'ARNt$^{Lys.3}$ a remplacé ces autres ARNt comme amorce durant les derniers stades de l'infection, pendant lesquels la production de progéniture virale a augmentée. L'engagement strict du VIH-1 à choisir l'ARNt$^{Lys.3}$ comme amorce de la réplication suggère fortement que le virus a évolué afin d'utiliser cet ARNt spécifique pour maintenir un avantage de croissance. En plus, nous avons démontré que la boucle riche en A et une

région de 7 nucléotides en aval du PBS n'étaient pas nécessaires pour la réplication virale, tandis qu'une séquence de 54 nucléotides située immédiatement en aval du PBS était indispensable pour la réplication virale. Cette séquence a été reconnu comme étant cruciale pour la transcription inverse et l'expression de l'ARN messager viral.

PREFACE

This Ph.D. thesis was written in order to conform to the requirements of the Guidelines Concerning Thesis Preparation from the Faculty of Graduate Studies and Research of McGill University. The main structural feature of this thesis is that it comprises a collection of papers that have a cohesive character, with connecting texts that provide bridges between the different papers. The major components of it include: a table of contents, an abstract in English and French, an introduction stating the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough reference list.

I have included, as chapters of this thesis, the texts of two original paper which have been published, one original paper that is in press and one paper that has been submitted for publication. Each chapter (2-5) includes its own Abstract, Introduction, Materials/Methods, Results and Discussion sections. A general discussion and Discussion to the thesis have been included and represent chapters 1 and 6, respectively. In order to bridge connecting papers, Chapters 3, 4 and 5 each contain a preface. The manuscripts presented in the thesis are the following.

1. X. Li, Z. Gu, R. Geleziunas, L. Kleiman, M. A. Wainberg, and M.A. Parniak. 1993. Expression, purification, and RNA-binding properties of HIV-1 nucleocapsid protein. Protein Expression and Purification. 4:304-311.

2. X. Li, Y. Quan, E. Arts, B. Preston, H. De Rocquigny, B. Roques, L. Kleiman, M. A. Parniak and M. A. Wainberg. 1996. HIV-1 nucleocapsid protein (NCp7) directs specific initiation of minus strand DNA synthesis primed by human $tRNA^{Lys.3}$ in vitro. J. Virol. (In press).

3. X. Li, J. Mak, E. Arts, Z. Gu, L. Kleiman, M. A. Wainberg, and M. A. Parniak. 1994. Effects of alterations of primer-binding site sequence on HIV-1 replication. J. Virol. 68: 6198-6206.

4. X. Li, C. Liang, Y. Quan, R. Chandok, L. Kleiman, M. A. Parniak, and M. A. Wainberg. 1996. Effects of sequences flanking the primer binding site on HIV-1 replication. J. Virol. (Submitted).

The candidate was responsible for all of the research described in chapters 2, 3, 4, 5. Drs. C. Liang, Y. Quan, E. Arts, Z. Gu, R. Geleziunas, L. Kleiman, B. Preston, H. DeRocqigny, B. Roques, B. Preston, J. Mak. R. Chandok provided important assistance in devising experiments and analyzing results. J. Mak was responsible in analyzing tRNA species in chapter 4. Dr. C. Liang actively participated in work presented in chapter 5, especially in mRNA analyses.

The contributions of co-authors to submitted manuscripts or published articles and the journals of submission appear on the title page of each Chapter. Papers that are not included in this thesis but provide important background information leading to our projects are the following:

Boulerice, F., X. Li, and M. A. Wainberg. 1991. Recovery of infectious HIV-1 after fusion of defectively infected clones of U-937 cells. J. Virol. 65:5589.

Gao, Q., Z. Gu, M.A. Parniak, X. Li, M.A. Wainberg. 1992. In vitro selection of nucleoside-resistant variants of HIV-1. J. Virol. 66: 12.

Gu, Z., Q. Gao, X. Li, M.A. Parniak, M.A. Wainberg. 1992. Novel mutation in the HIV-1 reverse transcriptase gene that encodes cross-resistance to ddI and ddC. J. Virol. 66:7128.

Li, X., E. Amandoron, M.A. Wainberg, M.A. Parniak. 1993. Generation and characterization of unique murine monoclonal antibodies reactive against N-terminal and other regions of HIV-1 reverse transcriptase. J. Med. Virol. 39:251.

Wu, J., E. Amandoron, X. Li, M.A. Wainberg, and M.A. Parniak. 1993. Monoclonal antibody-mediated inhibition of HIV-1 reverse transcriptase polymerase activity. Interaction with a possible deosynucleoside triphosphate binding domain. J. Biol. Chem. 268: 9980-9985.

Arts, E.J., X. Li, Z. Gu, M. A. Parniak, and M. A. Wainberg. 1994. Comparison of deoxy-oligonucleotide and $tRNA^{lys3}$ as primers in an endogenous HIV-1 in vitro reverse transcription/template switching reaction. J. Biol. Chem. 269: 14672-14680.

Gu, Z., X. Li, Y. Quan, M. A.Parniak, and M. A. Wainberg. 1996. Studies of a neutralizing monoclonal antibody to HIV-1 reverse transcriptase: antagonistic and synergistic effects in reactions performed in the presence of nucleoside & non-nucleoside inhibitors, respectively. J. Virol. (In press)

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Page **CHAPTER 5**

LIST OF ABBREVIATIONS

a.a. : amino acids

AIDS : ACQUIRED IMMUNODEFICIENCY SYNDROME

ALV : avian leukosis virus

AMV : avian myeloblastosis virus

b p : base pair

CA : capsid protein

dPR : deoxyribonucleotide primer (dPR)

g p : glycoprotein

HIV	:	human immunodeficiency virus
IN	:	integrase protein
kDa	:	kilo-dalton
LTR	:	Long terminal repeat
MA	:	Matrix protein
MMLV	:	Moloney murine leukemia virus
NCp	:	nucleocapsid protein
n t	:	nucleotides
PCR	:	polymerase chain reaction
PBS	:	primer binding site
PR	:	protease
R	:	repeat sequence
rPR	:	ribonucleotide primer (rPR)
RNase H	:	ribonuclease H activity
RRE	:	rev-responsive element
RSV	:	Rous sarcoma virus
RT	:	reverse transcriptase
SIV	:	simian immunodeficiency virus
TAR	:	trans-activation response element
U3	:	3' unique region
U5	:	5' unique region
(-)ssDNA	:	minus strand strong stop DNA

CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

The acquired immmunodeficiency syndrome (AIDS) is caused by a newly recognized member of the lentivirus family, the human immunodeficiency virus (HIV)(Popovic et al, 1984). This retrovirus family is distinct from the other subfamilies of the human retroviruses, the spumavirinae (human foamy virus) and oncovirinae (HTLV-1 and HTLV II). In particular, lentivirus genomes are large and contain several viral genes. The viruses frequently induce cytopathic effects in infected cells, and the disease they cause has a long incubation period resulting in immunologic disorders and neurological disease (Levy, 1988). The first viral species to be associated with AIDS were found in the Western world and in central Africa (Barre-Sinoussi et al 1983; Gallo et al., 1983). This viruses are called human immunodeficiency virus type 1 (HIV-1). Soon afterwards, another major and distinct species of HIV, called HIV-2, was isolated in West Africa (Clavel et al., 1986). The genomes of HIV-1 and HIV-2 are only 40% identical, but that of HIV-2 is nearly 80% identical to the genome of simian immunodeficiency virus (SIV). The proteins of HIV-2, accordingly, bear a closer resemblance to those of SIV than HIV-1. This chapter will deal mostly with HIV-1, with emphasis on viral structure and genetic organization, biogeneis of viral protein and steps in viral replication. Attention is directed to molecular mechanisms involved in reverse transcription, the focus of this thesis.

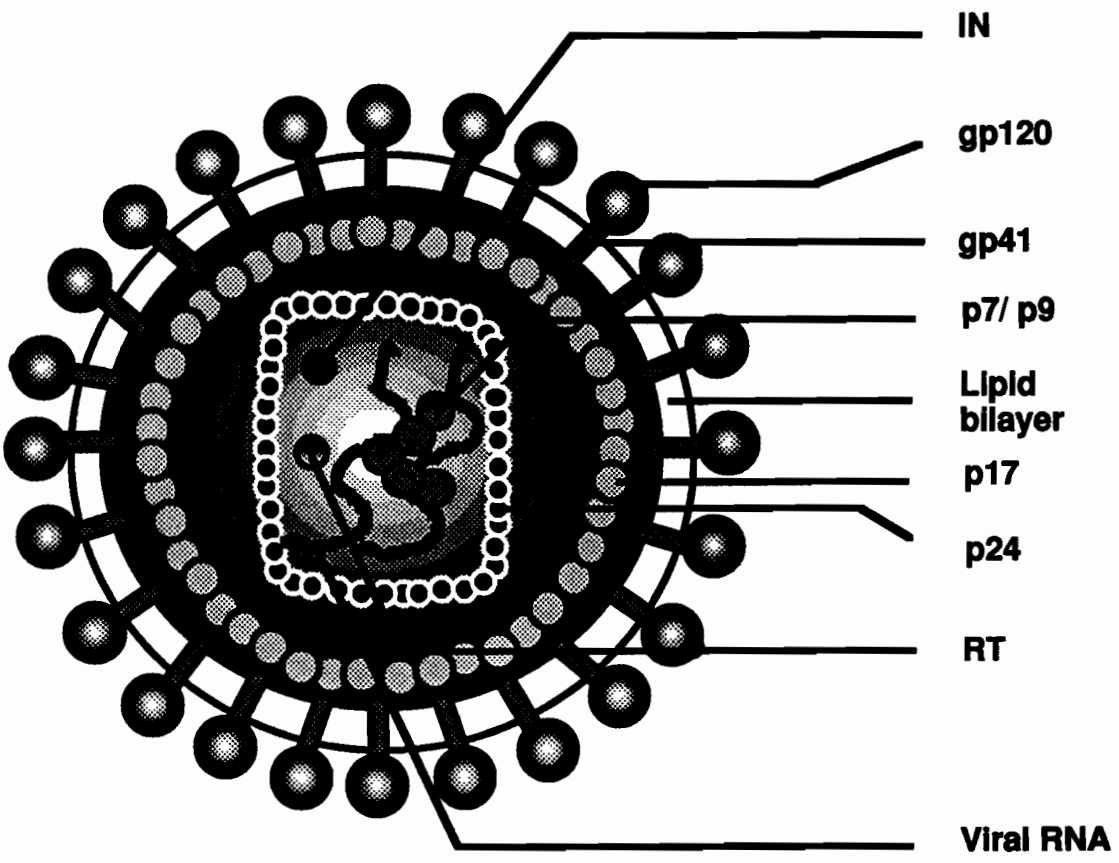
1.2 GENERAL DESCRIPTION OF HIV-1

1.2.1 The Virion

Fig.1 is a schematic representation of the HIV virion. HIV forms a icosahedral sphere with 72 projections consisting of gp120 and gp41, both of which are envelope glycoproteins. While only gp41 traverses the lipid bilayer, gp120 is loosely and non-covalently bound to gp 41. Under the lipid layer, the matrix protein (p17) covers the internal surface of the viral coat. The capsid protein (p24) constitutes the internal core shell, while p7 and p9 form part of the nucleoid. p7 and reverse transcriptase (p66/51) are associated with the two copies of the single-stranded genomic HIV RNA (Haseltine, 1991).

Fig.1: Graphic description of the HIV-1 virion

The glycoprotein gp120 constitutes the outer envelope of the virus and is non-covalently linked to the transmembrane protein, gp41. The matrix protein, p17, bridges the envelope protein with the cone-shaped structure formed by the capsid core (p24). The viral genomic RNA and processed *gag* and *pol* proteins are located inside the capsid core.

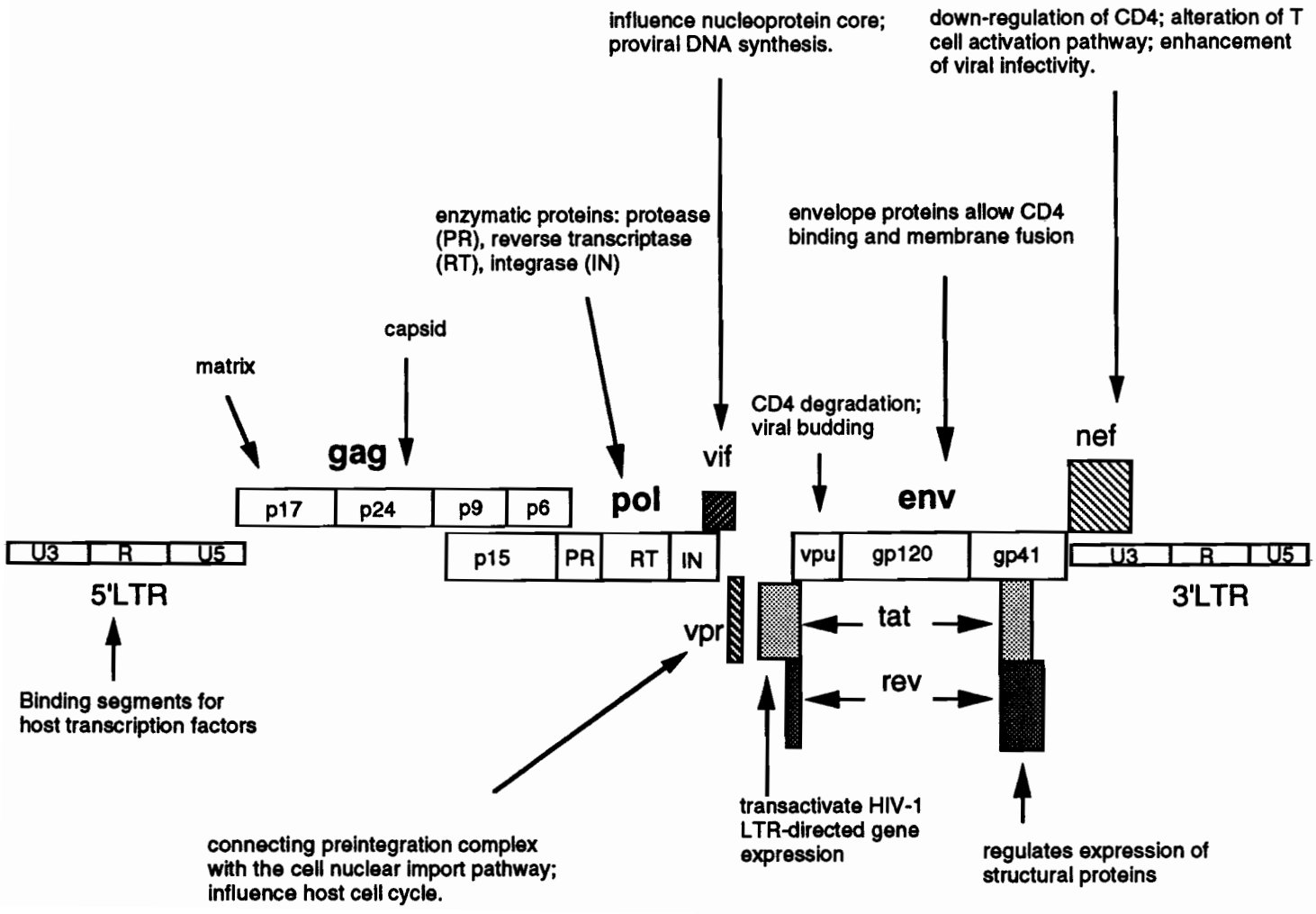

IN
gp120
gp41
p7/ p9
Lipid
bilayer
p17
p24
RT
Viral RNA

1.2.2 Genetic organization of HIV-1

The HIV provirus is about 9.5 kilobases long with long terminal repeats (LTRs) (634 base pairs), which flank sequences coding for viral proteins (Cullen 1991). Fig.2 is a schematic description of the HIV-1 genome and the known functions of its gene products. The LTRs contain *cis*-acting elements essential for viral integration and transcription. RNA synthesis is initiated within the 5'-LTR at the junction between the U3 and R regions, while the 3' LTR specifies the addition of poly-A tails to viral RNA at the junction between the R and U5 regions. The structural genes, i.e., *gag*, *pol*, and *env*, code for polyprotein precursors that are processed and assembled into virions. Within the HIV-1 genome, additional open reading frames that flank the *env* gene encode several regulatory proteins including Vif, Vpr, Tat, Rev, Vpu and Nef. (Fig.2)

Fig.2 Schematic description of the genetic organization of HIV-1 and known functions of these gene products

The structural genes are *gag*, *gag-pol* and *env*. Catalytic proteins are encoded by the *pol* gene. Regulatory proteins are translated from fully spliced mRNA.


influence nucleoprotein core; proviral DNA synthesis.
down-regulation of CD4; alteration of T cell activation pathway; enhancement of viral infectivity.
enzymatic proteins: protease (PR), reverse transcriptase (RT), integrase (IN)
envelope proteins allow CD4 binding and membrane fusion
capsid
matrix
CD4 degradation; viral budding
gag
vif
nef
pol
env
p17
p24
p9
p6
p15
PR
RT
IN
vpu
gp120
gp41
U3
R
U5
U3
R
U5
5'LTR
3'LTR
vpr
tat
rev
Binding segments for host transcription factors
connecting preintegration complex with the cell nuclear import pathway; influence host cell cycle.
transactivate HIV-1 LTR-directed gene expression
regulates expression of structural proteins

1.2.3 The *gag* and *gag-pol* gene encoded proteins

The *gag* open reading frame (1536 nucleotides) is translated directly into a 55 kDa precursor Pr.55 gag) (Fig.3). This polyprotein is further cleaved by the viral protease to yield several smaller polypeptides (Henderson et al, 1992; Henderson et al., 1993; Kaplan and Swanstrom, 1991; Sheng and Erickson-Viitanen, 1994; Veronese et al., 1987; Wondrak et al., 1993). While processing of p55 can be detected in the cytoplasm, it is generally believed that this processing of this *gag* precursor mainly takes place on the membrane of the host cell or inside the released viral particle (Gelderbloom, 1991; Henderson., et al, 1992; Henderson et al., 1993; Kaplan and Swanstrom, 1991; Sheng and Erickson-Viitanen, 1994; Veronese, et al., 1987; Wondrak et al., 1993; Kohl et al 1988). Processing of the precursor was also found to be accompanied by morphological rearrangement of virus particles; this can be visualized by electron microscopy (Gelderbloom., 1991). The final products include: i) p17 (matrix protein, MA), which comes from the amino-terminus of p55 and is myristylated at its N-terminus; ii) p24 (capsid protein, CA), which is derived from the central part of p55 and forms the cone-shaped shell underneath p17 (Fig.1); 3) p15 (nucleocapsid, NCp), which is further processed into four smaller fragments, p2, p7 (nucleocapsid [NC]), p1 and p6. (Gelderbloom, 1991; Sheng et al., 1994).

The matrix protein, p17, is N-myristylated and has been found to be important in viral assembly by directing the intracellular transport and membrane association of the *Gag* polyprotein (Varmus and Swanstrom, 1982). As part of the preintegration complex, p17 is also critical for transport of the complex into the nucleus (Burinsky et al., 1982; von Schwedler et al., 1994;

Gallay et al., 1995). p17 may be responsible for recruiting viral envelope proteins to the surface of the host cells (Dorfman et al., 1994; You et al., 1992). The capsid protein (CA), p24, has also been shown to be essential for viral assembly (Reicin et al., 1995).

Recent studies have indicated that the NCp15 may also be further processed to four additional peptides-p2, p7, p1, and p6 (Goff, 1990; Henderson et al 1992). The nucleocapsid protein, NCp15, consists of 149 amino acids (Ratner et al 1984) and contains two regions of zinc finger motifs, sequences associated with certain nucleic acid binding proteins (Berg 1986; South et al., 1990; Sheng and Erickson-Viitanen, 1994; Wondrak 1993). Of these, p7 is the only major form of nucleocapsid protein found in mature virions. p7 possesses the zinc finger motifs and may therefore represent the fully processed form of nucleocapsid protein. A number of functional roles of nucleocapsid protein p7 (NCp7) have been identified: 1) stimulation of reverse transcription; 2) to assist in viral genomic RNA packaging; 3) to promote dimerization of viral RNA (Darlix et al, 1993a; Darlix et al., 1993b; Gorelck et al., 1988; Li et al., 1993; Luban et al 1992; Prats et al, 1988; You and McHenry, 1994). p6 is involved not only in viral budding but also in association of Vpr with the virion (Gottlinger et al., 1991; Kondo et al., 1995). Little is known about possible functional roles of either p2 or p1.

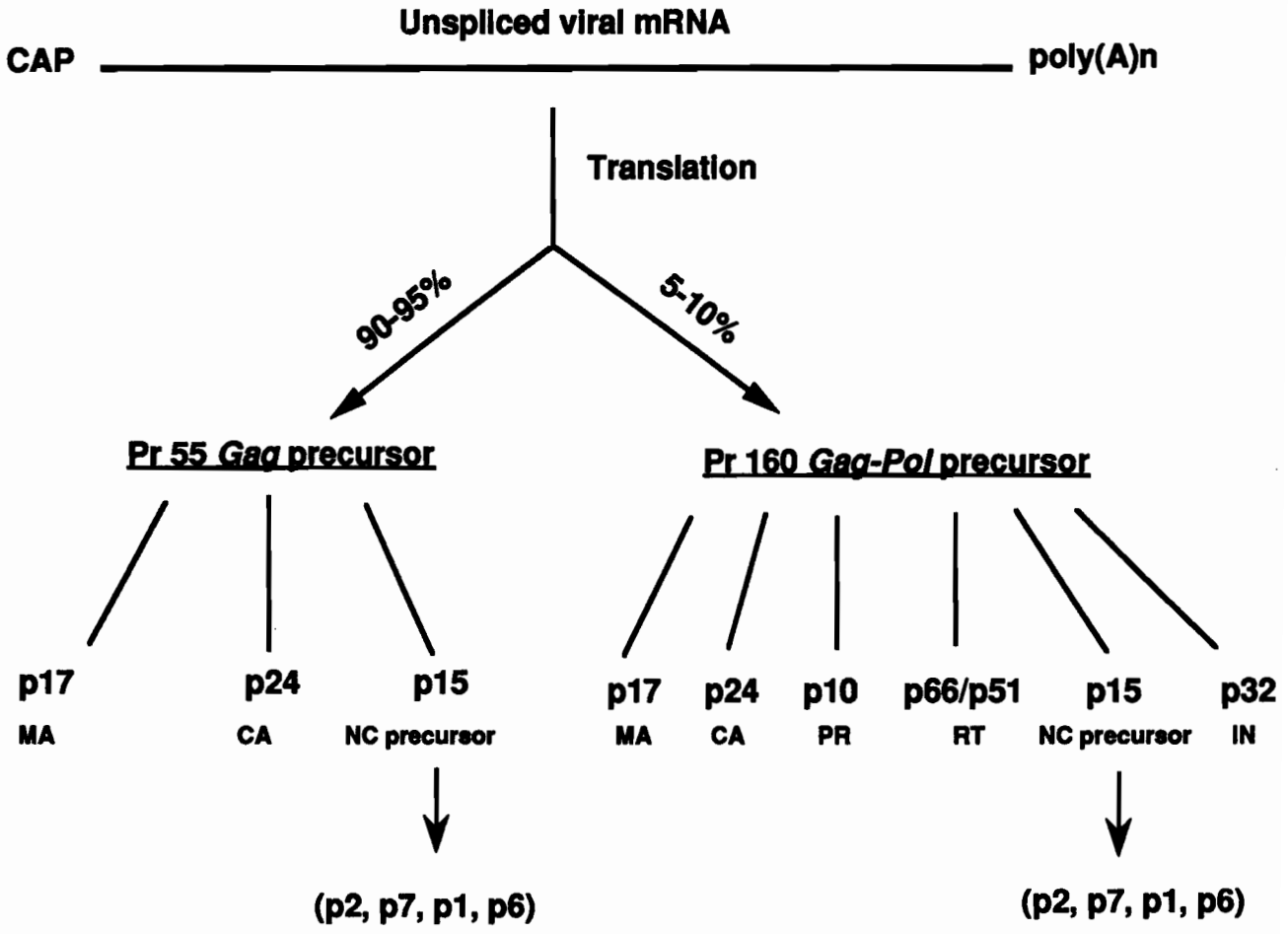
The *pol* open reading frame (3045 nucleotides) is translated only as a *gag-pol* fusion protein, Pr160, by a translational frameshift mechanism as ribosomes read full-length HIV transcripts. In mature virions, the *gag* and *pol* gene products are found in a ratio of about 20:1 (Jacks et al., 1988) (Fig.3). The *Pol* precursor is cleaved to produce protease (p10) [PR], reverse transcriptase (p66/51, RT) and integrase (p32, IN) (Goff, 1990; Ratner et al., 1985; Veronese et al., 1987). PR is responsible for processing the *Gag* and *Gag-Pol* precursors.

Mutations in the catalytic region of PR are lethal to the virus (Kohl et al., 1988). RT is responsible for catalyzing conversion of viral RNA into DNA (reverse transcription) (Goff, 1990), whereas IN plays a key role in inserting viral DNA into the host cell chromosome (Goff, 1991, Vink et al, 1993).

Fig.3: Processing of Gag and Gag-Pol precursor

The *gag* proteins are initially translated as a 55-kDa polyprecursor. Proteolytic processing of p55^{gag} generates several mature products. The catalytic proteins, including reverse transcriptase (RT), integrase (IN) and protease (PR) are produced as a gag-pol precursor (p160$^{gag\text{-}pol}$) through a translational frameshift mechanism.

Biogenesis of *Gag* and *Gag-Pol* proteins



1.2.4 The *env* gene encoded proteins

The *env* gene encodes a glycosylated precursor polyprotein of 160 kDa. A signal peptide (about 30 amino acid residues) at the amino-terminus of gp160 is removed and a subsequent proteolytic step yields the amino-terminal gp120 and the carboxy-terminal gp41 (Haseltine, 1991). While the external viral protein gp120 plays a key role in interacting with the CD4 receptor of susceptible cells, the transmembrane protein gp41 is responsible for anchoring gp120 through noncovalent interactions and mediating the fusion process between viruses and the target cells (Gallaher, 1987; Gallaher, 1989; Lifson et al., 1986; Kowalski et al., 1987; Sodroski et al., 1986; Veronese et al., 1985).

1.2.5 The regulatory proteins

The viral transactivator (*Tat* protein) belongs to a novel class of eucaryotic regulatory proteins (Sodroski et al., 1985). The protein is concentrated in the nucleus and nucleolus of infected cells and binds to a stem-loop structure at the 5'-end of nascent RNA (position 1-60) (Rappaport et al 1989; Ruben et al., 1989; Siomi et al 1990). This region is called TAR. Interaction of Tat and RNA may: i) increase the stability of the RNA polymerase to allow more efficient synthesis of full-length transcripts; ii) increase the frequency of RNA initiation (Haseltine, 1990). Interestingly, the sequence at the 5' end of the RNA, TAR, is recognized by some cellular proteins, suggesting the importance of potential interaction between Tat and host cell proteins. Several groups have, in fact, reported the identification of factors that bind specifically to either Tat or to TAR. None of these proteins has yet been shown to be definitively important for Tat function in vivo (Cullen 1995).

The regulator of virion protein expression (*Rev*) protein has a profound effect on the fate of primary RNA transcripts within the nucleus. Like *Tat, Rev* is located primarily in the nucleus and nucleolus of infected cells. A shuttling translocation of this protein between cytoplasm and the nucleus has been observed (Cochrane et al., 1989; Felber et al., 1989; Malim et al., 1994; Richard et al 1994; Venkatesh et al., 1990). This protein binds to a complex stem-loop structure, the rev responsive region (RRE), within the envelope glycoprotein encoding region of the HIV-1 *env* gene (Heaphy et al., 1990; Malim et al., 1990; Olsen et al., 1990; Zapp and Green, 1989). The RRE is present in all Rev-responsive viral mRNAs but is excluded, by splicing, from multiply spliced viral mRNAs. Consequently, in the absence of Rev only small multiply spliced RNAs (for regulatory proteins) accumulate in the cytoplasm. In the presence of Rev, both unspliced and singly spliced viral mRNAs (for capsid and glycoprotein) were found in the cytoplasm (Emerman et al., 1989; Farnet et al., 1991; Hammarskjold et al., 1989; Malim et al., 1989). Thus, the pivotal role played by this protein is to permit new viral particles to be made. While it is clear that Rev acts primarily at the level of nucleocytoplasmic RNA transport, no evidence exits for a direct effect on viral mRNA translation. Recently, efforts aimed at identifying cellular co-factors have received increased attention (Bogerd et al., 1995).

The product of the virion infectivity gene, *Vif*, is made from a singly spliced mRNA which accumulates late in infection (Garret et al., 1991). This late gene product acts during assembly to allow formation of particles competent to initiate new rounds of infection (Gabuzda et al., 1992; von Schwendler et al., 1993). Since only traces of this protein are found in virions, its effect are assumed to be indirect (Trono, 1995).

Vpu is made from the same singly spliced mRNA as is the envelop glycoprotein (Cohen et al., 1988; Schwartz et al., 1990; Strebel et al., 1988). At least two functional roles of Vpu have been identified, i.e., down-modulation of CD4 by stimulating degradation of CD4 and enhancement of virion release by a yet to be defined mechanism (Bour et al., 1995; Terwilliger et al., 1989; Willey et al., 1992).

Vpr is another regulatory protein that is packaged into mature viral particles (Cohen et al., 1990). Vpr was the first retroviral protein identified that is neither part of the capsid nor the capsid replicative enzyme precursor, but, rather, is included in the virion itself. Apparently, Vpr is packaged through interactions with p6 (Kondo et al., 1995). Vpr appears to be important in assisting transport of the preintegration complex from the cytoplasm into the nucleus (Heinzinger et al., 1994). Although Vpr displays a capability of disrupting the cell cycle (Levy et al., 1993), little is known about the mechanism involved or the implications for viral infection in vivo.

The *Nef* protein is encoded by the extreme 3' end of the viral genome (Ratner et al., 1985). This protein accumulates even earlier than do Tat and Rev in newly-infected cells (Kim et al., 1989; Gratelli et al., 1988;). The role of this earliest protein in viral replication is controversial. Initially, Nef was believed to be a transcriptional repressor of the HIV-1 LTR (Ahmad and Venkatesan, 1988; Niederman et al., 1989). Such ability to down-regulate HIV-1 LTR-directed gene expression *in trans* was questioned soon thereafter (Kim et al., 1989; Hammes et al., 1989). Recent evidence suggests that Nef is involved in modulating CD4 expression by triggering the rapid endocytosis and lysosomal degradation of this main virus receptor (Aiken et al., 1994). It may also be involved in reverse transcription (Schwartz et al., 1995; Aiken et al.,1995). The effects of Nef on down-regulation of CD4 and stimulation of proviral DNA

synthesis are specific; however, little is known of the mechanisms involved. Nef is not packaged into virions in significant quantities, suggesting that it may promote reverse transcription in an indirect way.

1.3 AN OVERVIEW OF HIV-1 REPLICATION

The life cycle of HIV-1 can be divided into several steps: virus entry, reverse transcription, integration, expression of the proviral genome, viral assembling/packaging, and budding mature particles. Fig.4 is a schematic description of the HIV-1 life cycle.

1.3.1 Virus Entry

HIV-1 primarily uses the CD4 receptor (a 58 kDa transmembrane protein) to gain entry into cells, through high-affinity interactions between viral envelope glycoprotein (gp120) and a specific region of the CD4 molecule (McDougal et al., 1986; Rosenberg & Fauci et al., 1991). CD4 is present in abundance on the surface of both immature T lymphocytes and mature CD4(+) T helper cells. It is present at lower concentrations on monocytes, macrophages, and antigen-presenting dendritic cells (Rosenberg and Fauci, 1991). Mounting evidence suggests that other cell surface moieties may also be important for viral entry. For example, CD4 negative brain-derived cells and human fibroblasts can be infected by HIV-1 (Levy, 1989). Moreover, mouse cells transfected with the CD4 protein to express this antigen cannot be infected by HIV-1 (Maddon et al., 1986). Entry likely occurs by a fusion of viral and cell membranes, mediated by the viral transmembrane protein (gp41) (Gallaher, 1987). Following fusion, the virion is uncoated by a proteolytic event likely mediated by the virion-encoded protease (Roberts and Oroszlan, 1990).

1.3.2 Reverse transcription

Following entry, viral RNA is converted into DNA, which is then integrated into host cell DNA. The process of reverse transcription usually occurs within 4-6 hr of infection, takes place mainly in the cytoplasm, and is catalyzed by virion encoded reverse transcriptase (RT) (Haseltine, 1991). The final products of reverse transcription are double-stranded DNA molecules that are longer at each end than is the viral RNA used as template due to duplication of the LTR (Giboa et al., 1979). The subject of reverse transcription will be addressed in greater detail below.

1.3.3 Integration

As stated above, reverse transcription products are mainly generated in the cytoplasm of the infected cells. These double stranded DNAs are then transported into the nucleus of the cell, where integration of viral DNA into host cell chromosomal DNA takes place (Roth et al., 1988; Varmus and Swanstrom, 1984). The preintegration complex of HIV-1 consists of IN, RT, p17, and reverse transcribed DNA (Bukrinsky et al; 1993, Gallay et al., 1995). The phosphorylated matrix protein, p17, plays a key role in targeting the preintegration complex into the nucleus of the host cell (Gallay et al., 1995). While transport of the preintegration complex of oncogenic retroviruses only takes place in dividing cells, active transport of preintegration complex in lentivirus infection has been observed in nondividing, terminally differentiated cells (Bukrinsky et al., 1992, 1993; Gallay et al., 1995).

The integration reaction is catalyzed by the virion-encoded integrase (IN). IN is found in the viral particle and, after reverse transcription of genomic viral RNA, IN remains associated with viral RNA as a high molecular weight nucleoprotein preintegration complex (Bukrinsky et al., 1992). IN first

removes two nucleotides from the 3' end of viral DNA and then cleaves target host DNA. This is followed by insertion of viral DNA into host cell DNA (Vink and Plasterk, 1993). The 5' gaps flanking the provirus as well as the two non-paired nucleotides are presumably repaired or removed by a cellular enzyme. The final products (provirus) are flanked by 5 bp direct target duplications, which have lost 2 bp from each end (Roth et al., 1988; Whitcombe and Hughes, 1991; Goff, 1992). Once integrated, viral DNA remains permanently associated with the host genetic material for as long as the cell is alive.

1.3.4 Expression of the HIV-1 proviral genome

The efficient transcription of the HIV genome requires a series of complex mechanisms involving both cellular and viral factors. Cellular activation and proliferation signals result in the binding of transcription factors to the LTR and lead to increased rates of initiation of transcription (Cullen, 1991). As mentioned above, Tat and Rev are two key virion encoded proteins that positively regulate viral gene expression and replication, whereas the accessory proteins including Nef, Vif, Vpu, and Vpr, are crucial determinants of HIV virulence (Cullen, 1995; Trono, 1995).

The primary RNA transcripts of the provirus are made by host cell RNA polymerase II. These transcripts serve as mRNA for the synthesis of viral polyproteins and as viral RNA that will be incorporated into new virions. Host cellular ribosomes translate proviral mRNA into viral proteins (Haseltine, 1991). As stated, all viral structural proteins are made as polyproteins. Regulatory proteins are made by transcribing spliced mRNA.

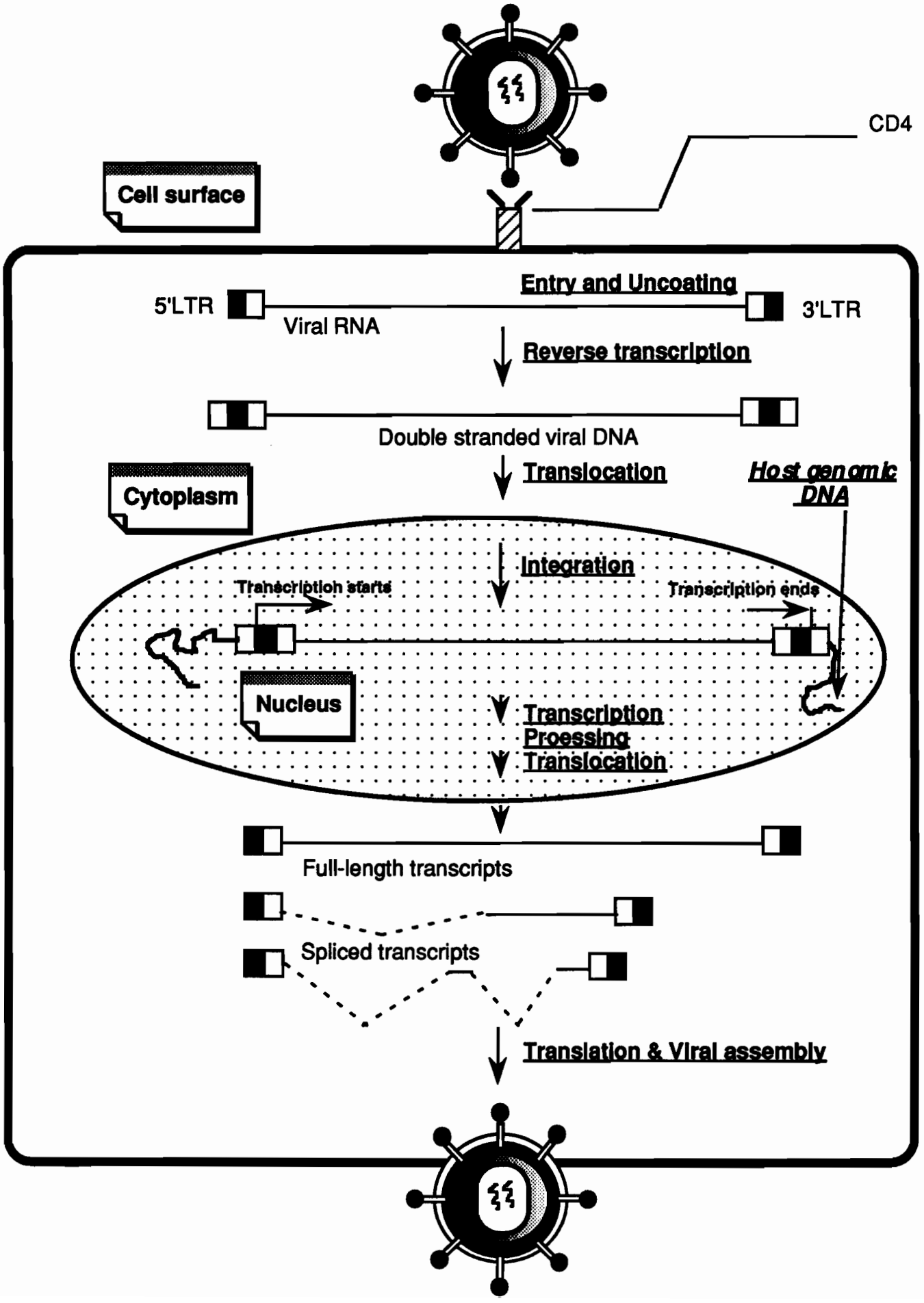
1.3.5 HIV Viral Packaging and Assembly

Post-translational modification of viral polyproteins by cellular enzymes is essential for viral assembly. Modifications include glycosylation of *Env* proteins and myristylation of the N-terminal residue of the p17 element in the *Gag* and *Gag-Pol* polyproteins (Haseltine, 1991; Gottlinger et al., 1988). N-terminal myristylation of *Gag* and *Gag-Pol* is required for attachment of these proteins to the cell membrane and, as well, for alignment of adjacent molecules. The *Gag* proteins play a central role in recruiting both viral proteins and host cell-derived elements into mature viral particles (Dorfman et al., 1993; Haseltine, 1991; Luban et al., 1993; Reicin et al., 1995; Yu et al 1992). Unspliced viral mRNA is believed to be specifically recruited for the viral assembly process by the *gag* precursor (Luban et al 1991). However, the location and nature of the HIV-1 packaging signal on the unspliced viral RNA remains largely unknown, despite several genetic and biochemical mutational analyses (Berkowitz et al., 1995). The replication primer, $tRNA^{Lys.3}$, is most likely selected by the viral *Gag-Pol* protein (Mak et al., 1993), yet it is not clear which region of tRNA binds to the viral protein.

In general, all viral components as well as elements derived from the host cell, such as cyclophilin and MHC antigen, are highly concentrated in an area on the host cell membrane from which release takes place (Haseltine 1991; Luban et al., 1993). However, ultimate maturation of the viral particle, most probably mediated by the viral protease to cleave polyproteins, may take place after the particles have been released (Kaplan and Swanstrom, 1991; Sheng et al., 1994).

Fig.4: Graphic description of HIV-1 life cycle

The diagram shows the various stages involved, including entry, oncoating, reverse transcription, integration, expression of proviral genome, viral assembly and particle release.


CD4
Cell surface
Entry and Uncoating
5'LTR
3'LTR
Viral RNA
Reverse transcription
Double stranded viral DNA
Translocation
Host genomic DNA
Cytoplasm
Integration
Transcription starts
Transcription ends
Nucleus
Transcription
Proessing
Translocation
Full-length transcripts
Spliced transcripts
Translation & Viral assembly

1.4 REVERSE TRANSCRIPTION: THE CURRENT MODEL

Reverse transcription is mediated by the virus encoded enzyme, reverse transcriptase (RT). RT has at least three enzymatic functions: RNA-dependent DNA polymerase activity, DNA-dependent DNA polymerase activity and ribonuclease H activity (RNase H) (Goff, 1990). All these activities are essential for reverse transcription.

Fig.5 is a graphic description of the major steps of reverse transcription (Giboa et al., 1979; Hu and Temin, 1989; Luo and Taylor, 1990; Peliska et al., 1992; Zack et al., 1990).

1.4.1 Step 1: Reverse transcription is initiated as the tRNA primer anneals to the PBS of viral RNA template

As in the case of other DNA polymerases, RT needs a primer carrying a free 3' hydroxyl to initiate DNA synthesis. All retroviruses utilize host-encoded tRNA as primer for reverse transcription (Leis et al., 1993). In the case of HIV-1, this role is played by $tRNA^{Lys.3}$. Annealing of the tRNA primer to viral RNA takes place in a region close to the 5' terminus of the unspliced full-length viral RNA (approximately 200 nt from the 5'-end) at a location referred as the primer-binding site (PBS). This 18-nt region is complementary to the 3'-end of host cell-derived $tRNA^{Lys3}$ (Ratner et al., 1985). Synthesis of minus strand DNA by RT proceeds towards the 5'-end of the RNA template. As RT reaches the end of the template, the RNA strand of the newly formed RNA/DNA hybrid is digested by the RNase H activity of the enzyme (Champoux et al., 1984), releasing the

newly-synthesized DNA. This DNA fragment is often called minus strand strong stop DNA [(-)ss DNA].

1.4.2 Step 2: The first strand transfer is made possible by repeat sequences (R) present on both ends of RNA template

The R region of nascent (-)ssDNA is complementary to the R region of the 3' end of the RNA template. This allows translocation of (-)ss DNA to the 3' end of the genomic RNA, where it base pairs to a repeat (R) sequence that exists on either end of the RNA template. Such strand transfer makes possible the continuation of minus strand DNA synthesis (Hu and Temin., 1989; Luo and Taylor., 1990; Peliska et al., 1992).

1.4.3 Step 3: Synthesis of plus-strand strong-stop DNA is initiated from a RNA fragment created by RNase H cleavage of the genomic RNA and is terminated by contact with the first modified residue of the tRNA primer.

As synthesis of minus strand DNA proceeds, a DNA/RNA hybrid forms between the newly synthesized DNA and RNA template. RNase H cleavage of the RNA template creates a purine-rich fragment that functions as a primer for plus strand DNA synthesis (Charneau and Clavel, 1991; Pullen and Champoux, 1990; Resnick et al; 1984). The purine-rich regions, highly resistant to RNase H degradation, are located on the RNA template just upstream of U3 and the middle region, respectively, and are often referred as the polypurine tract (PPT)

(Champoux, 1993). Before completion of minus strand DNA synthesis, plus strand DNA synthesis has already begun by using the nascent minus-strand DNA as template. The signal for the end of plus strand DNA lies within the tRNA primer. At this stage of reverse transcription, the tRNA that primed minus-strand DNA synthesis is still attached to the 5' terminus of the minus strand DNA (Champoux, 1993). When RT reaches tRNA, it continues to synthesize plus-strand DNA using tRNA as template.

The cellular synthesis of tRNAs involves posttranscriptional modification of some tRNA bases. During the initial stages of plus-strand DNA synthesis, DNA polymerization continues until the first modified tRNA base is encountered. The partial copy of tRNA regenerates the PBS at the 3' terminus of plus-strand strong-stop DNA. Since intracellular plus-strand strong-stop DNA appears to have a discrete 3' end, DNA synthesis must stop at the first modified residue, although errors may occur when the synthesis of plus strand DNA advances past the first modified residue (Pulsinelli and Temin, 1991). RNase H cleavage of the junction between tRNA primer and minus strand DNA (a single endonucleolytic cut) removes tRNA from the tRNA-DNA molecule, exposing the PBS copy on the plus-strand strong-stop DNA (Champoux et al. 1984; Furfine and Reardon 1991; Omer and Faras 1982; Smith et al., 1991; Whitcomb et al. 1990). While the fate of the free tRNA primer is unknown, removal of tRNA from the nascent minus strand DNA is necessary for the subsequent second template switch.

1.4.4 Step 4: The second strand transfer is facilitated by PBS sequences.

The repeat sequences used in the second strand-transfer are complementary copies of the 18-nt PBS. The first of these is a DNA copy of the PBS that is regenerated by using tRNA as template during the synthesis of plus-strand strong stop DNA (Hu and Temin, 1989; Papanigan and Fiore, 1988; Peliska et al., 1992; Rhim et al., 1990). The other is a complementary DNA copy that is formed when minus strand DNA synthesis advances into the PBS region (see step 2). Clearly, the PBS plays a dual role in reverse transcription, i.e., by providing an anchor for the tRNA primer at the initiation of reverse transcription (step 1) and by facilitating the second strand transfer (step 4). Thus in the case of HIV-1, $tRNA^{lys.3}$ serves as initiator for the synthesis of minus strand DNA (step 1) and as template for regeneration of the PBS (step 3 & step 4).

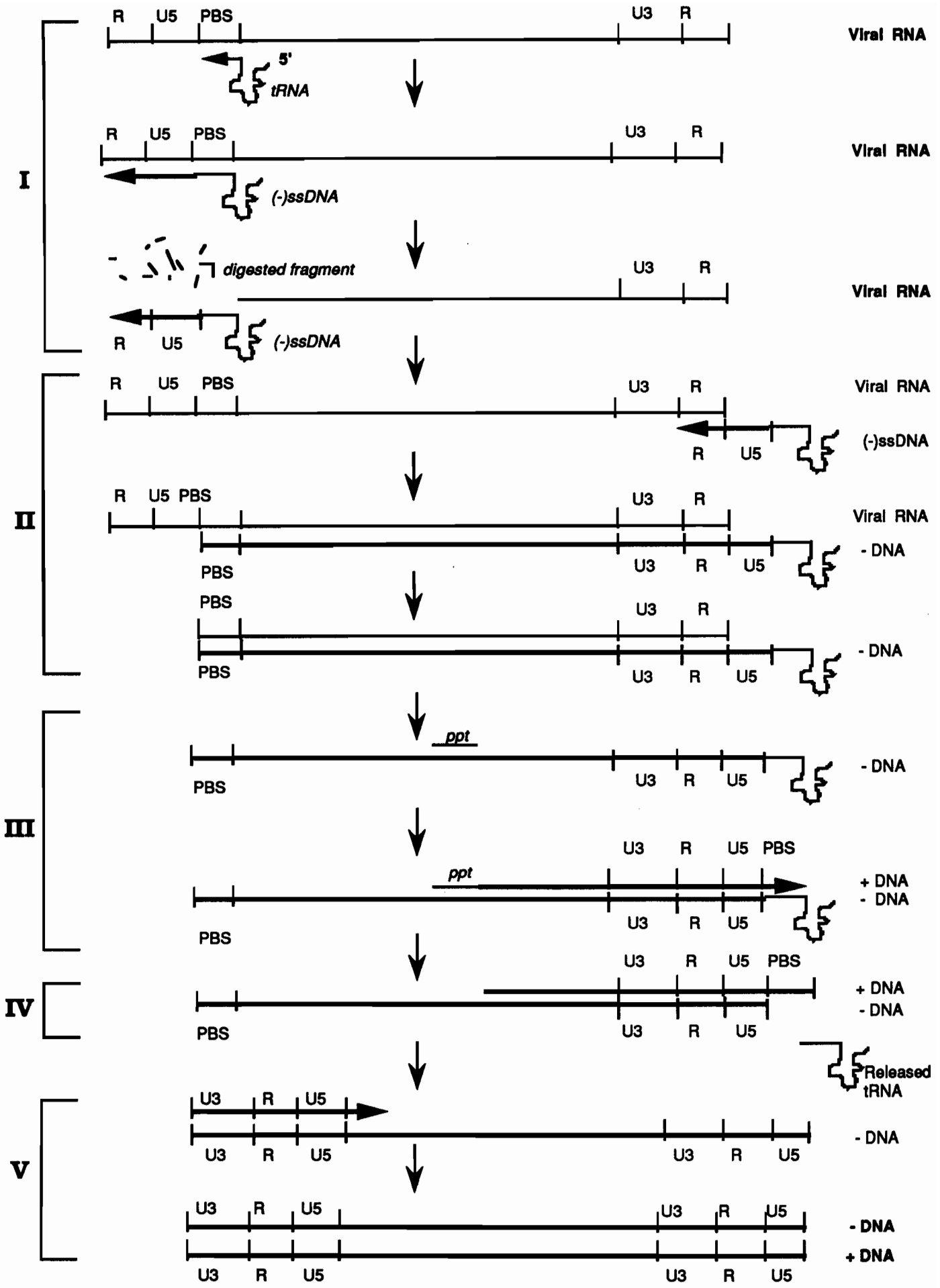
1.4.5 Step 5: Completion of synthesis of double stranded preintegrative DNA requires strand displacement of the short DNA stretch, while each strand serves as template for the other

When its synthesis is completed, the plus-strand strong-stop DNA contains an intact LTR that is engaged in a DNA/DNA hybrid with the 5' end of the minus strand DNA. It is likely that RT has strand-displacement activity to break up the DNA/DNA hybrid, allowing the minus and plus strand DNA to use each other as template to complete their synthesis (Boone and Skalka 1981; Huber et al 1989;

Hottiger et al 1994). As a consequence of two strand transfers, the provirus acquires a duplicated LTR at either end (Teletsky and Goff, 1993).

Fig.5 The current model of reverse transcription

Reverse transcription is a discontinuous process. The production of each strand of integration-competent DNA begins with the synthesis of short, discrete DNA products that can be elongated only after they are translocated to secondary template locations in a process called strand transfer. The initiation primer of HIV-1 reverse transcription is $tRNA^{Lys.3}$, which is positioned at the PBS. The first strand transfer, or template switch, is facilitated by the repeat (R) sequences present at either end of the genomic RNA. The second strand transfer is facilitated by complementary PBS sequences present on both the nascent plus and minus strand DNA. The final reverse transcribed products are longer in size than the RNA template due to these strand transfers.


I
II
III
IV
V
R
U5
PBS
U3
5'
tRNA
(-)ssDNA
digested fragment
Viral RNA
- DNA
+ DNA
ppt
Released tRNA

1.5 THE PARTICIPATING MACROMOLECULES IN REVERSE TRANSCRIPTION

1.5.1 Reverse transcriptase

In all retroviruses, reverse transcriptase is encoded downstream of the *gag* gene in a large coding region. In HIV-1, this large open reading frame (*pol*) encodes three enzymes: reverse transcriptase, protease and integrase (Fig.3) (Veronese at al., 1986; Ratner et al., 1985). The *gag* and *pol* reading frames overlap, with the latter located just in the -1 frame relative to the former. In unspliced, full-length mRNA, both reading frames are present (Jacks et al., 1988). Translation starts at the beginning of the gag open reading frame and, in most cases terminates at its end of this reading frame, generating a 55 kilodalton *Gag*-precursor. The latter is subsequently cleaved by the virus encoded protease to produce several small core proteins (p17, p24, p15). Infrequent "slip back" one nucleotides (5-10% frequency) during the process of translation of this long mRNA enables the translation machinery to gain access to the *pol* open reading frame, resulting in production of a fusion protein of the carboxy terminal truncated *Gag* precursor and the entire *Pol* polyprotein (Jacks et al., 1988). The amino terminus of protease is located 55 amino acids downstream of the frameshift site. The frameshift mechanism used to generate these proteins is of great advantage to the virus by: i) maintaining a fixed ratio of structural gag versus catalytic proteins (protease, integrase and reverse transcriptase); 2) making full use of the viral genome; 3) ensuring that viral catalytic proteins are assembled into the mature, budding virion by virtue of signals contained in the *gag* region. This results from a post-translational myristylation of glycine at the amino terminus of the *Gag-Pol* precursor.

Proteolytic processing of *Gag-Pol* precursor is mediated by the viral protease (Haseltine, 1991) and likely takes place both during viral assembly at the cellular membrane and inside the mature, budding virion (Kaplan and Swanstrom, 1991). The number of RT molecules per HIV particle is yet to be determined. In animal retroviruses, estimates of number of RT molecules range between 40 - 110 per viral particle (Bauer et al., 1980; Panet et al., 1975).

Based on sequence comparison between HIV-1 RT and *Escherichia coli* DNA polymerase, the RT molecule can be divided into several domains: i) The first 250 amino acids of the amino terminus of several retroviral reverse transcriptases show considerable homology with one another and with the α subunit of *Escherichia coli* polymerase, suggesting this region is involved in polymerase activity (Johnson et al., 1986; Larder et al., 1987); ii) the carboxyterminal region of retroviral RTs demonstrates significant homology with the RNase H region of *E.coli*; iii) the region between these areas, i.e., approximately 200 aa in length, referred to as the connecting domain. Genetic analysis has indicated that a fully functional RT containing both polymerase (primarily located in the aminoterminal region) and ribonuclease activity (primarily located in the carboxyterminal region) requires extensive interactions between the amino- and carboxy terminal regions. Structural modifications within one region may impose profound effects on the function of the other (Prasad and Goff 1988; Prasad and Goff, 1989; Prasad et al 1991; Schatz et al 1991; Boyer et al., 1992; Hizi et al., 1990). The connecting domain, especially the leucine-zipper motif (located between amino acid 281-310), also contributes to polymerase activity as change at position 283 or changes close to the motif may result in loss of polymerase activity. This domain is likely involved in dimerization of the enzyme -- a process of association of the two

subunits into a functionally active heterodimer (Prasad and Goff 1989; Becerra et al, 1991).

In mature HIV-1 viral particles, RT exists as a heterodimer consisting of a p66 and a p51 subunit (Veronese et al., 1986). Amino acid sequencing analysis showed the two subunits shared a common amino-terminal sequence (Le Grice et al., 1989; Lightfoote., et al, 1986). The p66 subunit retains both polymerase activity and ribonuclease activity. The p51 is generated by a protease-mediated cleavage of p66 subunit between phenylalanine 440 and tyrosine 441. (Becerra et al., 1990; Hansen et al., 1989; Hostomsky et al., 1992; Le Grice et al., 1989; Restle et al., 1989). Partial cleavage that produces the p51 subunits allows the p66/p51 heterodimer to adopt a conformation that maximizes both its polymerase and ribonuclease activities. The p51 subunit does not directly contribute to catalytic activity, a conclusion based on mutational analysis of the YXDD motif, a highly conserved structure in all polymerases comprising amino acids 183-186 in HIV-1 RT. Mutation of YXDD in the p51 subunit had little effect on polymerase activity of the p66/p51 heterodimer, whereas the same mutation in p66 completely destroyed polymerase activity (Le Grice et al., 1990). However, the importance of p51 probably lies in its ability to modulate the conformation and/or activities of the enzyme (Le Grice et al., 1991; Boyer et al., 1992). The p66 subunit, either as a monomer or dimer, has less polymerase activity than the heterodimeric p66/p51 form (Muler et al., 1989).

1.5.2 $tRNA^{Lys.3}$: the replication primer

All DNA polymerases, including RT, have an absolute requirement for 3' OH primers during DNA polymerization. However, retroviral RTs are unusual in

that they all utilize host cell-derived tRNA as their primer. The type of such tRNA depends on the virus. Table 1 lists tRNAs that are used by certain retroviruses. In regard to HIV-1, $tRNA^{Lys.3}$ has been identified as the replication primer through sequence determination of proviral DNA and isolation of tRNA species encapsidated in mature virion (Jiang et al., 1994; Ratner et al., 1985).

In mammalian cells, tRNAs constitute approximately 15 % of total cell RNA. There are about one hundred species of tRNA in a single cell (Rich and RajBhandary. 1976). Each tRNA molecule contains between 73 and 93 nucleotides (total molecular weight = 25 - 30 kDa) linked together in a single, covalently bonded chain. Although the exact sequence of residues varies, certain positions are conserved from one tRNA to another. For instance, the 3' end always terminates in a CCA sequences. The 5' end always carries a monophosphate group. A striking aspect of all tRNA sequences is their high content of modified bases. Many of these modified bases differ from the normal bases by the presence of one or more methyl (CH_3) groups that are added enzymatically after the nucleotides are linked by 3'-5' phosphodiester bonds. Other unusual bases arise by enzymatic modification of preexisting nucleotides. Unusual bases found in tRNA include 4-thiouridine (S^4U), inosine, methyguanosine (m^1G), methyadenosine, ribothymidine (T), pseudouridine (ψ), and dihydrouridine (D) (Rich et al., 1976).

A majority of the bases in tRNA are hydrogen-bonded to each other. Hairpin folds bring bases on the same chain into a double-helical arrangement where short stretches of nucleotides are complementary to each other. The common shape of all tRNAs, i.e., the cloverleaf configuration, is the thermodynamically stable form that folds the chain in two dimensions so as to maximize the number of base pairs. Each cloverleaf contains four hydrogen-

bonded stems and a number of non-hydrogen-bonded sections (Rich et al., 1976). Fig.6 depicts the cloverleaf structure of mammalian $tRNA^{lys.3}$, the replication primer for HIV-1. The main structural features of this 76-nucleotide long $tRNA^{Lys.3}$ are as follows (Litvak et al., 1993):

1. - The 3' end consists of CCA_{OH}, a sequence conserved in all tRNAs. This sequence plus one nucleotide (G) extends beyond the stem that is formed by base-pairing of the 5' end and 3' segments of the molecule. In protein synthesis, an amino acid always attaches to the 3'-terminal "A". This is also the site at which the first nucleotide is added during retroviral reverse transcription. The 18 nts found at the 3' end are complementary to the PBS of the HIV-1 RNA genome (Ratner et al., 1985).

2. - As one moves along the backbone from the 3' end, the TΨC loop (7 unpaired bases) is encountered. This is the first loop of the cloverleaf and consists of three modified bases (T, Ψ, m1A). In protein synthesis, this region is involved in binding to ribosomal bases. During retroviral reverse transcription, this loop likely plays an important role by interacting with the retroviral RNA template (Leis et al., 1993).

3. - Next is a loop called "variable loop", which is highly variable among tRNAs. In $tRNA^{lys.3}$, it consists of 4 nucleotides.

4. - The variable loop is followed by a base-paired stem called anticodon stem consisting of six base pairs.

5. - The third loop, i.e., the anticodon loop, also contains seven unpaired bases. The anticodon contains the 3 adjacent bases, flanked at the 5' end by a uridine and at the 3' end by a ribothymidine, and binds by base pairing to mRNA to direct the sequence specificity of protein synthesis. The anticodon loop may also be involved in reverse transcription (Isel et al., 1993).

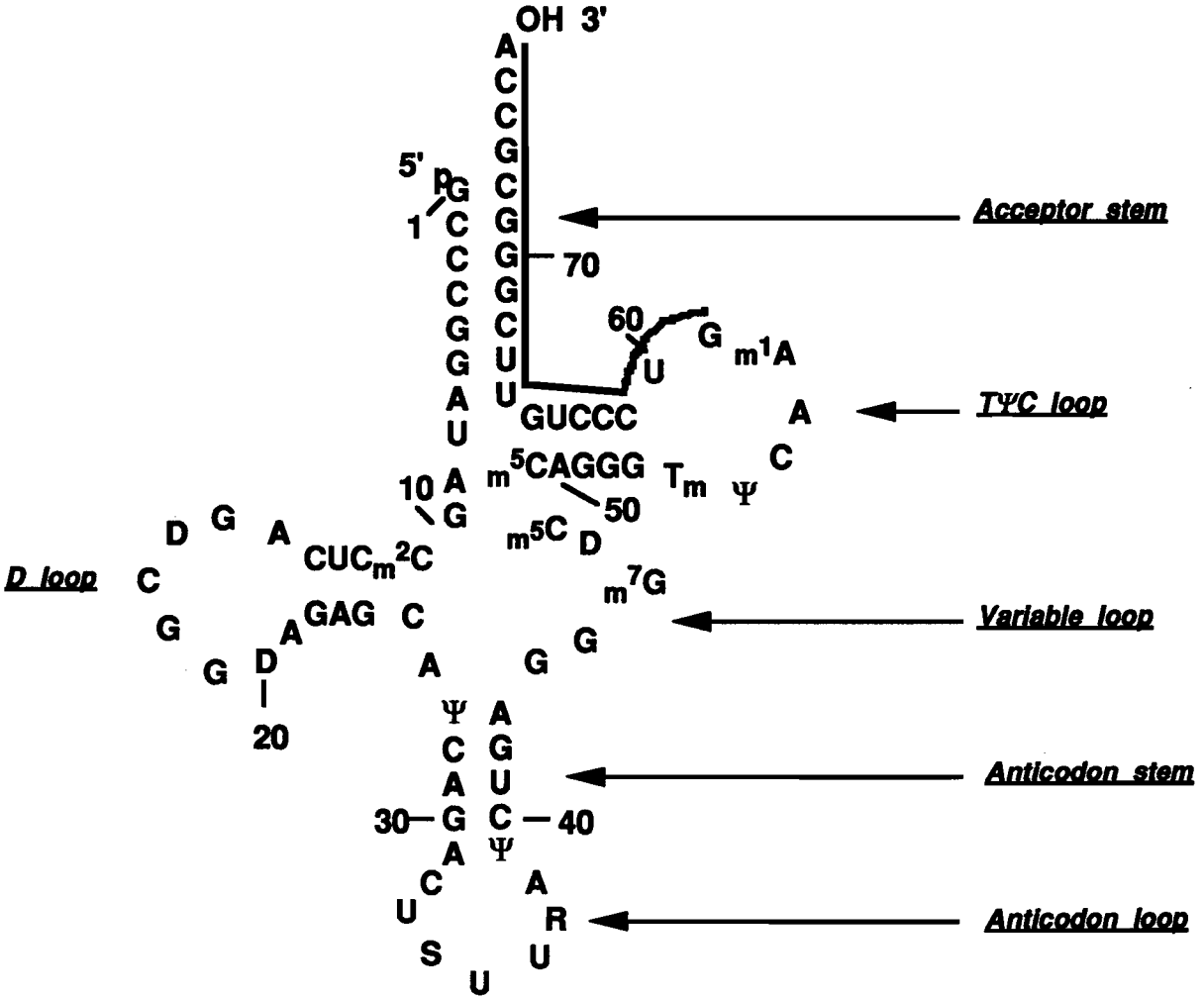
6. - The fourth loop contains eight unpaired bases and characteristically contains the modified base dihydro-uridine (hence the term D loop).

Fig.6 Cloverleaf structure of mammalian tRNALys.3

The 3' 18-nt sequence, complementary to the PBS of viral RNA, is indicated by a thick line. The loops and stems are also marked.

LEGEND:

Ψ: Pseudouridine
Tm: Ribothymidine
D: Dihydrouridine
R: ms^2t^6A
S: mcm^5s^2U



1.5.3 The viral nucleocapsid protein:

The HIV-1 p15 nucleocapsid protein (NCp15) consists of 149 amino acids (Ratner el al., 1984) and contains two regions of zinc finger motifs, sequences associated with certain nucleic acid binding proteins (Berg, 1986; South et al., 1990). Recent studies have indicated that the NCp15 may also be further processed to four additional peptides-p2, p7, p1, and p6 (Gelderbloom 1991; Henderson et al 1992, Wondrak 1993; Sheng and Erickson-Viitanen, 1994). Of these, p7 is the only major form of nucleocapsid protein found in the mature virions. p7 possesses the zinc finger motifs and may therefore represent the fully processed form of nucleocapsid protein.

A number of functional activities have been ascribed to HIV-1 nucleocapsid protein: i) Nucleic acid binding activity: NCp has been shown to bind single-stranded nucleic acid preferentially (Karpel., 1987; Sykora et al., 1981). The binding size of NCp7 has been estimated to be 7-15 nt (Kahn and Giedroc, 1992; You and McHenry 1994). ii) Unwinding of tRNA: denaturation of tRNA by NCp7 has been directly observed by circulation dichroism spectroscopy (Kahn and Giedroc, 1992) and probably occurs by virtue of its high affinity for single-stranded nucleic acids. iii) Stimulation of reverse transcription: NCp has been shown to both promote annealing between tRNA primer and RNA template (Prats et al 1988) and stimulates strand transfer (Alain et al., 1994; Darlix et al., 1993; You and McHenry, 1994). iv) promotion of viral RNA dimerization (Darlix et al., 1990). v) Recognition and packaging of viral genomic RNA (Aldovini et al., 1990; Gorelick et al., 1988; Jentoft et al., et al 1988). Mutations in NCp coding sequences resulted in decreased incorporation of viral RNA into virions. However, since the *Gag* polyprotein is involved in the initial assembly of HIV (Gottlinger et al., 1989; Gorelik et al., 1988; Jentoft et al.,

1988), it is likely that $p55^{gag}$ precursor is involved in selection and packaging of the viral RNA (Luban et al., 1992). As part of the precursor $p55^{gag}$, NCp might play a critical role in this activity, given that NCp demonstrates remarkably similar nucleic acids binding properties as its precursor (see Chapter 2)

1.6 REGULATION OF REVERSE TRANSCRIPTION

1.6.1 Initiation : Interactions among RT, $tRNA^{Lys.3}$, NCp and viral RNA template

A number of macromolecules participate in reverse transcription. These include viral RNA template, tRNA primer, RT and NCp. Gel retardation, UV crosslinking, and RNase footprinting studies revealed a complex formed between $tRNA^{Lys.3}$ and HIV-1 RT (Barat et al., 1989; Barat et al., 1993; Richter-Cook et al., 1992; Sallafranque-Andreola et al., 1989; Sarih-Cottin et al., 1992). The anticodon domain and the D-loop of $tRNA^{Lys.3}$ have been identified as he contact region for RT (Barat et al.; 1989; Sarih-Cottin et al., 1992), yet the exact RT sequences involved are not known. Independent studies showed that a specific binding site for the 5' end of $tRNA^{Lys.3}$ might exist in the C-terminal of the p66 RT subunit, implying that the 5' end of $tRNA^{Lys.3}$ is not completely mobile (Mishima and Steitz, 1995). Interestingly, interaction between $tRNA^{Lys.3}$ and RT results in conformational modulation for both of them (Litvak et al., 1994; Sarih-Cottin et al., 1992). Only the p66/p51 heterodimer has been shown to be the most efficient form for interaction with $tRNA^{Lys.3}$ (Litvak et al., 1994; Richter-Cook et al., 1992), even though the biological significance of this has yet to be defined. While binding between $tRNA^{Lys.3}$ and RT has been shown to be of considerable selectivity (Barat et al., 1989), data obtained from quantitative analysis argues against the notion of specific binding between these reagents (Arion, et al., 1993., B. Wohrl, personal communication).

For synthesis of minus strand DNA to occur, the tRNA primer must be positioned onto the PBS of the viral RNA template. However, both the viral RNA

template and tRNA are highly structured, folding into several stems and loops (Harrison et al., 1992; Baudin et al., 1992; Boukhout et al., 1993). Without being appropriately unfolded, these structures could potentially hinder the annealing process between tRNA and the viral RNA template. By virtue of its affinity to bind single strand nucleic acids (Kahn and Giedroc, 1993), NCp7 might destabilize the secondary structure of both tRNA and the viral RNA template. However, the mechanisms involved in annealing between the primer and the template remain largely unknown (Prats et al., 1988).

Recent evidence suggests that sequences that surround the PBS of the viral RNA template are necessary for efficient initiation of reverse transcription. Formation of a competent initiation complex between tRNA and RNA requires base pairing between the PBS and the 3' end 18-nt region of tRNA but also additional interactions between sequences around the PBS and the remaining portion of tRNA primer (Isel et al., 1993; Kohlsteadt and Steitz, 1992; Leis et al., 1993). In avian leukosis sarcoma virus (ALSV), seven bases located in the U5 region of the RNA template are known to interact with the TΨC loop of $tRNA^{Trp}$ (Aiyar et al., 1992). Disruption of such interaction resulted in a decrease in efficiency of initiation of reverse transcription both *in vitro* and *in vivo* (Aiyar et al., 1992; Leis et al., 1993). In HIV-1, a conservative "A" rich loop, located upstream of the PBS, has been shown to interact, through base-pairing, with the anticodon loop of $tRNA^{Lys.3}$ (Isel et al., 1993). The resulting loop-loop interaction between tRNA and RNA template, combined with normal PBS-tRNA binding, might give rise to significant alterations in secondary structure of the primer-template complex relative to that occurring when only the 18 nt of the PBS interact with tRNA (as in the case of the pPBS-Lys1,2 and pPBS-Phe mutants) (Li et al., 1994). The stability of additional tRNA/RNA template interactions might depend on particular base modifications found

only in $tRNA^{Lys3}$. Such interactions could play a role in formation of RT-tRNA/RNA template transcription complexes, thereby affecting transcription efficiency. In an independent study involving *in vitro* reverse transcription, six bases located immediately downstream of the PBS were suggested to be involved in specifying tRNA utilization (Kohlsteadt and Steitz, 1992). However, the biological significance of these studies is not clear.

1.6.2 Regulation of strand transfer

Since there are two RNA copies in each virion, the first strand transfer could be either an intra- or an inter-strand event (Panganiban and Fiore 1988; Hu and Temin, 1990b). The relative frequencies of these two types of first strand transfers are not known, nor are the factors that govern inter vs intra-strand transfers understood during retroviral replication. Only intramolecular transfer has been observed during the second strand transfer (Hu and Temin, 1990; Panganiban and Fiore, 1988).

Although the repeat sequences (R and PBS) are the basis for strand transfer, several other factors greatly influence this process. Strand transfer is likely to occur with RT poised at the 3' terminus of strong stop DNA. RT may play an active role in this process of strand transfer by acting as a bridge between the donor and acceptor template (Xu and Boeke 1987). Moreover, structural modifications of RT, i.e., mutations in the RNase H domain, resulted in a marked decrease of strand transfer, suggesting that removal of the RNA portion of the DNA/RNA hybrid was critical (Blain and Goff 1995).

Comparative studies of strand transfer, using either tRNA or deoxyoligonucleotide (dPR) as primer (Arts et al 1994), showed that strand transfer occurred more efficiently in reaction primed by tRNA than by dPR.

This suggests that $tRNA^{lys.3}$, together with RT, may be part of a replicating complex that influences the efficiency of strand transfer. The virus encoded nucleocapsid protein is also actively involved in strand transfer, presumably by stimulating annealing of the donor with the acceptor strand (Alain et al 1994; Darlix et al., 1993; Yu and McHenry, 1994).

The outcome of strand transfer is formation of a DNA/RNA duplex. The ability of NCp to stimulate strand transfer can not be solely explained by its preferential binding to single-stranded nucleic acids, and other factors must govern the stabilization of DNA/RNA hybrids. Further investigations will require use of reconstituted reverse transcriptions that include both viral and cellular factors (Takahashi et al., 1994).

CHAPTER 2

EXPRESSION, PURIFICATION, AND RNA-BINDING PROPERTIES OF HIV-1 NUCLEOCAPSID PROTEIN (NCp15)

This chapter was adapted from an article that appeared in Protein Expression and Purification (1993), Vol. 4, pp. 304-311. The authors of this paper were X. Li, Z. Gu, R. Geleziunas, L. Kleiman, M. A. Wainberg, and M. A. Parniak. The data presented in this chapter was largely performed by myself under the supervision of Drs. Wainberg and Parniak. Drs Z. Gu, R. Geleziunas, L. Kleiman have provided assistance in devising the experiments and analyzing the results.

2.1 PREFACE TO CHAPTER 2

The retroviral nucleocapsid protein (NCp) has been implicated to play important roles in viral replication. The mature form of HIV-1 NCp (NCp7) has been prepared by chemical synthesis, an expensive method beyond the scope of many laboratories (De Rocquiny et al., 1991). No attempts have been made to chemically synthesize the larger form of NCp (NCp15), which should be more technically demanding and expensive. We reported here a simple and efficient method to purify NCp15 from a bacterial over-expression system and characterization of its RNA binding properties.

2.2 Abstract

We have cloned and expressed HIV-1 gag p15 nucleocapsid protein (NCp15) in the form of a 41-kDa fusion polypeptide with the glutathione-S-transferase (GST-NCp). The recombinant protein was rapidly degraded in bacterial lysates unless Zn^{2+} and Cd^{2+} were present in the extraction buffer. Inclusion of these metals stabilized the protein, allowing facile purification of GST-NCp by affinity chromatography. The native NCp15 was readily prepared from the GST-NCp by proteolytic cleavage with thrombin. Both GST-NCp and the processed NCp15 were able to bind RNA containing sequences from the 5' end of the HIV-1 genome. This binding was unaffected by the absence or the presence of Zn^{2+}; however, the binding of RNA was absolutely dependent on K^{+}. The GST-NCp fusion protein was nonselective in the binding of RNA, with all transcripts, including antisense and non-HIV RNA, binding with equal efficiency. In contrast, NCp15 was highly selective in binding of RNA. Sequences within nucleotides 1244-1421 of the HIV-1 proviral genome were found necessary for maximal binding of RNA to NCp15.

2.3 INTRODUCTION

The internal structural proteins of HIV-1 comprise the major protein components of the virus. These proteins, which are encoded by the gag gene, are initially synthesized as a 55-kDa polyprotein precursor which participates in virion assembly at the cell membrane. After virus budding, this polyprotein is cleaved by the viral protease to yield the processed viral gag structural proteins: p17, the matrix protein derived from the N-terminus of p55; p24, the major core protein which constitutes the shell of the cone-shape core structure of the native protein; and p15, the nucleocapside protein (NCp15) derived from the C-terminus of the p55 precursor (Haseltine, 1991). This postbudding processing is essential for the synthesis of replicative virus, since viral particles released in the absence of a functional protease contain only polyprotein and are noninfectious (Karpel et al., 1987; Krausslich and Wimmer, 1988; Peng et al., 1989; Skalka, 1989).

A number of functional activities have been ascribed to HIV-1 nucleocapsid protein. This protein has been shown to bind single-stranded nucleic acids preferentially (Karpel et al., 1987). NCp sequences in the p55 precursor polypetide may be involved in the packaging of viral genomic RNA during viral assembly. Mutational studies have indicated alterations or deletions in the NCp Zn^{2+} -finger sequences can inhibit the packaging of HIV-1 genomic RNA, leading to blockage of viral replication despite normal expression of viral RNA and protein in such systems (Aldovini and Young, 1990; Clavel and Orenstein, 1990; Gorelick et al., 1988; Gorelick et al., 1989; Lever et al., 1989). In addition, NCp facilitates the dimerization of HIV-1 genomic RNA (Darlix et al., 1990), a process which may be important in the functional maturation of encapsidated viral RNA. Finally, NCp may participate in the

placement of specific replication initiation primer $tRNA^{Lys.3}$ onto the primer binding site (PBS) of the viral genomic RNA (Prats et al., 1988).

A number of important questions pertaining to the role of NCp in specific recognition and association with HIV-1 genomic RNA remain unanswered. For example, neither the specificity nor the stoichiometry of NCp binding to HIV-1 RNA is well characterized. Although approximately 2000 copies of each of the gag proteins are thought to be incorporated into mature virions, extensive study of the structure-function relationships of NCp have been hampered by the difficulty and expense in purifying suitable quantities of the protein from isolated virions. NCp has also been prepared by chemical synthesis (De Rocquigny et al., 1991), an expensive method beyond the scope of many laboratories. Although a form of HIV-1 NCp has been cloned and expressed in *Escherichia coli* (Fitzergerald et al., 1991), this recombinant protein possessed an additional 22 non-NCp N-terminal amino acid residues, including 15 residues derived from T7 gene 10. We report here the cloning and expression of HIV-1 NCp as a cleavable fusion protein with glutathione-*S*-transferase. The recombinant is inducible to high levels in *E. coli*, and is readily purified by affinity chromatography on glutathione-Sepharose. Subsequent treatment of the protein with thrombin results in the preparation of native p15 NCp. Our data indicate that although the GST-NCp fusion protein binds RNA in a nonselective manner, the processed NCp15 binds specifically to HIV-1 RNA. This binding is dependent on the presence of potassium, and appears to require a region located between nucleotides 1244 and 1412 in the HIV-1 gag gene, in addition to previously identified packaging signals.

2.4 MATERIALS AND METHODS

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pBENN7 and pNL4-3 from Dr. Malcolm Martin and pBH10 from Drs Beatrice Hahn and George Shaw. Plasmid pTB4, which contains sequences encoding cellular CD4 (Maddon et al., 1985), was a gift from Drs Paul J. Maddon and Richard Axel. The TA cloning System was obtained from Invitrogen (San Diego, CA), and expression vector pGEX-2T and glutathione-Sepharose were products of Pharmacia. Plasmids pGEM3Z and pGEM4Z for the preparation of *in vitro* RNA transcripts were from Promega. *E. coli* strains DH1, TG1, HB101, and Y1090 were obtained from Clontech. A synthetic peptide corresponding to amino acid residues 64-79 of the NCp sequences (Ratner et al., 1985) was obtained from American Biotechnologies, Inc. (Boston, MA). Restriction enzymes were products of New England Biolabs. All other biologicals and reagents were obtained from local suppliers and were of the highest grade obtainable.

2.4.1 Preparation of Monospecific Anti-NCp Antiserum

A peptide with the sequence YKGRPGNFLQSPEPTA, residues 64-79 of HIV-1 NCp15 (Ratner et al., 1985), was conjugated to keyhole limpet hemocyanin using gluteraldehyde as cross-linking agent (Zeggers et al., 1990). Monospecific antiserum was prepared in rabbits using standard procedures. Antiserum was tested for the ability to recognize NCp by ELISA and Western blot analyses. All procedures with animals were conducted according to protocols approved by the Faculty and University Animal Care Committees, McGill University, accredited by the Canadian Council on Animal Care.

2.4.2 Molecular Cloning and Expression of NCp15

The sequence encoding NCp15 in plasmid pBH10 (nucleotides 1465-1869) was amplified by PCR using 5'-CCGGATCCATGCAGAGAGGCAATTTT-3' as forward primer and 5'-GGGAATTCTTATTGTGACCGAGGGTCGTTCCCA-3' as reverse primer. The latter oligonucleotide imparts a restriction site for *Eco*RI, and the forward primer provides a restriction site for *BamH*I, in addition to sequences complementary to the NCp coding region. PCR amplified a fragment of about 450 base pairs as determined by agarose gel electrophoresis; this fragment was excised from the gel and inserted into the TA cloning vector, designed for efficient cloning of PCR amplified DNA (Invitrogene). Positive clones containing the PCR-derived inserts were selected in the presence of kanamycine and verified by agarose gel electrophoresis after *BamHI/Eco*RI digestion of plasmid minipreps. A number of recombinant plasmids were then sequenced in order to identify a clone containing an error-free sequence for HIV-1 NCp15. This sequence was excised using *BamHI/EcoR*I and inserted into the same sites in the expression vector pGEX-2T (Smith and Johnson, 1988) to give pGEXNCP. The resulting recombinant expression plasmid was expected to produce a thrombin-cleavable fusion protein of approximately 41 kDa containing glutathione-S-transferase at the N-terminus and HIV-1 NCp at the C-terminus. Proteolytic cleavage with thrombin provides intact 15-kDa HIV-1 NCp, modified only by an additional Gly-Ser sequence at the N-terminus.

pGEXNCP was used to transform four different strains of *E. coli*: TG-1, HB101, Y1090, and DH1. Antibiotic-resistant transformants were tested for recombinant protein expression after induction with IPTG (0.1-1 mM) for 1-16

h. Aliquots of the IPTG-induced cultures were harvested by centrifugation (4500 rpm in a Beckman JA-13 rotor, 15 min at 4°C), mixed with 1 vol of 2x SDS-PAGE sample buffer and subjected to electrophoretic analysis.

2.4.3 Purification of Recombinant HIV-1 NCp15

Bacteria were grown in 2x YT medium to a cell density of approximately 1.5 $OD_{600\ nm}$, and then incubated with 0.5 mM IPTG for 2 h. The induced cultures were harvested by centrifugation, and the cell pellets were resuspended in cold degassed extraction buffer (50 mM Tris-Cl, pH 7.5, containing 10 mM dithiothreitol, 0.1 mM $ZnCl_2$, 0.1 mM $CdSO_4$, and 1 mM phenylmethysulfony fluoride). The cells were disrupted by sonication (3x 60-s pulses) on ice. The supernatants obtained after centrifugation (12,000 rpm in a Beckman JS-13 rotor, 30 min at 4°C) were applied to a column of glutathione-Sepharose preequilibrated with extraction buffer containing 1% Triton X-100 at 4°C. The column was then washed with 10 vol of extraction buffer. The 41-kDa GST-NCp fusion protein was obtained by warming the column to room temperature and then eluted with extraction buffer containing 10 mM reduced glutathione. In order to obtain pure HIV-1 NCp15, the column was then washed with an additional 5 column vol of thrombin cleavage buffer (50mM Tris-Cl, pH 7.5, containing 150 mM NaCl, and 2.5 mM $CaCl_2$). The column was warmed to room temperature, and thrombin (50 µg/ml in thrombin cleavage buffer) was applied. Column flow was stopped , and the column was left to stand at room temperature for variable times (usually 30 min). Pure NCp15 was then obtained by elution with extraction buffer containing 150 mM NaCl. The protein-containing fractions were concentrated using a Centricon-10 device (Amicon) and stored at -70°C until further use.

2.4.4 Preparation of RNA transcripts

Plasmid pBENN7 contains HIV-1 sequences corresponding to the entire 5'-LTR and a portion of the *gag* gene of the HIV-1 proviral genome, as well as about 0.45 kb of host cell-derived 5'-flanking sequences (Gendelman et al., 1986). These sequences were excised by digestion with *Eco*RI/*Pst*I, and the resulting 1.8 kb fragment was inserted into the appropriately digested pGEM4Z to give plasmid pPBS2. This plasmid allows in vitro preparation of sense HIV-1 RNA transcripts using SP6 polymerase and antisence transcripts using T7 polymerase. The HIV-1 sequence in pPBS2 has several unique restriction sites which were used for preparation of HIV-1 RNA transcripts of varying sizes. For example, use of SP6 polymerase after linearization with *Pst*I gave a sense transcript (designated RNA-Pst) comprising nucleotides 1-1411 of the HIV-1 proviral genome, whereas use of T7 polymerase after linearization with *Eco*R I gave the corresponding antisense transcript. In addition to the antisense RNA transcripts from pPBS2, two other control RNA transcripts were also used. Plasmid pSP-*env*13 was used to generate transcripts containing a message for the HIV-1 *env* protein gp120. This plasmid was constructed by digesting HIV-1 molecular clone pNL4-3 with *Sal*I/*Xho*I to yield a 3.1-kb fragment containing genes for tat, rev, rev, vpu, and env proteins. This fragment was inserted into the *Sal*I site of pGEM3Z (Promega). Linearization of pSPenv13 with *Xba*I and treatment with SP6 polymerase produced an RNA transcript which, when added to an in vitro rabbit reticulocyte lysate translation reaction, generated a product that was specifically precipitated by anti-gp120 antibodies. Linearization of pT4B (Maddon et al., 1986) with *Hin*dIII and use of SP6 polymerase gave non-HIV RNA comprising cellular CD4 sequences.

RNA transcripts were synthesized in vitro at 37°C using linearized pPBS2 in 20 µl reaction mixture comprising 40 mM Tris-Cl, pH 7.5, containing 6 mM $MgCl_2$, 10 mM NaCl, 10 mM DTT, 2 mM spermidine, 1 mM nucleoside triphosphates (with or without added tracer [^{32}P]CTP), 1 µg plasmid template, and 15 U of either SP6 or T7 RNA polymerase. Transcription was allowed to proceed at 37°C for 5-30 min, the DNA template was removed by digestion with DNase, and the RNA transcripts were extracted using phenol/chloroform saturated with 10 mM Tris-Cl, pH 8.0, containing 1 mM EDTA. Transcripts were precipitated with ethanol, redissolved in sterile water, and stored in aliquots at -70°C. All transcripts were assessed for integrity by agarose gel electrophoresis prior to use in binding assays.

2.4.5 Analysis of HIV-1 NCp-RNA Interactions

Interaction of GST-NCp or NCp15 with RNA was assessed by immobilizing the protein on nitrocellulose (0.1 µ) after SDS-polyacrylamide gel electrophoresis and irradiated with short-wave UV light for 1 min. The immobilized proteins were incubated with renaturation buffer (50 mM Tris-Cl, pH7.5, containing 200 mM KCl, 0.1 mM $ZnCl_2$, 10 mM DTT, and 0.5 mg/ml heparin) with shaking at 4°C for 48 h. The various RNA transcripts were added in equal amounts on the basis of either mass (20 ng) or molar concentration (30 pmol), using identical specific radioactivities (cpm/pmol). After incubation at room temperature for 1 h, the nitrocellulose membranes were washed three times with renaturation buffer, air-dried, and analyzed by autoradiography. In order to quantitate the binding, regions of the nitrocellulose with the 15-kDa NCp or the 41-kDa GST-NCp fusion protein were excised and counted in a liquid scintillation counter.

2.5 RESULTS

2.5.1 Expression of HIV-1 NCp

Four strains of *E. coli* were transformed with pGEXNCP: TG-1, Y1090, HB101, and DH1. Induction with IPTG resulted in essentially no expression of 41-kDa fusion protein in Y1090, intermediate amounts of expression in TG-1 and HB101, and the highest levels of expression in DH1 (Table 1). The latter strain transformed with pGEXNCP was therefore used as a source of recombinant protein. As shown in Fig.1, the 41 kDa recombinant GST-NCp fusion protein was expressed to high levels within 2 h following induction with IPTG. A additional 36 kDa and smaller amounts of a 32 kDa protein were also formed; it is not known whether these species arise from premature termination or from proteolytic degradation of the 41-kDa protein.

Initial attempts to isolate the recombinant protein resulted in low yields of NCp, due to proteolytic degradation even in the presence of high concentrations of protease inhibitors. SDS-PAGE analysis indicated rapid disappearance of the 41 kDa GST-NCp fusion protein, leaving only the 26 kDa glutathione-S-transferase (data not shown). Since HIV-1 NCp has metal binding Zn-finger domains, a variety of divalent metal cations were added to the extraction buffer in an attempt to stabilize the NCp sequences during purification. As indicated in Table 1, both Zn^{2+}, and Cd^{2+} were effective in this respect; indeed, the highest yields of purified NCp15 were obtained only when both Zn^{2+} and Cd^{2+} were present during the isolation process.

Western analysis using monospecific rabbit anti-NCp15 peptide antiserum confirmed the expression of recombinant HIV-1 NCp (Fig.2). In addition to the intact 41 kDa GST-NCp fusion protein, two smaller molecular weight species were purified by affinity chromatography on glutathione-Sepharose. Coomassie staining indicated that these species were present in much lower amounts than the intact 41-kDa recombinant, and are presumed to be NCp15 C-terminal truncations since intact 26 kDa GST was produced from both of these species upon thrombin cleavage (data not shown). Efficient cleavage of NCp15 from the 41-kDa GST-NCp fusion protein occurs within 15 min of exposure to thrombin (Fig.2). The final product prepared as described under Materials and Methods consists almost entirely of the intact 15 kDa HIV-1 nucleocapsid protein.

TABLE 1
Expression of HIV-1 p15 Nucleocapsid Protein
in Different strains of E. coli

Additions to Extraction buffer[a]	Yield pf pure NCp(μg)[b]			
	DH-1	TG-1	HB101	Y1090
None	—	—	—	—
Zn2+(0.1 mM)	550	75	84	—
Cd2+(0.1 mM)	764	48	93	—
Zn2+(0.1 mM) + Cd2+ (0.1 mM	1150	111	92	—

Table 1: (legend):

a: Extraction buffer was 50 mM Tris-Cl, pH 7.5, containing 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin, antipain, leupeptin, and pepstatin.

b: Bacteria were grown in 500 ml LB medium at 37°C to an OD_{600} of 1.5, and then protein expression was induced by 0.5 mM IPTG for 2 hr. The protein amounts listed are those of pure NCp15 released after on-column thrombin cleavage as described in Materials and Methods.

Fig.1 Expression of HIV-1 nucleocapsid protein as a fusion protein with glutathione-S-transferase

Control (vector only) and pGEXNCP-transformed E. coli were grown in 2xYT to cell densities of approximately 1.5 $OD_{600\ nm}$, at which time cells were subjected to induction with IPTG. At various time bacteria were pelleted and boiled in SDS sample buffer, and the proteins were fractionated on 7.5% SDS-polyacrylamid gels and visualized by Coomassie staining. Lanes 1, 3, 5: pGEXNCP-transformed cells at 0, 1, and 2 h after addition of IPTG. Lanes 2, 4, 6: Control cells at 0, 1, and 2 h following addition of IPTG. The arrows indicate the two major protein products expressed in pGEXNCp-transformed cells.

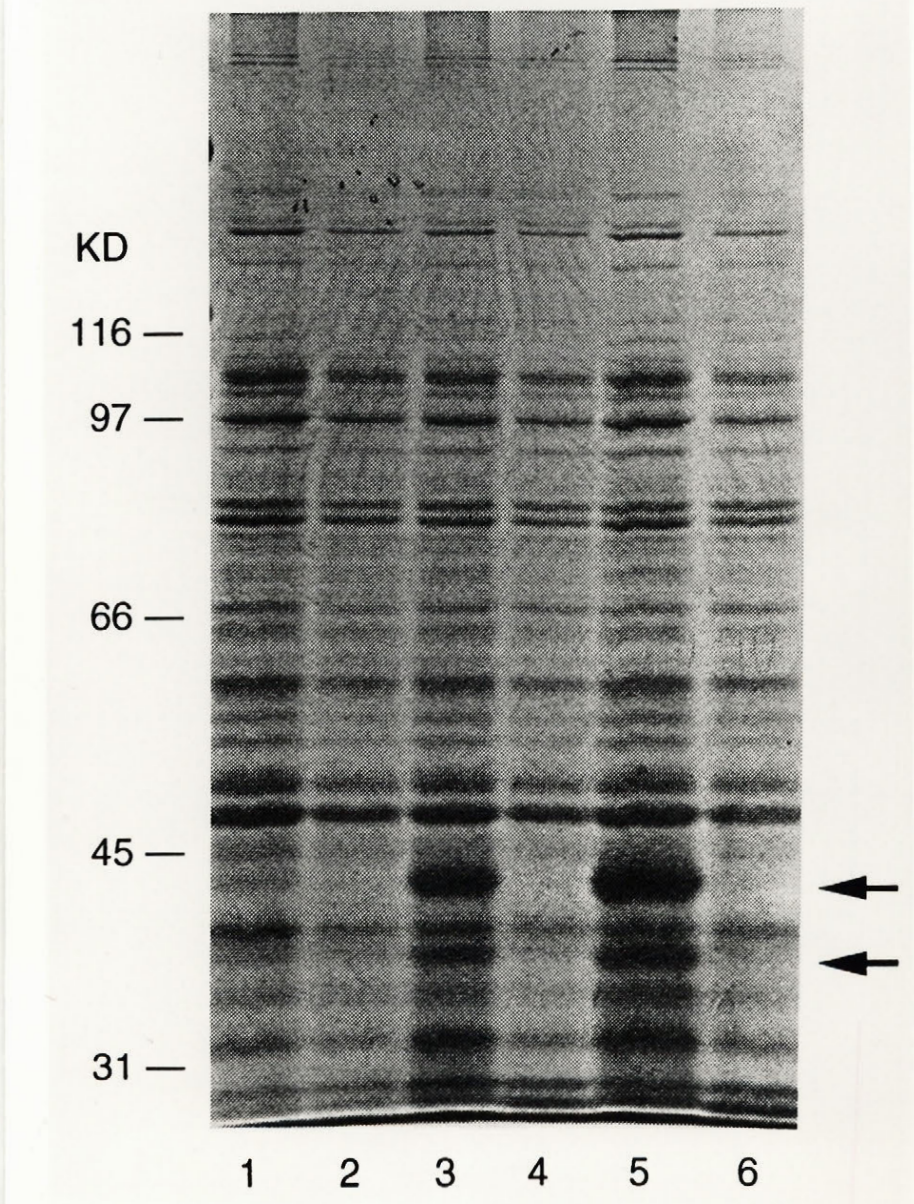
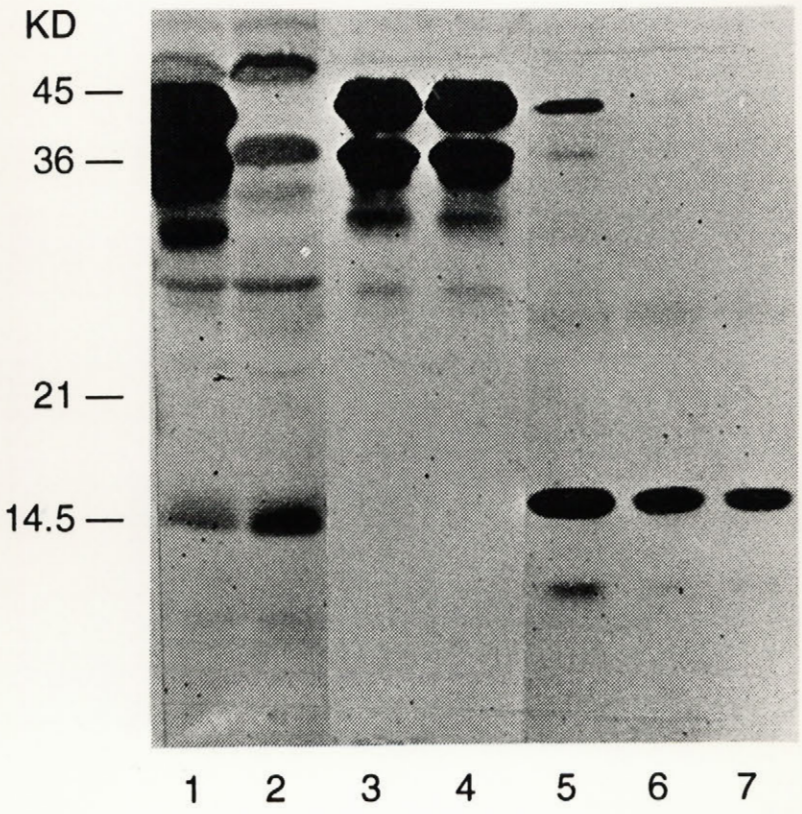

KD
116
97
66
45
31
1
2
3
4
5
6

Fig.2 Western blot analysis of HIV-1 nucleocapsid protein using rabbit monospecific anti-HIV-1 NCp

Lane 1, lysate of pGEXNCp-transformed E. coli DH1, 2 h following induction by IPTG. Lane 2, lysate of control E. coli DH1 (vector only), 2 h following induction by IPTG. Lane 3, GST-NCp fusion protein, purified by affinity chromatography on glutathione-Sepharose. Lanes 4-7, formation of NCp15 by thrombin cleavage of GST-NCp at 0, 15, 30, and 120 min, respectively.


KD
45
36
21
14.5
1
2
3
4
5
6
7

2.5.2 Interaction of NCp with RNA

Lysates of pGEXNCP-transformed bacteria, and affinity-purified GST-NCp and NCp15 were fractionated by SDS-PAGE, transferred to 0.1-μ nitrocellulose filters, and incubated in renaturation buffer as described under Materials and Methods. These filter-immobilized proteins could be stored at -70°C for up to three months without significant loss of binding activity. No binding of RNA was noted to proteins in lysates from either control bacteria or noninduced pGEXNCP-transformed bacteria (data not shown). In contrast, significant binding of RNA transcripts was noted by a 41-kDa protein in lysates from IPTG-induced pGEXNCP-transformed bacteria, as well as by the less abundant 36-kDa and 32-kDa species.

Both the purified GST-NCp fusion protein and NCp15 were able to bind RNA (Table 2). Binding to the 41-kDa GST-NCp was independent of RNA sequences, with all transcripts, including antisense and non-HIV RNA, binding to the same extent. In contrast, the processed p15 nucleocapsid protein was more selective. Only the largest transcripts (the 1.8-kb RNA-Pst) exhibited strong binding; much lower levels of binding by NCp15 were noted to the smaller transcripts as well as to the antisense and non-HIV RNA transcripts.

Heating viral RNA transcripts containing dimer linkage site at 95°C for 3 min prior to use decreased binding both to the GST-NCp fusion protein and to NCp15 by about 50% (data not shown). Evaluation of the non-heated transcripts by nondenaturing gel electrophoresis revealed both dimer and monomer species, in approximately equal proportions. This suggests that spontaneous dimerization of these RNA transcripts had occurred during *in vitro* synthesis and manipulation, an observation also noted by other

investigators (Bieth et al., 1990; Darlix et al., 1993; Marquet et al., 1991). Only RNA monomers were noted after heating.

TABLE 2

Binding of Various RNA Transcripts

to ~~of~~ GST-NCp and NCp15

Transcripts[a]	Size of transcripts (kb)	RNA bound (cpm)	
		GST-NCp	NCp15
RNA-Nar (1-637)	1.09	2370	720
RNA-BssH(1-708)	1.16	3400	830
RNA-Acc(1-956)	1.40	2750	690
RNA-Nsi(1-1244)	1.69	3010	750
RNA-Pst(1-1414)	1.86	2950	3490
RNA-Eco[b]	1.86	2880	640
pSPenv(5819-8930)	3.11	3730	460
pT4B[c]	1.87	2870	280

Table 2 (legend): Binding of various RNA transcripts of GST-NCp and NCp15

a: RNA transcripts were prepared and labeled as described in Materials and Methods. Transcripts were adjusted to identical specific radioactivity (cpm/pmol) by mixing appropriate amounts of labeled and unlabeled material. All transcripts showed a single band on denaturing gel electrophoresis. The numbers in parentheses indicate the HIV sequences contained in the transcripts. In the experiment illustrated, GST-NCp and NCp15 were incubated with equivalent molar amounts (30 pmol) of each of the transcripts.

b: Antisense RNA transcripts corresponding to RNA-Pst.

c: A CD4 sequence.

2.5.3 Influence of Metal Ions and pH on NCp-RNA Interactions

Both the GST-NCp fusion protein and the processed NCp15 show similar pH dependence profiles for RNA binding (Fig.3), with a broad pH maximum between pH 6 and 7.5. Very little binding was noted above pH 8.0.

Although Zn^{2+} and Cd^{2+} were required in the NCp extraction buffer in order to stabilize the recombinant protein, neither metal was necessary to maintain strong binding to RNA by either GST-NCp or NCp15 which had been renatured in the presence of Zn^{2+} (Table 3). However, if the protein was renatured in the absence of Zn^{2+}, substantial binding of RNA was noted only if Zn^{2+} was included in the incubation buffer. Interestingly, Co^{2+} was also effective in this regard, whereas addition of Cd^{2+} to the proteins which had been renatured in the absence of divalent cations was unable to promote the binding of RNA. In contrast, the ability of both GST-NCp and NCp15 to bind RNA was absolutely dependent on the presence of potassium. Neither Li^{+} nor Na^{+} was able to replace potassium in the binding reactions.

Fig.3 Effects of pH on the binding of RNA-Pst (1-1412) to GST-NCp and NCp15.

Proteins, immobilized on nitrocellulose were renatured at pH 7.5, then incubated with ^{32}P-Labeled RNA-Pst at the indicated pH in buffers containing 200 mM KCl and 0.1 mM ZnCl2. The extent of binding was assessed by liquid scintillation counting of excised bands corresponding to the 41-kDa GST-NCp fusion protein and the 15-kDa NCp protein.

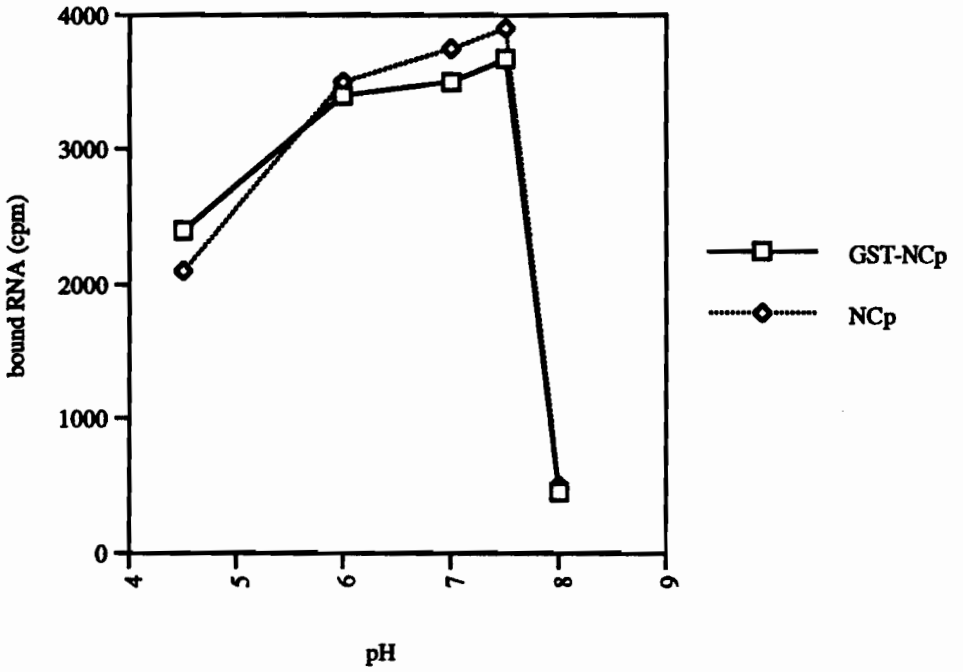

4000
3000
2000
1000
0
bound RNA (cpm)
4
5
6
7
8
9
pH
GST-NCp
NCp

TABLE 3

Effects of Monovalent and Divalent Cations on Interaction of RNA with GST-NCp and NCp15

Incubation condition	RNA bound (cpm)	
	GST-NCp	NCP15
Control[a]	3690	3860
Effect of monovalent cations[b]		
- K^+	60	50
-K^+, + Li^+(0.2 M)	210	250
-K^+, + Na^+(0.2 M)	800	620
Effect of divalent cations[c]		
Experi. 1: Renaturation in presence of Zn^{2+}		
-Zn^{2+}	3500	3660
-Zn^{2+}, + Mg^{2+}(0.1 mM)	3440	3000
-Zn^{2+}, + Co^{2+}(0.1 mM)	3420	2970
-Zn^{2+}, + Cd^{2+}(0.1 mM)	2700	2620
Experi. 2: Renaturation in absence of Zn^{2+}		
-Zn^{2+}	1500	620
+Zn^{2+} (0.1 mM)	3000	3090
-Zn^{2+}, + Mg^{2+} (0.1 mM)	1600	890
-Zn^{2+}, + Co^{2+} (0.1 mM)	3300	1920
-Zn^{2+}, + Cd^{2+} (0.1 mM)	920	450

Table 3 (legend): Effects of monovalent and divalent cations on interaction of RNA with GST-NCp and NCp15

Note. Purified GST-NCp and NCp15 were fractionated by SDS-PAGE and transferred to nitrocellulose. The immobilized proteins were incubated with renaturation buffer in the absence or the presence of 0.1 mM $ZnCl_2$ as described in Materials and Methods, then incubated with ^{32}P-labeled RNA-Pst for 1 hr at 20°C. After gentle washing to remove excess RNA, bound RNA was assessed by liquid scintillation counting.

a : Control incubation comprised 30 mM Hepes, pH 7.5, containing 0.2 M KCl and 0.1 mM $ZnCl_2$.

b : All incubation contained 0.1 mM $ZnCl_2$.

c : All incubation contained 0.1 M KCl.

2.6 DISCUSSION

Nucleocapsid proteins are essential components of replication-competent retroviruses. These proteins appear to have multiple functions including specific recognition of retroviral genomic RNA during viral assembly (Dupraz et al., 1990; Gorelick et al., 1988; Jentoft et al., 1988; Leis et al., 1984; Meric et al., 1984; Meric et al., 1988; Meric et al., 1989), dimerization of genomic RNA during the postbudding viral maturation (Darlix et al., 1990), and positioning of the specific replication tRNA primer onto the viral genomic RNA primer binding site (Prats et al., 1988). However, little is known concerning the specificities and affinities of NCp-RNA interactions, especially those in HIV. Such biochemical characterization requires relatively large amounts of HIV NCp protein. Although between 2000 and 3000 copies of NCp are thought to be present in the mature virion (Karpel et al., 1987), isolation of the protein from HIV-1 involves considerable expense, and potential risk, due to the need for large-scale virus cultivation. Certain groups have used solid-phase peptide synthesis for the preparation of shorter form of HIV-1 NCp (NCp7) (De Rocquigny et al., 1991); however, this method is also expensive and time-consuming, yields only finite amounts of materials. Synthesis of the larger form of nucleocapsid protein (NCp15) should be more technically demanding and expensive.

In order to obtain large amounts of HIV-1 NCp15, appropriate sequences of pBH10 were amplified by PCR and cloned into vector pGEX-2T, and the recombinant NCp was expressed in *E. coli* as a cleavable fusion protein with glutathione-S-transferase. Although the recombinant appeared to be relatively stable in *E. coli* (Fig.1), isolation of purified intact GST-NCp was initially problematic, due to rapid degradation of NCp sequences in the bacterial

extracts. Neither the presence of high concentration of protease inhibitors nor the use of protease-deficient *lon* mutants (*E. coli* Y1090) was able to prevent this degradation. However, addition of Zn^{2+} and Cd^{2+} to the extraction buffer stabilized the recombinant protein, enabling the isolation of large quantities of intact GST-NCp, and subsequently the 15-kDa HIV-1 NCp (Table 1). These metal ions have been found to prevent oxidation of NCp sulfhydryl residues during the aerobic manipulation (Fitzergerald and Coleman, 1991); our data imply that these metal cations may also induce significant changes in NCp conformation, rendering the protein less susceptible to proteolytic degradation during purification. It is also possible that these metal cations work in more general manner, stabilizing recombinant fusion proteins by an as yet unknown mechanism. It would be interesting to determine whether other recombinant fusion proteins expressed by the pGEX-2T system are also stabilized by Zn^{2+} and Cd^{2+}.

In addition to the full-length 41-kDa GST-NCp fusion product, lesser amounts of smaller protein products were also expressed (Fig.1 and 2). These shorter recombinant proteins were presumed to be C-terminal degradation products, since intact 26-kDa GST was formed from them after thrombin cleavage. Smaller C-terminal-truncated proteins have also been noted when HIV-1 $p55^{gag}$ polyprotein was expressed in *E. coli* (Luban and Goff, 1991).

A central question in retrovirology is how viral RNA is specifically recognized and packaged in virions. Given its nucleic acid binding characteristics, NCp presumably plays a significant role. HIV-1 NCp is initially expressed as part of a 55-kDa precursor polypepetide that is subsequently processed by the viral protease into the mature *gag* proteins. Since the *gag* polyprotein is involved in the initial assembly of HIV-1 (Gelderbloom, 1991, Gottlinger et al., 1990; Gottlinger et al., 1991), it is likely that $p55^{gag}$ is involved

in the selection and packaging of retroviral RNA. Recent studies have demonstrated specific binding of viral genomic RNA to HIV-1 $p55^{gag}$ *in vitro* (Luban and Goff, 1991). The function of processed p15 NCp is less certain. It has been proposed that within the viral particles, the 2000-3000 copies of nucleocapsid bind to dimeric retroviral RNA (Darlix et al., 1990; Karpel et al., 1987), implying a histone-like function. Studies using avian myeloblastosis virus (AMV) nucleocapsid indicated that the binding size for NCp is approximately 5-6 nucleic acid residues (Karpel et al., 1987), indicating that approximately 2000 copies of NCp could bind to 10 kb genomic RNA (Karpel et al., 1987; Meric et al., 1984). Such binding would imply a lack of specificity in the interaction of mature processed NCp with RNA. Although AMV NCp may lack specificity in its interaction with RNA, our data indicate that the binding of HIV-1 NCp15 to HIV-1 genomic RNA involves substantial sequence specificity, similar to that reported for the $p55^{gag}$ polyprotein (Luban and Goff, 1991). Interestingly, the GST-NCp fusion protein showed no specificity in the binding of RNA (Table 2), implying that the tertiary conformation of NCp may play an important role in the specificity of NCp-RNA interactions. The lack of specificity in RNA binding by NCp-GST is unlikely due to incorrect refolding of this protein during renaturation, since both GST-NCp and NCp15 bound equivalent amounts of RNA-Pst (Table 2). However, quantitative studies concerning binding of RNA to GST-NCp and NCp15 in solution will be required to adequately resolve this question. The largest transcript used in our studies (RNA-Pst) contains the previously identified packaging sequence, as well as a large portion of the *gag* coding region. Deletion of about 200 nucleotides from the 3'-end of the latter region significantly diminished binding by NCp15, but did not affect binding by GST-NCp. We do not yet know whether NCp15 binds directly to RNA sequences in this region, or whether these sequences are

necessary to maintain RNA in a configuration appropriate for specific binding by NCp15. In support of the latter possibility, heating denaturation of the RNA transcript prior to incubation with NCp15 decreased binding by about 50%.

As noted with HIV p55^{gag} (Luban and Goff, 1991), HIV-1 NCp15 bound RNA over a wide pH range (pH 4-8). This contrasts significantly with Rous sarcoma virus p12 NCp, which binds RNA only over a narrow pH range (pH 6-7) (Meric et al., 1984). We also found that K^+ was essential for the binding of RNA either to GST-NCp or NCp15. Neither lithium nor sodium, nor divalent cations such as Mg^{2+}, could replace potassium in this respect. Interestingly, although Zn^{2+} and Cd^{2+} were important for the isolation of undegraded NCp, only Zn^{2+} and not Cd^{2+} appeared to be necessary for the interaction of HIV-1 NCp with RNA when the protein was renatured in the absence of any divalent metal ion. This positive effect of Zn^{2+} may be due in part to assistance of this metal in the continued refolding of NCp15 during the subsequent incubation with RNA.

In conclusion, we have successfully cloned and expressed HIV-1 NCp15 in a biologically active form. The RNA binding characteristics of NCp15 are strikingly similar to those described for recombinant p55^{gag} polyprotein, especially with respect to the specificity of NCp-RNA interactions. This specificity appears to be a function of NCp conformation, since the GST-NCp fusion recombinant shows no RNA binding specificity. This implies that the conformation of NCp sequences in the p55^{gag} polyprotein must be remarkably similar to that of mature processed 15 kDa NCp. We have also identified an additional region with nucleotides in 1244-1412 of the HIV-1 sequence that appears to be necessary for strong binding to NCp. Other transcripts, even those containing previously identified packaging sequences (nt 288-344), were bound only weakly by NCp.

CHAPTER 3

HIV-1 NUCLEOCAPSID PROTEIN (NCP7) DIRECTS SPECIFIC INITIATION OF MINUS STRAND DNA SYNTHESIS PRIMED BY HUMAN TRNALYS.3 IN VITRO

This chapter was adapted from an article submitted to the Journal of Virology. The autors of this paper were Xuguang Li, Yudong Quan, Eric J Arts, Zhou Li, Bradley D. Preston, Hugues de Rocquigny, Bernard P. Roques, Lawrence Kleiman, Michael A. Parniak and Mark A. Wainberg. This work was largely performed by myself under the supervision of Drs. Wainberg, Parniak and Kleiman. Drs. Y. Quan and E. Arts helped in devising the experiments and analyzing the results. Dr. B. Preston provided recombinant NCp7 and critical review of the manuscript. Drs. De Rocquigny and B. Roques provided synthetic NCp7.

3.1 PREFACE TO CHAPTER 3

In Chapter 2, we have investigated the role of the larger form of HIV-1 nucleocapsid protein (NCp15) in binding to HIV-1 RNA. One of the functional activities of nucleocapsid protein is its ability to promote reverse transcription. As stated in chapter 1, processing of the larger form of NCp (NCp15) is dependent on viral RNA and most likely occurs in the released, mature virions. It is of interest to study the fully-processed form of nucleocapsid, i.e., NCp7, for its role in reverse transcription.

3.2 ABSTRACT

Retroviral reverse transcription starts near the 5' end of unspliced viral RNA at a sequence called the primer binding site (PBS), at which the tRNA primer anneals to the RNA template for initiation of DNA synthesis. We have investigated the role of NCp7 in annealing of primer $tRNA^{Lys.3}$ to the PBS and in reverse transcriptase (RT) activity, using a cell-free reverse transcription reaction consisting of various 5' viral RNA templates, natural primer tRNALys.3 or synthetic primer, HIV-1 nucleocapsid protein (NCp7), and human immunodeficiency virus type 1(HIV-1) RT. In the presence of $tRNA^{Lys.3}$, NCp7 was found to stimulate synthesis of minus-strand strong-stop DNA [(-) ss DNA], consistent with previous reports. However, specific DNA synthesis was only observed at a NCp7: RNA ratio similar to that predicted to be present in virions. Moreover, at these concentrations, NCp7 inhibited synthesis of non-specific reverse transcribed DNA products, which are probably initiated due to self-priming by RNA templates. In contrast to results obtained with $tRNA^{Lys.3}$ as primer, NCp7 inhibited synthesis of (-) ss DNA products primed by a 18 nt ribonucleotide (rPR), complementary to the PBS, even though rPR can initiate synthesis of such material in the absence of pre-annealing with NCp7. Primer placement bandshift assays showed that NCp7 was necessary for efficient formation of the tRNA/RNA complex. In contrast, NCp7 was found to prevent formation of the rPR/RNA complex. Since NCp7 appears to exert opposite effects (annealing versus dissociation) on $tRNA^{Lys.3}$ and rPR substrates, the non-PBS binding regions of the $tRNA^{Lys.3}$ molecule may play a role in the annealing of tRNA to the template. We also investigated the roles of an A-rich loop upstream of the PBS, a 7 nt region immediately downstream of the PBS, and a 54 nt deletion further downstream of the PBS in interactions with $tRNA^{Lys.3}$. We

found that deletions in the 54 nt region, that may prevent formation of the U5/leader stem, prevented $tRNA^{Lys.3}$ placement and priming, while deletions in the A-rich loop or the 7 nt sequence had relatively minor effects in this regard.

3.3 INTRODUCTION

Both the 5' end of retroviral RNA and tRNA are highly structured, folding into several stems and loops. Therefore, partial unfolding of these molecules is prerequisite for annealing between them. Although denaturation of tRNA by viral nucleocapsid protein (NCp) has been observed by circulation dichroism spectroscopy, the mechanism of annealing between tRNA primer and its viral RNA template is not well understood, even if NCp has been shown to stimulate synthesis of initial DNA products. Therefore, it is important to use modified templates and primers to further elucidate the nature of these interactions and to understand their specificity.

Reverse transcription involves the conversion of retroviral single-stranded RNA into double-stranded DNA and is carried out by the virion-encoded enzyme, reverse transcriptase (RT). All retroviral RTs utilize host cell-derived tRNAs as an initiation primer but the specific type of tRNA employed may vary, depending on the virus (Litvak et al., 1993). In the case of HIV-1, this role is played by $tRNA^{Lys.3}$ (Jiang et al., 1993; Ratner et al., 1985).

The binding site for $tRNA^{Lys.3}$ is positioned about 180 nt from the 5' end of unspliced viral RNA i.e. a sequence termed the primer binding site (PBS) (Ratner et al., 1985; Rhim et al., 1991). The PBS is 18 nt long and is complementary to the 3' end of tRNALys.3. Computer modeling, in conjunction with chemical and enzymatic studies, suggests that the 5' region of viral RNA is highly structured and can fold into several stems and loops (Baudin et al., 1993; Berkhout et al., 1993; Darlix et al., 1980; Harrison et al., 1992). If left unfolded, these structures could potentially hinder annealing of primer tRNA to the viral RNA template. Likewise, although tRNA is uni-chained, a majority of bases are hydrogen-bonded to each other. Hairpin folds bring bases on the same chain

into a double-helical arrangement whereby short stretches of nt are complementary to each other. Only four of the 18 nt in $tRNA^{Lys.3}$, complementary to the PBS, are not base paired (Litvak et al., 1993). Thus, appropriate unwinding of both tRNA and the RNA template must be prerequisite for correct annealing between these structures in order for efficient reverse transcription to occur. It is unclear how this unwinding takes place, although reverse transcriptase (RT) may itself be able to disrupt secondary structure of the 5' viral RNA and the tRNA primer. Another viral protein of interest is the viral nucleocapsid protein (NCp), which bears strong similarity to other nucleic acid-binding proteins; notably it is highly basic and contains one or two copies of a conserved sequence, i.e. a so-called Cys-His box (Berg., J 1986; Bess et al., 1992; Fitagerald et al., 1991; South et al., 1990; Surovoy et al., 1993). Physicochemical studies suggest that NCp binds preferentially to single-stranded nucleic acids (Karpel., 1987; Surovpy et al., 1993) and unwinds primer tRNA in vitro (Khan and Giedroc, 1992). NCp also facilitates transition between single-stranded and double-stranded nucleic acids as well as renaturation of nucleic acids (Darlix et al., 1995; Khan and Giedroc et al., 1992; Tsuchihashi and Brown, 1994; You and McHenry, 1994), properties which could be responsible for stimulatory effects on strand transfer during DNA polymerization (Allain et al., 1994; Darlix et al., 1993; Rodriguez-Rodriguez et al., 1995; You and McHenry, 1994).

Addition of viral nucleocapsid protein (NCp) into reverse transcription reaction mixtures greatly stimulated synthesis of minus strand DNA, probably through enhancement of annealing between the tRNA primer and the viral RNA template (Barat et al., 1989; Prats et al., 1988). Interactions between the tRNA primer and the viral RNA template may not be limited to the 18-nt complementary regions between them, since sequences outside the PBS of

the viral RNA template are thought to be necessary for efficient initiation of retroviral reverse transcription (Aiyar et al., 1992; 1994; Cobrinik et al., 1988; 1991; Isel et al., 1993; 1995; Kohlsteadt and Steiz, 1992 Leis et al., 1993). For example, chemical and enzymatic footprint analysis showed that a conservative " A " rich loop, located 10nt upstream of the HIV-1 PBS, may be able to base pair with the anticodon loop of $tRNA^{Lys.3}$ (Iset et al., 1993; 1995); however, direct analysis of RT activity was not performed in this study. In separate experiments, six bases located downstream of the PBS were shown to be involved in specifying tRNA utilization (Kohlsteadt and Steiz et al., 1992), but the templates used were non-physiological and lacked significant secondary structure. Neither of these studies examined the role of NCp.

To further investigate this subject, we performed cell-free reverse transcription reactions that included various modified 5' HIV-1 RNA templates, nucleocapsid protein (NCp7), recombinant RT, human $tRNA^{Lys.3}$ or synthetic primer and radiolabeled substrates. Consistent with previous observations (Prats et al., 1922), NCp7 was found to stimulate minus-strand strong-stop DNA synthesis [(-) ssDNA] in our system. However, this effect was obtained only if the protein: nucleic acid ratio was one NCp7 molecule per 6-20 tRNA/RNA nt residues. Moreover, a maximal effect was obtained at a ratio of one NCp7 per 6 tRNA/RNA nt residues, i.e. close to the predicted NCp:nucleic acid ratio in virions (Karpel et al 1987; Khan and Giedroc, 1992). Remarkably, at these concentrations, NCp7 suppressed synthesis of non-specific reverse transcribed DNA products, which were probably initiated due to self-priming by RNA templates. We also found that synthesis of (-)ss DNA, initiated by a 18 nt ribo-oligonucleotide primer (rPR), complementary to the PBS, was inhibited by NCp7 in a concentration-dependent manner. Using a primer placement assay, NCp7 was found to be necessary for efficient formation of the $tRNA^{Lys.3}$-RNA

complex, but prevented formation of a rPR-RNA complex. Finally, a 54 nt sequence, located downstream of the PBS, was essential for efficient formation of the $tRNA^{Lys.3}$/RNA complex and synthesis of (-) ss DNA.

3.4 MATERIALS AND METHODS

3.4.1 Reagents

All chemicals were purchased from Sigma Inc. (St Louis, Mi) unless specified. Radioisotopes were obtained from Dupont Inc, (Mississauga, Ontario, Canada). Restriction enzymes, modifying enzymes and RNA^{guard} (RNase inhibitor) were obtained from Pharmacia, Inc. (Montreal, Qc). $tRNA^{Lys.3}$ was purified from human placenta as described (Jiang et al., 1993). The 18-mer ribooligonucleotide (rPR), complementary to the PBS, was obtained from General Synthesis Diagnosis, Inc. (Toronto, ON). The sequence of the synthetic rPR is 5'- GUCCCUGUUCGGGCGCCA-3'. HIV-1 RT (p66/p51) was a gift of Dr. Stuart F J Le Grice of Case Western Reserve University, Cleveland, OH. HIV-1 nucleocapsid protein (NCp7, 72 amino acids in length) was chemically synthesized as described (De Rocquigney et al., 1991).

3.4.2 Construction of RNA expression plasmids and preparation of HIV-1 RNA transcripts

Construction of PBS/WT, a plasmid for making a 483-nt 5' HIV-1 RNA transcript comprising R, U5, PBS, and part of the gag encoding region, has been described (Arts et al., 1994), as has the PBS (-) plasmid (with a complete deletion of the 18-nt PBS) (Li et al., 1994). Deletions of either four nt, i.e., "AAAA", 10 nt upstream of the PBS, positions 169-172) (Ratner et al., 1985) or seven nt, immediately downstream of the PBS (positions 202-208 in PBS/WT) (Ratner et al., 1985) were

made by a modified PCR-based mega-primer mutagenesis method (Picard et al., 1995) and designated PBS/del-A and PBS/del-7, respectively. A deletion of 54 nt immediately downstream of the PBS (positions 202-255) (Ratner et al., 1985) was generated by cutting PBS-WT with Nar I and BssH II, following which the PBS was rebuilt using the same mutagenesis strategy described above. Templates containing this modification were designated PBS/del-LD. For RNA transcript preparations, plasmids were linearized by AccI and used as templates in an Ambion Mega-scripts kit (Austin, TX) according to the manufacturer's instructions. The integrity of RNA transcripts was routinely checked using a denaturing gel (5% polyacrylamide/7 M urea) before their use in reverse transcription assays.

3.4.3 Reverse transcription

Reverse transcription reactions (unless otherwise specified) were performed in a volume of 10 µl containing 50 mM Tris-Cl, pH 7.2, 50 mM KCl, 5 mM MgCl2, 10 mM DTT, 200 µM dATP, dGTP and dTTP, 50 µM dCTP, 1.5 ml of α-^{32}p labelled dCTP (specific activity 3000 Ci/mmol), 5 units of RNAguard, 100 nM of RNA template, 100 nM of primer (either tRNALys.3 or rPR), and various amounts of NCp7 depending on the individual experiment. NCp7 had been prepared by either peptide synthesis (De Rocquigny et al., 1991) or by expression of recombinant clones (Ji et al., 1996) as described; identical results were obtained with both preparations in these studies. Reaction mixtures were preincubated at 37°C for 30' prior to addition of RT (final concentration 50 nM). The reaction mix was then incubated at 37°C for 15'-30' before termination by addition of EDTA (final concentration 50 mM). These reactions yielded linear results up to 60 min after initiation, most likely due to continuous denaturation and

refolding of the nucleic acids involved. After phenol/chloroform extraction, the reaction products were precipitated with ethanol and boiled for 5 min in formamide denaturing buffer, before being fractionated in a 5% denaturing polyacrylamide gel containing 7 M urea. Finally, the gel was dried and exposed to Kodak film at -70°C. In some cases, the reaction products were treated with NaOH to remove the RNA primer before being fractionated on a denaturing gel (Blain et al., 1995).

3.4.4 Placement of primers onto RNA template by NCp7

5' end-labelled primer (tRNA or rPR) was incubated with RNA template in the absence of dNTPs but in the presence of various amounts of NCp7 under the same reverse transcription conditions described above. Reaction products were treated with 200 µg/ml of proteinase K at 37°C for 30 min followed by extraction with phenol/chloroform. To the resultant aqueous phase, we added an equal volume of 2X sample loading buffer, consisting of 50 mM Tris-Cl, pH7.2, 25 mM EDTA, 25% glycerol, 2% SDS, 0.01% bromphenol blue (You and McHenry, 1994). This was followed by fractionation on a 1.5% agarose gel. The gel was dried and exposed to Kodak film at -70°C.

3.5 RESULTS

3.5.1 Effects of NCp7 on reverse transcription primed by human $tRNA^{Lys.3}$

To investigate the effects of NCp7 on (-) ss DNA synthesis, we reconstituted a reverse transcription reaction consisting of HIV-1 template, human tRNALys.3, RT, and NCp7. As shown in Fig 1, the template and tRNA were preincubated with various concentrations of NCp7 prior to addition of enzyme. The final product is 259 nt in length, consisting of (-) DNA (183 nt) and the attached primer $tRNA^{Lys.3}$ (76 nt) at the 5' end. Fig. 2 shows that (-) strand ss DNA was clearly detected when NCp7 was present at concentrations of 14, 9.3, 6.2, 4.1, and 2.7 μM. This corresponds to a ratio of one NCp7 molecule to 4, 6, 9, 14, and 21 nucleotide residues, respectively (lanes 1, 2, 3, 4, 5). The strongest (-) ss DNA bands were observed at a NCp: nt ratio of 1:6 (lane 2). Bands smaller than 259 nt probably represent incomplete DNA products (Arts et al., 1994; Huber et al., 1989; Klarmann et al., 1993; Li et al., 1994). Note that no specific (-) ss DNA products were detected when a template deleted of the PBS [i.e. PBS(-)] was used in either the presence of NCp7 (one NCp per 4 nucleotides, lane 9) or its absence (lane 10). Indeed, the 259 nt (-)ss DNA product was never present under these conditions, no matter how much NCp7 or $tRNA^{Lys.3}$ was added (data not shown). Thus, the 259 nt reverse transcribed DNA product results from tRNA priming at the PBS. Unexpectedly, we found that non-specific reverse-transcribed DNA products (mostly >259 nt) accumulated when lower concentrations of NCp7 were employed (lanes 4, 5, 6, 7). Indeed, maximal amounts of these DNA products were detected in the absence of NCp7 (lane 8). An equivalent amount of non-specific DNA product to that in reactions containing $tRNA^{Lys.3}$ (lane 8) was also detected in reactions that contained PBS/WT template, RT and dNTPs but excluded NCp7 and tRNALys.3 (lane 12). This suggests that these products might be due to self-priming of the RNA template itself, and that this could be inhibited by NCp7 (lane 11). Pre-treatment of RNA template with cytidine 3', 5'-bisphosphate (pCp) (Jiang et al.,

1993) to block the hydroxyl group at its 3' end, or denaturing the RNA template (92°C for 2 min, followed by quick chilling on ice), before adding it back into the reverse transcription reaction resulted in the disappearance of non-specific DNA products (data not shown). These observations provide additional evidence that synthesis of non-specific reverse transcribed products was due to self-priming of the RNA template. The fact that other investigators did not observe non-specific reverse transcribed DNA products may be due to their use of 5' end labelled tRNA (Barat et al., 1989; Prats et al., 1988; Weis et al., 1992), rather than our use of radiolabeled dNTPs to enhance sensitivity. Overall, we found that increased concentrations of NCp7 led to decreased accumulation of non-specific reverse transcribed DNA products and increased generation of specific (-) strand ss DNA (lanes 1-8).

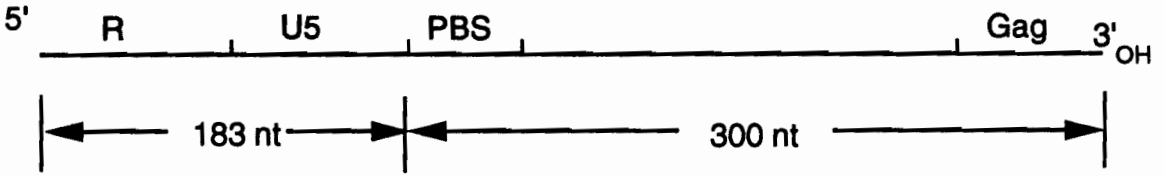
Fig. 1: Graphic description of reverse transcription reaction:

1. The RNA template consists of R, U5, PBS, and a 300 nt 3' flanking sequence. The position and size of each region are indicated.

2. RNA template and $tRNA^{Lys.3}$ were pre-incubated with variable amounts of NCp7 at 37°C for 30', followed by addition of RT (final concentration, 50 nM) and additional incubation at 37°C for up to 30'. Reactions were terminated and products fractionated on 5% polyacrylamide gels containing 7 M urea.

3. The expected full-length (-) ss DNA products initiated by $tRNA^{Lys.3}$ consist of a 183 nt (-) cDNA and a 76 nt (-) tRNA. The use of an 18-nt ribonocleotide (rPR) (complementary to the PBS) as primer instead of tRNALys.3 is expected to yield full-length (-) ss DNA of 183 nt and a 18 nt dPR.

1 5' viral RNA template consisting of R, U5, PBS, and a 300 nt 3' flanking sequence



2 Procedure for reverse transcription :

Preincubation:

RNA template + tRNA + NCp7

37°C, 30 min

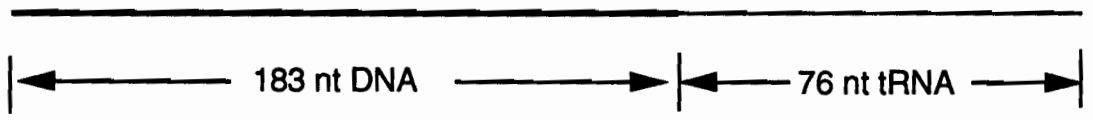
+

RT

37°C, 60 min

Termination of reaction and analysis of products by denaturing gel

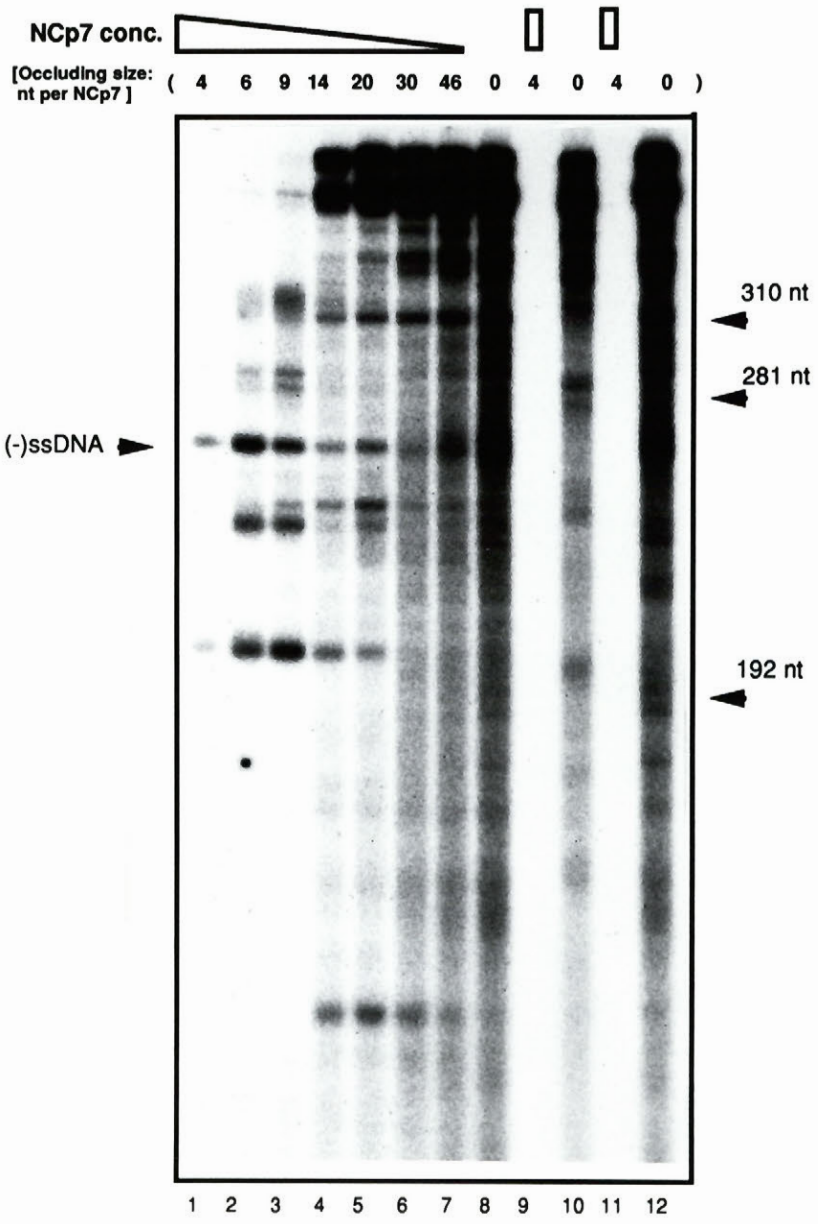
3. The expected minus-strand strong-stop DNA products [(-)ss DNA] include the 183-nt reverse transcribed DNA and the attached 76 nt tRNA



(Length of (-) ss DNA: 183 + 76 = 259 nt)

Fig. 2: Effect of NCp7 concentration on DNA synthesis.

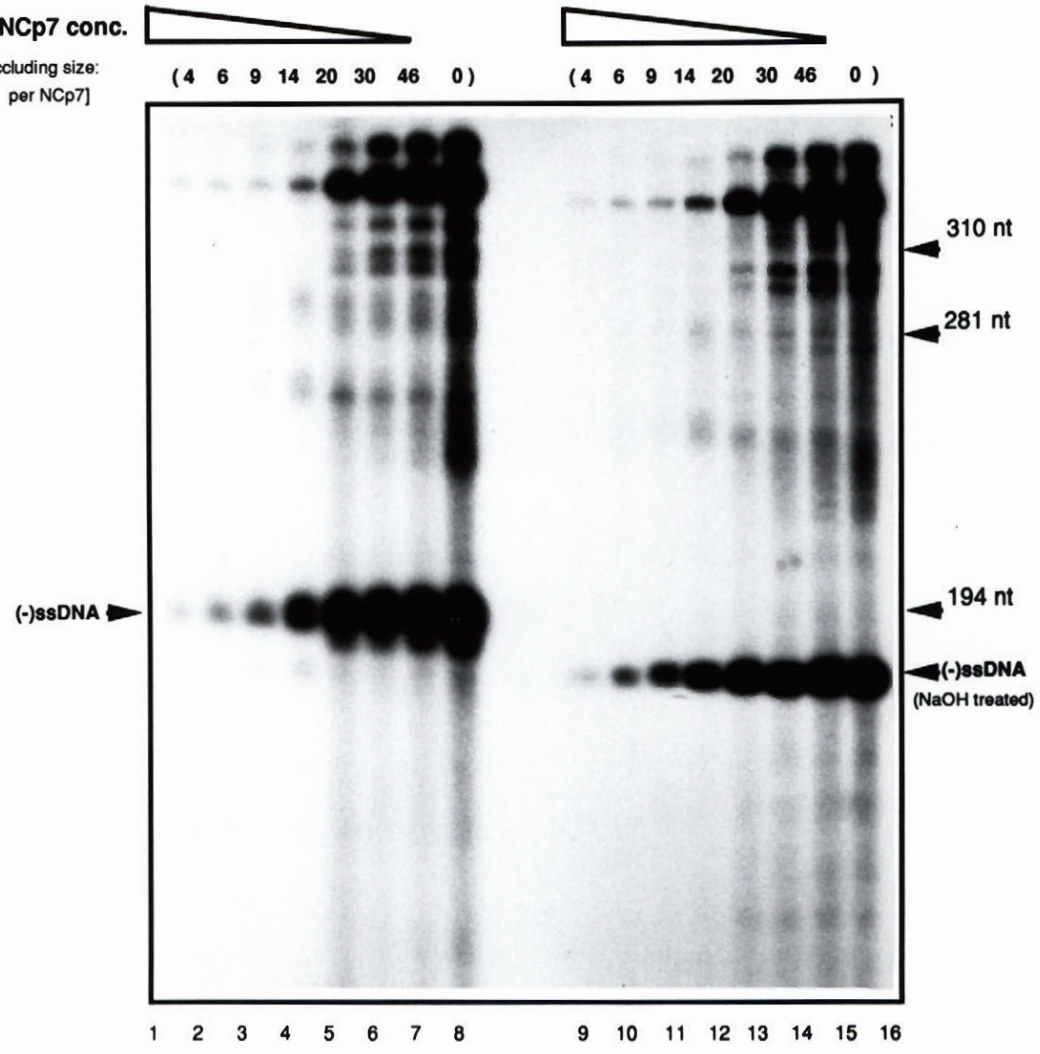
Lanes 1-7 represent reverse transcription reactions performed with wild-type template (PBS/WT), tRNALys.3, RT and decreasing amounts of NCp7, i.e., 14, 9.3, 6.2, 4.1, 2.7, 1.8, and 1.2 μM(corresponding to one NCp7 molecule per 4, 6, 9, 14, 20, 30, and 46 nt residues). Lane 8 represents a reaction performed without NCp7. Lane 9 shows results from a reaction that included PBS (-) template, $tRNA^{Lys.3}$, RT and 14 M NCp7 (one NCp7 for 4 nucleotide residues). Lane 10 included only PBS (-) template and RT. Lane 11 included PBS/WT template, RT, and 12 μM NCp7 to achieve a ratio of one NCp7 per 4 nt (in order to compare with lane 1 in which $tRNA^{Lys.3}$ was added). Lane 12 contained PBS/WT template and RT. No reverse transcribed products were detected in a control experiment performed with $tRNA^{Lys.3}$, RT, and dNTP, but without RNA template and NCp.


NCp7 conc.
[Occluding size: nt per NCp7]
(4 6 9 14 20 30 46 0 4 0 4 0)
310 nt
281 nt
(-)ssDNA
192 nt
1 2 3 4 5 6 7 8 9 10 11 12

3.5.2. Inhibition effect of NCp7 on reverse transcription primed by either viral template or by ribooligonucleotide (rPR) complementary to the PBS.

We next investigated reverse transcription using a 18-mer ribooligonucleotide (rPR) complementary to the PBS, as primer in the place of $tRNA^{Lys.3}$. In this instance, NCp7 displayed dose-dependent inhibitory rather than stimulatory effects on rPR-primed synthesis of (-) ss DNA(Fig. 3, lanes 1-8). Indeed, the strongest (-) ss DNA signal was obtained in the absence of NCp7 (Fig. 3, lane 8). Fig. 3 also shows that NCp7 inhibited formation of non-specific DNA products of reverse transcription (lanes 1-8), as described above. No specific (-) ss DNA products were detected, if PBS (-) template was used in place of the PBS-containing PBS/WT template in the reverse transcription reaction (not shown). Treatment of the reaction products with NaOH to digest the RNA portion of the reaction products resulted in a decreased size of (-) ss DNA(approximately 18 nt shorter), confirming that these products were indeed initiated by rPR (Fig. 3, lanes 9-16). The decreased size of non-specific DNA products after NaOH treatment also confirmed that these products were due to self-priming of RNA templates (Fig. 3, lanes 9-16).Thus, NCp7 appears to inhibit formation of reverse transcribed DNA products initiated either by rPR or by RNA template.

Fig. 3: Effect of NCp7 concentration on rPR initiated reverse transcription. Lanes 1-8 represent experiments performed with PBS/WT template, rPR, RT and decreasing amounts of NCp7, i.e., 12.5, 8.3, 5.5, 3.7, 2.4, 1.6, and 1 μM, to achieve the same ratios of NCp:nt described in Fig. 2. The experiment in lane 8 was performed without NCp7. Lanes 9-16 represent the same reactions as lanes 1-8, but with NaOH treatment of reaction products to digest the RNA portion of the reaction products (Blain et al., 1995).


NCp7 conc.
[Occluding size:
nt per NCp7]
(4 6 9 14 20 30 46 0)
(4 6 9 14 20 30 46 0)
310 nt
281 nt
194 nt
(-)ssDNA
(-)ssDNA
(NaOH treated)
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16

3.5.3 Effect of NCp7 on placement of primers onto RNA template

To initiate synthesis of (-) ss DNA, the primer must be annealed onto the primer binding site (PBS) of the RNA template. Since NCp7 had differential effects on tRNALys.3 versus rPR-initiated DNA synthesis, it was of interest to investigate how NCp7 could influence annealing between primer and RNA template. Toward this end, 5' end-labelled primer was incubated with RNA template and NCp7, under the same conditions as that of reverse transcription reactions (see Materials and Methods). Following incubation at 37°C for 30 min, the reaction complex was fractionated on a 1.5% agarose gel. As expected (Prats et al., 1988), increasing concentrations of NCp7 gave rise to increasingly intense signals representing the tRNALys.3/RNA complex (Fig.4). In the absence of NCp7, barely detectable annealing was observed (lane 8). The amount of NCp7 needed for optimal annealing between $tRNA^{Lys.3}$ and the RNA template, i.e. one NCp per 6-20 nt (Fig.4), correlated reasonably well with the amount of NCp7 required for specific $tRNA^{Lys.3}$ primed synthesis of (-) ss DNA (Fig.2). No annealing of $tRNA^{Lys.3}$ onto the template was observed when the PBS (-) RNA template was used in a control reaction (not shown here but shown below). The addition of RT, BSA or other basic proteins, e.g., histone, into the primer placement reaction did not result in significant changes with regard to formation of the primer/template complex. This shows that the effects of NCp7 on formation of the primer/template complex are specific. In contrast to the results of the primer placement experiment with $tRNA^{Lys.3}$, different findings were obtained if rPR was used as primer (Fig. 5). In this case, increasing concentrations of NCp7 prevented formation of a complex between rPR and the RNA template. In the absence of NCp7, the RNA template annealed less

efficiently to tRNA (Fig. 5, lane 8) than to rPR (Fig. 6b, lane 8). The ability of NCp7 to inhibit annealing between rPR and viral RNA correlated with its inhibitory effect on synthesis of (-) ss DNA (Fig. 3).

Fig. 4: Effect of NCp7 concentration on formation of complexes between tRNALys.3 and RNA template. Lanes 1-8 represent experiments performed with RNA template, 5' end labeled tRNALys.3, and decreasing concentrations of NCp7, i.e., 14, 9.3, 6.2, 4.1, 2.7, 1.8, and 1.2 μM. Lane 8 excluded NCp7. Lane 9 is a 5'-end-labeled tRNALys.3 control. The presence of the tRNA/RNA complex is indicated. Note that the upper bands represent complexes between tRNA and the dimeric RNA template, while the lower bands represent complexes between tRNA and monomeric RNA (Lapadat-Tapolsky et al., 1995; Prats et al., 1988).

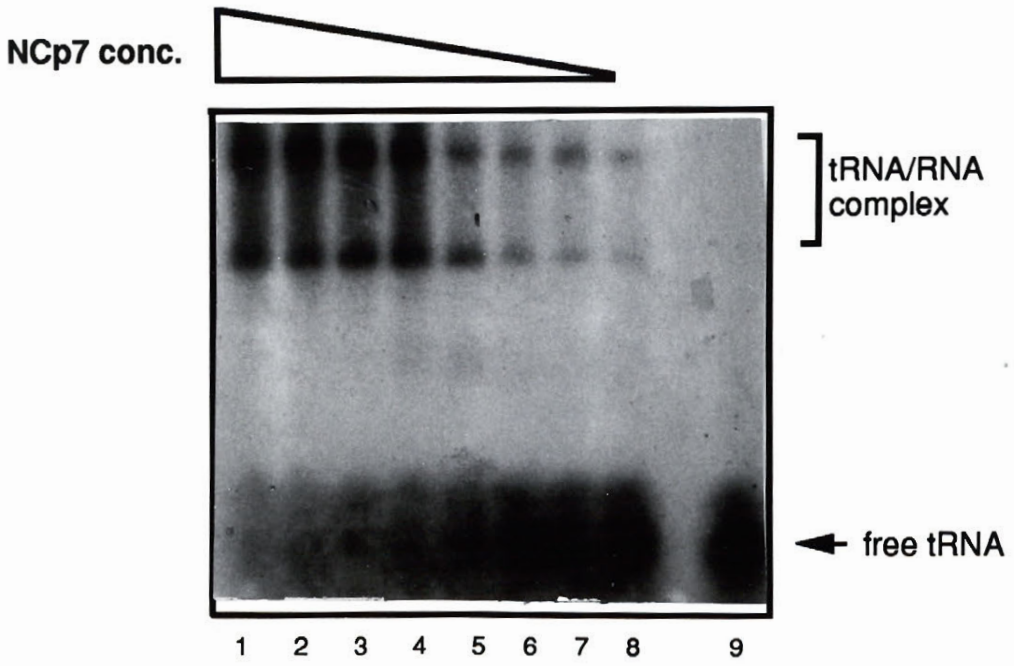
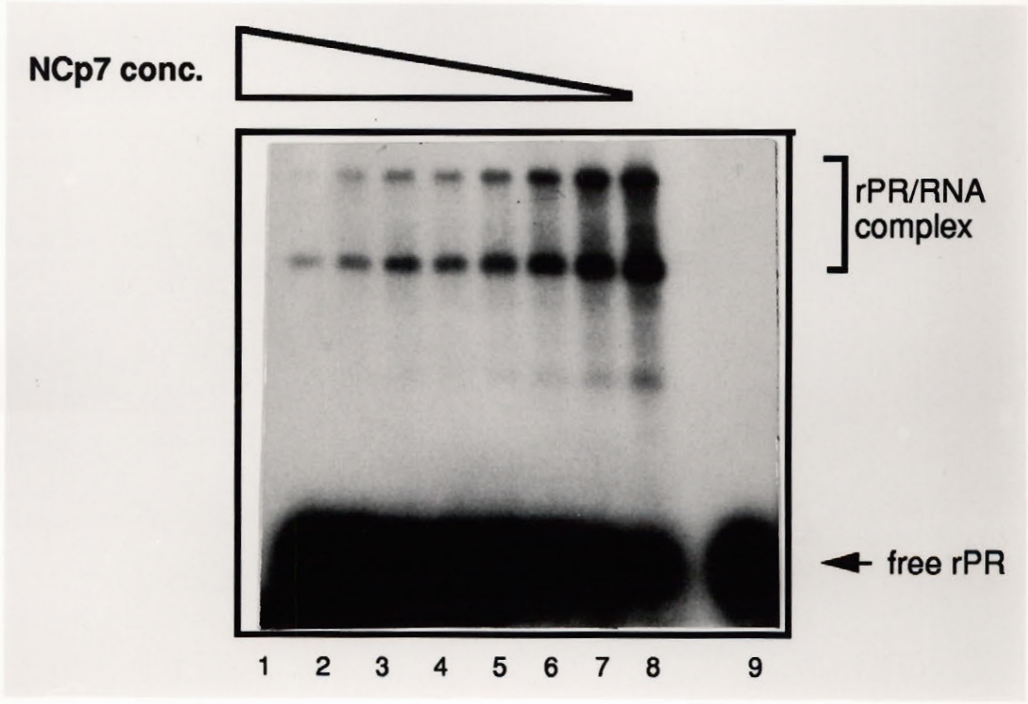

NCp7 conc.
tRNA/RNA
complex
free tRNA
1 2 3 4 5 6 7 8 9

Fig. 5: Effect of NCp7 concentration on formation of complexes between rPR and the RNA template. Lanes 1-8 represent results from reactions including RNA template, 5'-end-labeled rPR, and decreasing amounts of NCp7, i.e., 12.5, 8.3, 5.5, 3.7, 2.4, 1.6, 1 μM.


NCp7 conc.
rPR/RNA
complex
free rPR
1 2 3 4 5 6 7 8 9

3.5.4 Effects of NCp7 on reverse transcription involving mutant RNA template

Previous investigations have shown that HIV RNA sequences that flank the PBS may be important in interactions between tRNA and the RNA template but did not examine the potential role of NCp7 in this regard (Isel et al., 1993; 1995; Kohlsteadt et al., 1992). To test how NCp7 might influence such interactions, we created RNA templates that contained deletions of the A-rich loop (PBS/del-A) (nt 69-172) or of seven nucleotides located immediately downstream of the PBS (PBS/del-7) (nt 202-208). In addition, a deletion of 54 nt, located immediately downstream of the PBS, (PBS/del-LD) (nt 202-255), was constructed to disrupt the U5/leader "stem" of viral RNA (Leis et al., 1993). Fig. 6A shows the results of reverse transcription reactions performed with these mutant RNA templates using $tRNA^{Lys.3}$ as primer. As expected, if the template contained a deletion in the PBS, i.e., PBS (-) template, no (-) ss DNA was generated (lane 1). Furthermore, within the time frame of these experiments (15-30 min), del-A and del-7 had only a minor effect on tRNALys.3 placement and priming. Deletion of the A-rich loop (PBS/del-A) or of a 7-nt sequence, downstream of the PBS, (PBS/del 7), had a minor effect on synthesis of (-) ss DNA (lanes 3, 4) compared to wild-type (lane 2). However, the larger deletion located downstream of the PBS (PBS/del-LD) resulted in a significant reduction in amount of (-) ss DNA. This result was obtained in each of three separate experiments. These data were further confirmed by testing these mutant templates in time course reverse transcription assays (not shown).

To further investigate the mechanisms involved, tRNA primer placement studies were performed. As expected, no significant differences regarding formation of a tRNA/RNA template complex, promoted by NCp7, were

observed among reactions performed with PBS/WT, PBS/del-A and PBS/del-7 (Fig. 6B, lanes 2, 3, and 4). However, less primer/template complex was formed if PBS/del-LD was employed (lane 5), while no primer/template could be detected in the case of the PBS(-) template (lane 1). Similar results were obtained when NCp7 concentrations in these reactions corresponded to one NCp7 molecule per 4, 8 or 14 nt residues (data not shown).

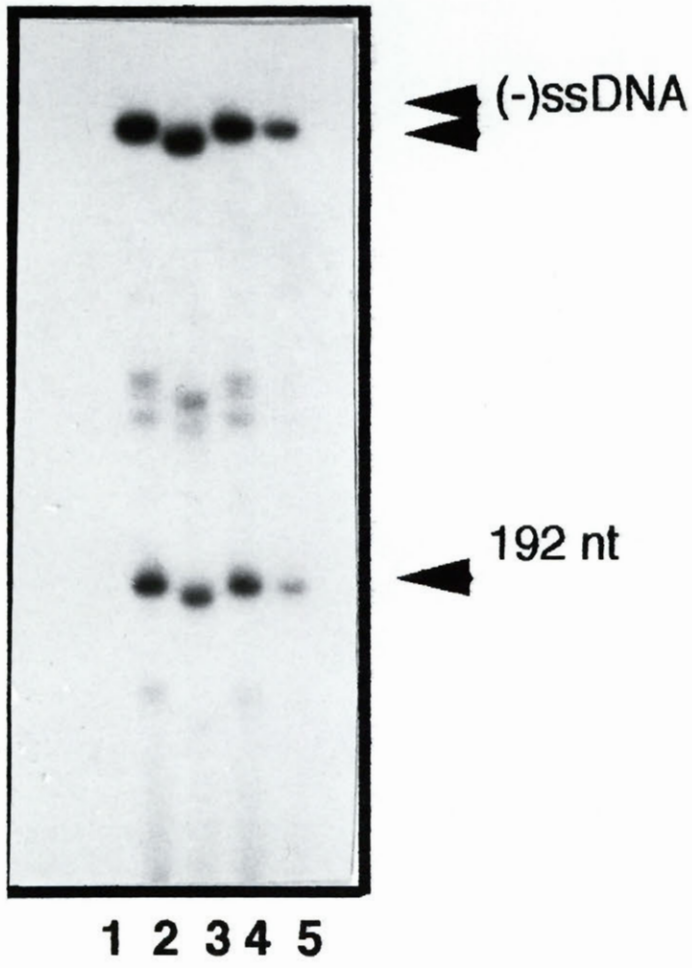
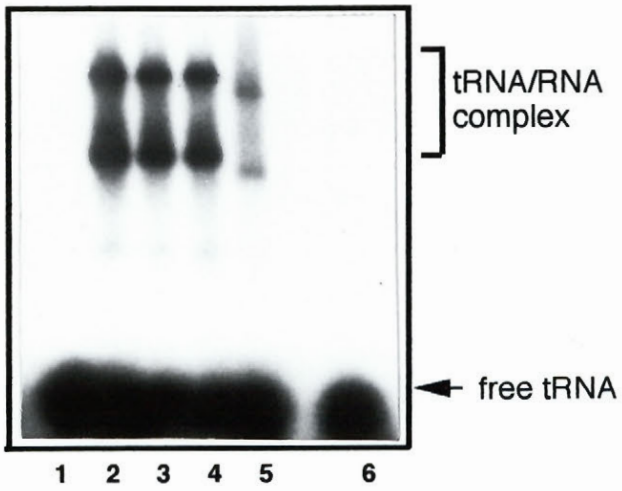

(-)ssDNA
192 nt
1 2 3 4 5

Fig.6B: Effect of NCp7 on formation of complexes between $tRNA^{Lys.3}$ and various RNA templates. Lane 1: PBS (-); lane 2: PBS/WT; lane 3: PBS/del-A; lane 4: PBS/del-7; lane 5: PBS/del-LD; lane 6: 5'-end-labeled $tRNA^{Lys.3}$ as a control marker.


tRNA/RNA
complex
free tRNA
1 2 3 4 5 6

3.6 DISCUSSION

Initiation of retroviral reverse transcription requires primer tRNA to be annealed to the 5' end of the viral RNA template at a region called the PBS. As stated above, both tRNA and the PBS-containing 5' end of the viral RNA are highly structured and fold into several stems and loops (Baudin et al., 1992; Berkhout et al., 1993; Harrison et al., 1992; Darlix et al., 1980). Therefore, appropriate unfolding or denaturation of these structures is necessary for annealing between the tRNA primer and the RNA template to occur. The fact that little RT-catalyzed reverse transcribed DNA products were observed in the absence of other proteins, suggesting the enzyme alone may not be sufficient for destabilizing secondary structures of these nucleic acid sequences (Alain et al., 1994; Barat et al., 1989; Prats et al., 1988; Weis et al., 1992). While it is likely that NCp7 may melt tRNA by virtue of its high affinity for single-stranded RNA (Karpel et al., 1987; Khan et al., 1992), the nature of the annealing between tRNA and the viral RNA template is not well understood.

Interactions between the viral RNA template and tRNALys.3 are not limited to base-pairing between the PBS and a 18 nt segment at the 3' end of the latter molecule (Aiyar et al., 1992; 1994; Isel et al., 1993; 1995; Kohlsteadt et al., 1992). It is unclear whether NCp7 can induce melting of viral template RNA, that possesses extensive secondary and tertiary structure, in spite of its ability to facilitate transitions between single- and double-stranded DNA (Tsuchihashi and Brown, 1994). The use of altered RNA templates and primers lead themselves to these issues.

We have both confirmed and extended previous observations (Alain et al., 1994; Barat et al., 1989; Prats et al., 1988; Weis et al., 1992), by using a cell-free reverse transcription assay consisting of 5' viral RNA, tRNALys.3, RT and

NCp7. This system also enabled us to study primer placement. Consistent with the data of others, we found that NCp7 stimulated generation of (-) strand ss DNA (Alain et al., 1994; Barat et al., 1989; Prats et al., 1988; Weis et al., 1992). A novel observation, however, is that NCp7 conferred exquisite specificity to reverse transcription, as shown by the supression of non-specific initiation from self-primed RNAs and the concomitant stimulation of specific initiation by tRNALys.3. Moreover, the most efficient generation of specific (-) ss DNA occurred at NCp concentrations close to those predicted to be present in virions (Karpel et al., 1987; Khan et al., 1992). The fact that previous investigators failed to observe non-specific DNA products of RT reactions may be due to their use of less sensitive conditions, e.g. use of end-labeled tRNA primer rather than radiolabeled dNTPs as employed in this study.

Self-priming most likely resulted because the 3' end of the RNA template folded back to initiate DNA synthesis. This may be due to a tendency of the RNA template to form secondary structure (Baudin et al., 1993; Harrison et al., 1992). NCp7-mediated inhibition of synthesis of non-specific DNA may be due to destabilization of the RNA helix (Herschlag et al., 1994; Ji et al., 1996; Khan et al., 1992; Tsuchihashi and Brown, 1994), and/or the coating of viral template RNA in a manner that renders it non-recognizable by RT. Further work will be necessary to clarify the mechanisms involved as well as to determine whether NCp7 might inhibit the generation of non-specific DNA in vivo. Virion genomic RNA contains nicks that might initiate synthesis of non-specific reverse transcribed products (Coffin, 1979; 1985; Darlix et al., 1995; Tanchou et al., 1995; Temin, 1993).

In sharp contrast to tRNALys.3 primed synthesis of (-) ss DNA, reverse transcription initiated by the 18 nt rPR complementary to the PBS was inhibited by NCp7 in a dose-dependent manner (Fig. 3). This result was

apparently due to NCp7-mediated prevention of annealing between rPR and the viral RNA template (Fig. 5). Indeed, in the absence of NCp7, rPR was modestly annealed onto the RNA template during an incubation period of 30 min at 37°C. In contrast, little $tRNA^{Lys.3}$ was found to be annealed with the RNA template in the absence of NCp7, and addition of NCp7 stimulated this process more than 50-fold (molecular imaging analysis not shown) (Fig.4). Therefore, it appears that the remaining portion of the tRNA molecule (i.e. sequences other than the 18 nt that base-pair with the PBS) might be the driving force for an equilibrium that favours formation of a stable tRNA/RNA duplex mediated by NCp7. This might occur through interaction with sequences outside the PBS.

Removal of the remaining portion of the tRNALys.3 molecule (as in the case of rPR) resulted in poor hybridization between rPR and the RNA template in the presence of NCp7. Thus, NCp7 apparently confers specificity by stabilizing and/or mediating the formation of the $tRNA^{Lys.3}$/RNA complex that is recognized by RT for initiation of (-)ssDNA synthesis. Indeed, mutant retroviruses, containing a PBS replaced by sequences complementary to other tRNAs, reverted back to wild-type in culture, even though these mutant viruses can use alternate tRNAs as replication primers during early stages of infection (Das et al., 1995; Li et al., 1994; Wakefield et al., 1995; Whitcomb et al., 1995). These results indicate that the NCp-mediated configuration of the wild-type tRNA/RNA complex is essential for recognition by RT and that NCp can cause more efficient annealing between viral template RNA and $tRNA^{Lys.3}$ than between viral RNA and other tRNA isoacceptor species. Protein-protein interactions involving both RT and NCp7 may also be important in this regard (Khan et al., 1992; Tanchou et al., 1995).

Recent genetic and biochemical evidence suggests that interactions at multiple sites between tRNA and 5' viral RNA (not merely complementary

sequences between the PBS and the 3' end of the tRNA primer) may be required for efficient reverse transcription (Aiyar et al., 1992; 1994; Isel et al., 1993; 1993; Kohlsteadt et al., 1992). In the avian retroviral system, interactions between the T C loop of tRNAPro and sequences located upstream of the PBS can help to stabilize the tRNA/RNA complex (Aiyar et al., 1992; 1994). In HIV-1, the A-rich loop located upstream of the PBS and a 6-nt sequence located immediately downstream of the PBS have been shown to interact with the anticodon region of $tRNA^{Lys.3}$ (Isel et al., 1993; Kohlsteadt et al., 1993). In our system, deletion of either of these regions had little effect on synthesis of (-) ss DNA. Any discrepancies in results might be attributable to the use of different viral strains and/or experimental conditions (Isel et al., 1993; Kohlsteadt et al., 1993). However, we found that a larger deletion, located downstream of the PBS (PBS/del-LD), did result in decreased formation of the primer/template complex as well as as synthesis of (-) ss DNA (Fig. 6). We have also tested this modification in the context of an infectious molecular clone, and found that it greatly reduced infectivity, while the other two deletions tested, i.e., PBS/del-A and PBS/del-7, had little or no effect in this regard (submitted for publication). Determination of nascent reverse transcribed DNA products from infected cells using a quantitative PCR revealed no differences among PBS/del-A, PBS/del-7 and wild-type virus, but a > 10-fold reduction in DNA sythesis was observed in the case of the PBS/del-LD mutants. We are currently investigating the role of the latter region in maintaining an appropriate configuration of template and in whether specific sequences within it are necessary for direct interaction with $tRNA^{Lys.3}$.

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CHAPTER 4

EFFECTS OF ALTERATIONS OF PRIMER-BINDING SITE SEQUENCES ON HIV-1 REPLICATION

This chapter was adapted from an article that appeared in the Journal of Virology (1994), Vol 68 (10), pp. 6198-6206. The authors of this paper were X. Li, J. Mak, E. J. Arts, Z. Gu, L. Kleiman, M. A. Wainberg, and M. A. Parniak. This work was largely performed by X. Li under the supervision of Drs Wainberg and Parniak. J. Mak analyzed tRNA species in the virion. Drs E. Arts, Z. Gu and L. Kleiman helped in devising the experiments and analyzing the results,

4.1 PREFACE TO CHAPTER 4

In Chapter 3, we have investigated the effects of HIV-1 nucleocapsid protein (NCp7) on initiation of reverse transcription. We found NCp7 is essential for efficient and specific generation of minus strand DNA primed by human $tRNA^{Lys.3}$. Sequences surrounding the PBS are important for efficient initiation of reverse transcription. HIV-1 utilizes the host cell derived $tRNA^{Lys.3}$ as replication primer. Now, the questions are: Why HIV-1 choose a $tRNA^{Lys.3}$ as primer? Can tRNAs other than $tRNA^{Lys.3}$ be used as replication primer by HIV-1? If so, how could this affect viral replication? We thereby decided to test a hypothesis that *in vivo* interaction between $tRNA^{Lys.3}$ and the viral RNA template at multiple sites could be critical to maintain efficient viral replication.

4.2 ABSTRACT

The immunodeficiency virus type 1 (HIV-1) genomic RNA primer binding site (PBS) sequence comprises 18 nucleotides complementary to those at the 3'-end of the replication initiation primer $tRNA^{Lys3}$. To investigate the role of PBS in viral replication, we either deleted or replaced the original wild type PBS (complementary to $tRNA^{Lys3}$) with sequences complementary to $tRNA^{Lys1,2}$ or $tRNA^{Phe}$. Transfection of COS cells with such molecular constructs yielded similar levels of viral progeny, that were indistinguishable with regard to viral protein and tRNA content. Virus particles derived from PBS deleted molecular clones were non-infectious for each of MT-4, Jurkat and CEM cells. However, infectious viruses were derived from constructs in which the PBS had been altered to sequences complementary to either $tRNA^{Lys1,2}$ or $tRNA^{Phe}$, although mutated forms showed significant lags in replication efficiency in comparison with the wild-types. Molecular analysis of reverse transcribed DNA in cells infected by the mutated viruses indicated that both $tRNA^{Lys1,2}$ and $tRNA^{Phe}$ could function as primers for reverse transcription during the early stages of infection. During subsequent rounds of infection, reversion of the mutated PBS to wild type sequences was observed, accompanied by increased production of viral gene products. Such reversion was confirmed by both specific PCR analysis, using distinct primer pairs, and by direct sequencing of amplified segments. Data from *in vitro* reverse transcription experiments indicated that initiation with $tRNA^{Lys1,2}$ and $tRNA^{Phe}$ occurred much less efficiently than with $tRNA^{Lys3}$ when (-)ss DNA synthesis was primed from a synthetic RNA template containing a PBS complementary to the respective tRNA isoacceptors.

4.3 INTRODUCTION

An early, critical step in the HIV-1 life cycle is reverse transcription of RNA into proviral DNA, which can then be integrated into the infected host cell genome. This process is carried out by the multifunctional viral enzyme reverse transcriptase (RT) and requires a primer annealed to a single stranded template to initiate DNA synthesis. All retroviruses use a host cell-derived specific tRNA as primer, which is packaged along into the mature virions (Weiss et al., 1985). Eighteen nucleotides (nt) at the 3' end of $tRNA^{Lys3}$ are complementary to a 18 nt sequence of HIV-1 genomic RNA, termed the primer binding site (PBS). The PBS is found approximately 180 nt from the 5'-end of the HIV-1 viral genome (Ratner et al., 1985). It is believed to both provide a site for binding of primer tRNA, thereby allowing initiation of reverse transcription, and to facilitate the second template switch (strand transfer) (Gilboa et al., 1975). Neither of these functions is well understood. It is believed that viral proteins, perhaps in the form of *Gag-Pol* precursor play an important role in the selective incorporation of tRNALys3 (Mak et al., 1993); yet it is unclear whether PBS also plays a role in this process.

To study these multiple functions, we altered the wild-type PBS sequences (complementary to $tRNA^{Lys3}$) in an HIV-1 infectious clone by either deleting or replacing them with sequences complementary to $tRNA^{Lys1,2}$ or $tRNA^{Phe}$. Such PBS mutant viruses were analyzed in infectivity study as well as determination of viral tRNA contents. The rationale for choosing such PBS modification was (i) $tRNA^{Lys1,2}$ is utilized as primer for reverse transcription in other retroviruses, e.g. Mason-Pfizer monkey viruses (Leis et al., 1993), whereas $tRNA^{Phe}$ has never been identified as a transcription initiation primer, and (ii) while $tRNA^{Lys1,2}$ is packaged into wild-type HIV-1 in amounts

even greater than those of the wild-type primer $tRNA^{Lys3}$, $tRNA^{Phe}$ is present at much lower levels (Jiang et al., 1993). Thus it is of interest to see whether the abundance of a tRNA isoacceptor could influence its utility as primer.

We found that the quantities and patterns of tRNA species incorporated into virions were unaffected by either the absence of a PBS or the presence of altered PBS sequences, indicating that the PBS does not play a significant role in the selection and incorporation of primer tRNA during HIV-1 assembly. However, deletion of the 18 nt wild type PBS completely abolished viral infectivity, whereas its replacement with sequences complementary to either $tRNA^{Lys1,2}$ or $tRNA^{Phe}$ impaired but did not abort viral infectivity. Interestingly, the mutant PBS sequences reverted to wild-type during infection. The ability of these various viruses to replicate was closely related to the status of the PBS. During the early stages of infection, the two PBS mutants, in conjunction with $tRNA^{Lys1,2}$ and $tRNA^{Phe}$, apparently functioned as primers for reverse transcription, although less efficiently than the wild-type primer $tRNA^{Lys3}$.

4.4 MATERIALS AND METHODS

4.4.1 Cells, viruses, plasmids and other reagents.

The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: the HXB2D infectious clone of HIV-III_B, containing the full-length HIV-1 proviral genome (provided by Drs. G. Shaw and B. Hahn); MT-4 cell line (contributed by Dr. D. Richman). Other cells, including the simian-derived COS-7 cell line, CEM and Jurkat cell lines were purchased from the American Type Culture collection, Rockville, MD. Cells were routinely maintained either RPMI-1640 (for MT4, Jurkat and CEM cells) or DMEM (for COS-7 cells), supplemented with 10% fetal calf serum. Both cell culture medium and fetal calf serum were obtained from Gibco-BRL Laboratories, Toronto, Canada. pSCV21/BH10, a eucaryotic expression vector containing the full-length HIV-1 genome with a simian virus-40 (SV-40) origin of replication, was a gift of Dr. E. Cohen, Universite de Montreal, Canada. pSVK3 and pSP72 were purchased from Pharmacia (Montreal, Quebec) and Promega (Nepean, Ontario) respectively. Recombinant HIV-1 reverse transcriptase (p66/51 heterodimer) was kindly provided by Dr. Casey Morrow, University of Alabama at Birmingham. Restriction enzymes and other modifying enzymes were obtained from Pharmacia (Montreal, Quebec). Other chemicals were obtained from Fisher chemicals or Sigma chemicals.

4.4.2 Construction of plasmids with altered primer binding site sequences

The PBS of HIV-1 molecular clones was altered using a combination of linker replacement and site-directed mutagenesis (Sambrook et al., 1989). Briefly, HXB2D was cut with SmaI and ApaI to generate a 3.7 kb fragment comprising the 5'-region of the HIV-1 proviral genome and cellular flanking sequences. This fragment was subcloned into SmaII/ApaI digested pSVK3 to give pSVPBS, which was used for subsequent construction of PBS mutants. pSVPBS was digested with NarI, followed by treatment with a mung bean nuclease to generate blunt ends. The plasmid was then cut with BssHII to remove 70 HIV nucleotide sequences including the PBS and downstream sequences. We then ligated various 70 olignucleotide sequences into the gap created by the NarI and BssHII digestion described above. These inserts contained PBS-like sequences complementary to either $tRNA^{Lys1,2}$ or $tRNA^{Phe}$ to yield pSVPBS-Lys1,2 and pSVPBS-Phe, respectively. Standard site-directed mutagenesis was used to delete the entire 18 nt region of pSVPBS to yield pSVPBS(-). The SmaI /ApaI fragments from each of pSVPBS, pSVPBS(-). pSVPBS-Lys1,2 and pSVPBS-Phe were then cloned into the appropriately digested pSCV21 molecular clone of HIV-1 to yield expression plasmids containing full-length HIV-1 proviral DNA with the wild-type PBS (pPBS-WT) and mutated PBS [pPBS(-), pPBS-Lys1,2 and pPBSPhe]. All constructs were sequenced to verify that correct modifications in PBS sequences had been achieved. The sequences in the PBS region (18 nt) of these molecular clone constructs are:

5'-TGG CGC CCG AAC AGG GAC-3' (pPBS-WT);

5'-TGG CGC CCA ACG TGG GGC-3' (pPBS-Lys1,2);

5'-TGG TGC CGA AAC CCG GGA-3' (pPBS-Phe);

pPBS(-) has a 18 nucleotide deletion in the PBS region (nucleotide position 183-201)(Ratner et al., 1985). Each of the above plasmids with the altered primer binding sit sequences were cut with Bgl II and Pst I to generate a fragment of 947 base pairs (473 - 1420) comprising PBS/U5/R region of HIV-1 proviral sequences (Ratner et al., 1985). Such fragments were then ligated into RNA expression vector pSP72 cleaved with Pst I and Bgl II to generate various HIV-1 RNA expression plasmids containing altered PBS sequences.

4.4.3 In vitro reconstituted reverse transcription assay

The above HIV-1 RNA expression vectors were linearized by Acc I (nt 9560) (Ratner et al., 1985) and used in the Promega Riboprobe Gemini Core System to generate run-off transcripts of 483 ribonucleotides with alterations in the PBS region. In vitro reverse transcription assays were carried out in a volume of 20 µl containing 10 mM dithiothreitol, 50 mM Tris-HCl, pH 7.8, 100 mM KCl, 10 mM $MgCl_2$, and 0.2 mM of each of the four dNTPs as described (Arts et al., 1994). $tRNA^{Lys1,2}$, $tRNA^{Lys3}$ and $tRNA^{Phe}$ were purified from human placenta (Jiang et al., 1993; Roe, 1975). Reactions generally contained 1 pmol of RNA template, 5 pmol of tRNA primer and 0.75 µCi/µl of both (α-^{32}P)dATP and (α-^{32}P)dCTP. Reaction mixtures were first denatured at 84°C for 5 min, cooled to 55°C for 10 min to allow specific annealing of primer to template, and then cooled further to 37°C for 10 min to allow renaturation of RNA secondary structure. 0.3 µg of recombinant HIV-1 RT was then added together with 200 units of RNasin to reaction mixture and incubated at 37°C for up to 15 min. Reactions were terminated at various time points by adding EDTA to the reaction at a final

concentration of 100 mM. The terminated reaction mixtures were then extracted with phenol:chloroform and chloroform and passed through a Sephadex G-25 (Pharmacia) column to remove unincorporated free radioactive nucleotides. The products of these reactions were boiled for 4 min in formamide gel loading buffer (Maniatis et al., 1982) and chilled on ice for 5 min before loading onto the 5% denaturing polyacrylamide gel. The full-length (-) ssDNA synthesized in these reactions is 249 nt long.

4.4.4 Infectivity study

COS-7 cells were transfected with the PBS constructs by calcium phosphate precipitation method (Jiang et al., 1993). After 60 hr incubation, cell-free virus stocks were prepared by centrifugation of culture supernatants at 3000 rpm at 4°C for 30 min in a Beckman bentch-top centrifuge, followed by filtration through a 0.2 μ sterile membrane (Becton-Dickinson, Oxnard, CA). To remove possible contaminating plasmid DNA which could interfere with our PCR assays (see below), the viruses stock was treated at 37°C for 30 min with excess DNase I at a final concentration of 100 u/ml in the presence of 10 mM $MgCl_2$ (Panacino et al., 1993). The virus-containing supernatants were aliquoted and stored at -70°C until use.

Infectivity of virus particles produced by transfection of COS-7 cells was determined using MT-4 cells as target. Briefly, 5 x 10^5 cells were harvested during exponential growth, washed once by centrifugation, and incubated in virus-containing medium (5 ng of viral p24), supplemented with 10 mg/ml polybrene, at 37°C for 3 hr with occasional gentle shaking. Unbound viruses were removed by washing 4 times with PBS and the cells were resuspended in fresh medium. To ensure complete removal of contaminating plasmid DNA, PBS

from the fourth cell wash were checked by PCR using primer pairs specific for HIV-1 RT gene (Gu et al., 1992). Medium from infection study cultures was changed thereafter after 3-4 day intervals. Samples of cell-free culture supernatants were collected at regular intervals and assayed for virus content by indirect immunofluorecence assay and RT assay (Boulerice et al., 1990). Samples from MT-4 cells infected by heat-inactivated pPBS-WT virus (60°C, 30 min) served as negative control.

4.4.5 PCR analysis and DNA sequencing

Total cellular DNA was obtained by suspending infected cells (5 x 10^5 cells) in 0.5 ml TE buffer (50 mM Tris-HCl; EDTA 1 mM), pH 8.0, containing 10% sodium dodecyl sulfate (SDS) and 0.5 mg/ml pronase, and incubated at 37°C for 5-8 h with gentle shaking. The samples were then extracted with TE-saturated phenol and chloroform/isoamyl alcohol. High molecular weight DNA was isolated by standard methods (Maniatis et al., 1982) and used for determination of integrated HIV-1 proviral DNA. Samples were then analyzed by polymerase chain reaction (PCR).

Selected primer pairs were used in PCR analysis of the PBS sequences of various viral DNA species (unintegrated intermediates or integrated forms). The sequences, locations and orientations of the primer pairs, designed to detect DNA species with contiguous R and PBS region, as well as a description of the products formed, are illustrated schematically in Fig.1. These three sets of primer pairs, including PS/Lys1,2, PS/Phe, and PS/Lys3, were chosen to distinguish the three types of PBS studied. Primer pair PS/PA amplifies full-length proviral DNA and therefore detects completion of reverse transcription (Zack et al., 1990). To distinguish PBS forms, high stringent PCR reactions were

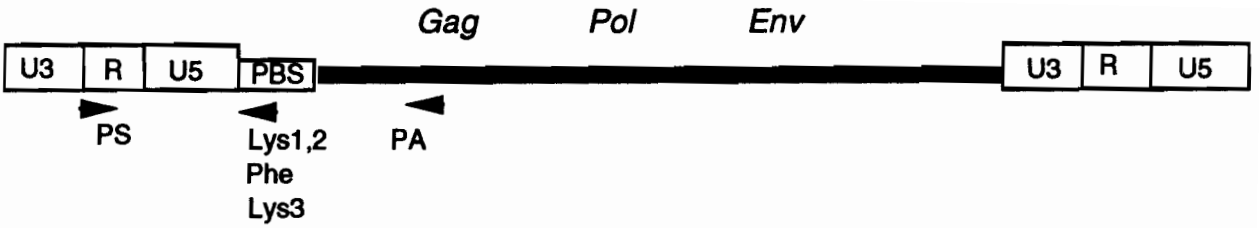
used: 50 µg sample DNA, 50 mM Tris-Cl, pH8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 5 pmols of ^{32}P end-labeled sense primer, and 20 pmols of cold antisense primer. The reaction was run for 25 cycles of 94°C (2 min) and 65°C (2 min). Other PCR reactions were essentially the same except that 50 mM of cold primers (sense and antisense) were used and the reactions were run for 30 cycles of 94°C (2 min), 60°C (2 min) and 72°C (2 min). Reactions were usually standardized by simultaneous amplification of ß-globin DNA (Zack et al., 1990). For direct sequencing of R/U5/PBS region, PS/PA amplified fragments were resolved by electrophoresis, purified by electroelution and sequenced using PCR-based dsDNA cycling sequencing system (Gibco-BRL Laboratories, Toronto, Ontario).

Figure 1. Primer location and strategy for detection of viral DNA by PCR.

A: Sequences of primers used in PCR.

B: PCR strategy. Primer pairs were chosen to distinguish the three types of PBS studied (PS/Lys1,2; PS/Phe; PS/Lys3), or to detect full-length proviral DNA (PS/PA). The later primer pair was used to amplify a region flanking the PBS. Amplified fragments were subsequently analyzed by using the PS primer to sequence (-) DNA and the PA primer to sequence (+) DNA using a double-stranded (ds) DNA cycling sequencing system (Gibco-BRL, Toronto, Ontario).

A



Sequences

PS	5'-AGA CCA GAT CTG AGC CTG GGA-3'
Lys3	5'-GTC CCT GTT CGG GCG CCA-3
Lys1,2	5'-GCC CCA CGT TGG GCG CCA-3'
Phe	5'-TCC CGG GTT TCG GCA CCA-3'
PA	5'-CCC ATC GAT CTA ATT CTC CCC-3'

B

Primer pair	Size of products	meaning of products
PS/Lys3	185bp	PBS complementary to $tRNA^{Lys3}$
PS/Lys1,2	185bp	PBS complementary to $tRNA^{Lys1,2}$
PS/Phe	185bp	PBS complementary to $tRNA^{Phe}$
PS/PA	367bp	Full length proviral DNA

4.4.6 Identification of tRNA species in virus particles

A dot blot assay using DNA oligonucleotides complementary to the 3'-end of $tRNA^{Lys3}$ (probe sequence 5'-TGGCGCCCGAACAGGGAC-3'), $tRNA^{Lys1,2}$ (probe sequence 5'-TGGCGCCCAACGTGGGGC-3') or $tRNA^{Phe}$ (probe sequence 5'-TGGTGCCGAAACCCGGGA-3') was used to identify specific tRNA species. The positive controls, including $tRNA^{Lys1,2}$, $tRNA^{Phe}$ and $tRNA^{Lys3}$ were purified from human placenta (Jiang et al., 1993). Total RNA was purified from viruses as described (Boulerice et al., 1990), and the viral RNA was normalized according to copy numbers of HIV-1 genomic RNA. Total RNA corresponding to 4×10^8 copies of viral genomic RNA was used in each analysis. RNA samples were blotted onto Hybond N filters (Amersham, Toronto, Ontario) and hybridized separately with each of the three probes. Following high stringent washing (Boulerice et al., 1990; Jiang et al., 1993), the filters were air-dried and exposed to X-ray film at -70°C.

4.5 RESULTS

4.5.1 Effects of alterations in PBS on viral infectivity

The infectious HIV-1 clone pPBS-WT, which possesses a wild type PBS complementary to $tRNA^{Lys3}$, was altered (i) by deleting the 18 nucleotide PBS to give pPBS(-) or (ii) by replacing the wild-type PBS with sequences complementary to $tRNA^{Lys1,2}$ or $tRNA^{Phe}$ to give pPBS-Lys 1,2, respectively. Northern and western blots were performed to study expression of the proviral genome. No differences regarding viral RNA transcripts and protein patterns were noted following transfection of COS-7 cells with these various clones (data not shown). We also transfected the various PBS constructs into the CD-4- RD cell line, which permits only one round of viral replication (Nagashunmugam et al., 1992). No significant differences were observed with regard to levels of RT activity in culture fluids after various times following transfections with these constructs (data not shown). These results are not surprising, considering that neither the PBS nor reverse transcription is involved in viral replication following transfection with proviral DNA.

Viral particles were harvested from COS-7 transfection cultures after 60 hr, normalized according to p24 contents (approximately 5 ng), and used to infect MT-4 cells. Culture supernatants were regularly monitored for virion-related reverse transcriptase activity over 4 weeks. Infection of MT-4 cells with pPBS-WT resulted in the rapid emergence of RT activity, syncytia, p24 Ag(+) cells as measured by indirect immunofluorescence assays (IFA), and other cytopathic effects (CPE) within 2 days. Virtually, all cells were p24 Ag(+) after 7 - 9 days, at which time RT activity had peaked (Fig.2A). The RT activity would

gradually decline with death of infected cells. In contrast, only 5 - 10% of cells infected by pPBS-Lys1,2 or pPBS-Phe were p24 Ag(+) after this period and RT activity was low in the culture (Fig.2A). Virus particles produced from COS-7 cells transfected with pPBS(-) were unable to infect MT-4 cells. No CPE, p24 antigen or RT were detected even after 30 days. We noted that virus production by cells infected with the two PBS mutants reached their peak by 15 days, followed by a gradual decrease (due to death of the infected cells). Similar virus production kinetics was noted when either Jurkat or CEM cells were used as targets (data not shown). This suggests that cell type differences were not responsible for the observed results with the mutated PBS-containing viruses.

We next used the progeny of these MT-4 infections obtained after 9 days (pPBS-WT infection) or 18 days (pPBS-Lys1,2 and pPBS-Phe infection) in a second round of replication in MT-4 cells. Figure 2B shows that high rates of replication, equivalent to those obtained with wild type viruses, were observed when the progeny of pPBS-Lys1,2 or pPBS-Phe obtained 18 days were studied for ability to infect MT-4 cells. In contrast, if the progeny of these MT-4 infections obtained after 6 days were compared in terms of infectivity in a second round infection, a similar infection kinetics curves as Figure 2A were observed (data not shown). This suggests that wild-type PBS forms had preferentially emerged during the 24 day period of study; this subject will be considered in the Discussion.

Figure 2A. Infection of MT-4 cells with viruses harvested from COS-7 transfections.

Viral inocula were equalized on the basis of either p24 (5 ng) content or RT activity (800,000 cpm) to infect 5 x 10^5 cells. Cultures were regularly monitored for HIV-1 production by RT assay following infection by pPBS(-) ( ),pPBS-Lys1,2 (•), pPBS-Phe ( ), pPBS-WT (O). ( ) designates mock infection.(MT-4 cells were infected by pPBS-WT which had been heated at 60°C for 30 min). No fresh cells were added during the 24 day period study in order to observe viral particle accumulation in culture.

Figure 2B Second round of infection of MT-4 cells by viruses obtained from initially-infected cultures (9 days following pPBS-WT infection and 24 days following pPBS-Lys1,2 or pPBS-Phe infection). For symbol code, see Figure 2A.

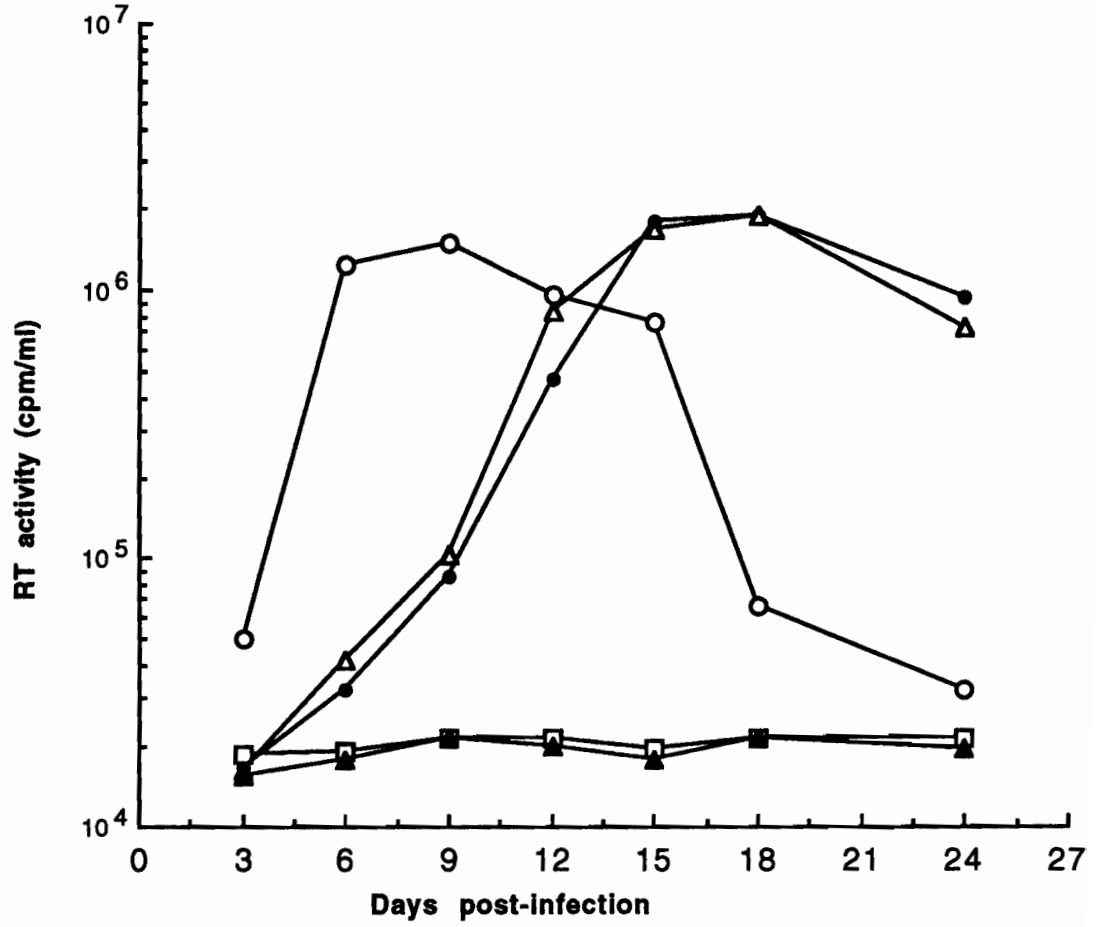
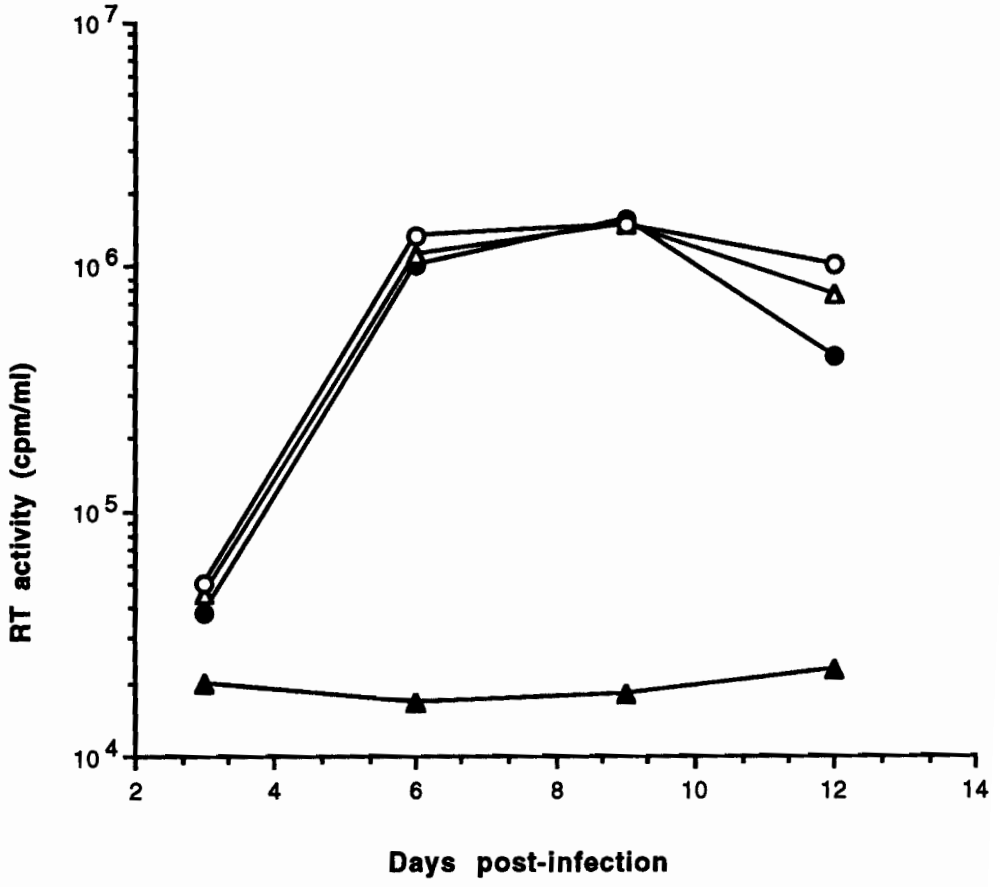


Figure 2A, Li et al


10^7
10^6
10^5
10^4
RT activity (cpm/ml)
2
4
6
8
10
12
14
Days post-infection

4.5.2 PCR analysis of the PBS sequence of proviral DNA in infected cells.

The specificity of our PCR assay was monitored by mixing 0.1 ng of cloned HIV-1 plasmid (wild-type or mutated) with 50 μg MT-4 DNA from uninfected cells. The R/U5/PBS region of each type of HIV-1 genomic DNA was amplified using the three sets of primer pairs, PS/Lys1,2, PS/Phe or PS/Lys3, described in Fig.1. Each HIV-1 clone could only be amplified by its own specific primer pair, e.g., pPBS-Lys1,2 by PS/Lys1,2 (Fig.3A, lane 2) and not PS/Lys or PS/Phe. Nor could primer pair PS/Lys1,2 amplify any of the R/U5/PBS region of PBS(-) (Fig.3A, lane 1), pPBS-Phe(Fig.3A, lane 3) or pPBS-WT (Fig.3A, lane 4).

The three sets of primer pairs were used to analyze PBS sequences in DNA harvested from MT-4 cells at various times after infection by the various viral clones. Total cellular DNA was used to simultaneously detect PBS in intermediate viral species as well as in full-length integrated proviral DNA. At all times, we detected only wild-type PBS sequences in DNA extracted from MT-4 cells exposed to pPBS-WT virions, starting 3 days after infection (Fig.3B, panel I). 24 days after infection, the intensity of the band decreased, apparently as a result of virus-induced cytopathicity. In contrast, only mutated PBS forms were present in infected with pPBS-Lys1,2 or pPBS-Phe 6 days after infection (Fig.3B, panel II and III). 9 days after infection, the wild type PBS began to emerge. It is clear that mutated PBS forms gradually disappeared beyond this time point, concomitant with increasing emergence of wild type PBS in these cells. This is about the same time at which the these cultures began to release high levels of infectious progeny viruses (Fig.2A). These PCR data were later confirmed by sequencing analysis of the PBS region. Table 1 is a summary of DNA sequencing with respect to the PBS. The above data suggested that

replicating rates of the two mutant viruses appeared closely related to the PBS status.

Figure 3A Specificity of PCR for differentiation of PBS sequences. Primer pairs PS/Lys1,2, PS/Phe and PS/Lys3 were used to detect PBS complementary to each of $tRNA^{Lys1,2}$, $tRNA^{Phe}$, and $tRNA^{Lys3}$, respectively. Linearized PBS constructs [pPBS(-), pPBS-Lys1,2, pPBS-Phe and pPBS-WT] were mixed with 50μg of uninfected cellular DNA and subjected to PCR in separate analyses using the above primer pairs. Amplified products were fractionated on 5% polyacrylamide gel. The dried gels were then exposed to X-ray film. The sensitivity of the three primer pairs were similar and the specificity were present over a range of plasmid concentrations. Fig.3A shows the intensity of reaction products using 0.1 ng plasmid and exposure time of 2 h at -70°C.

A

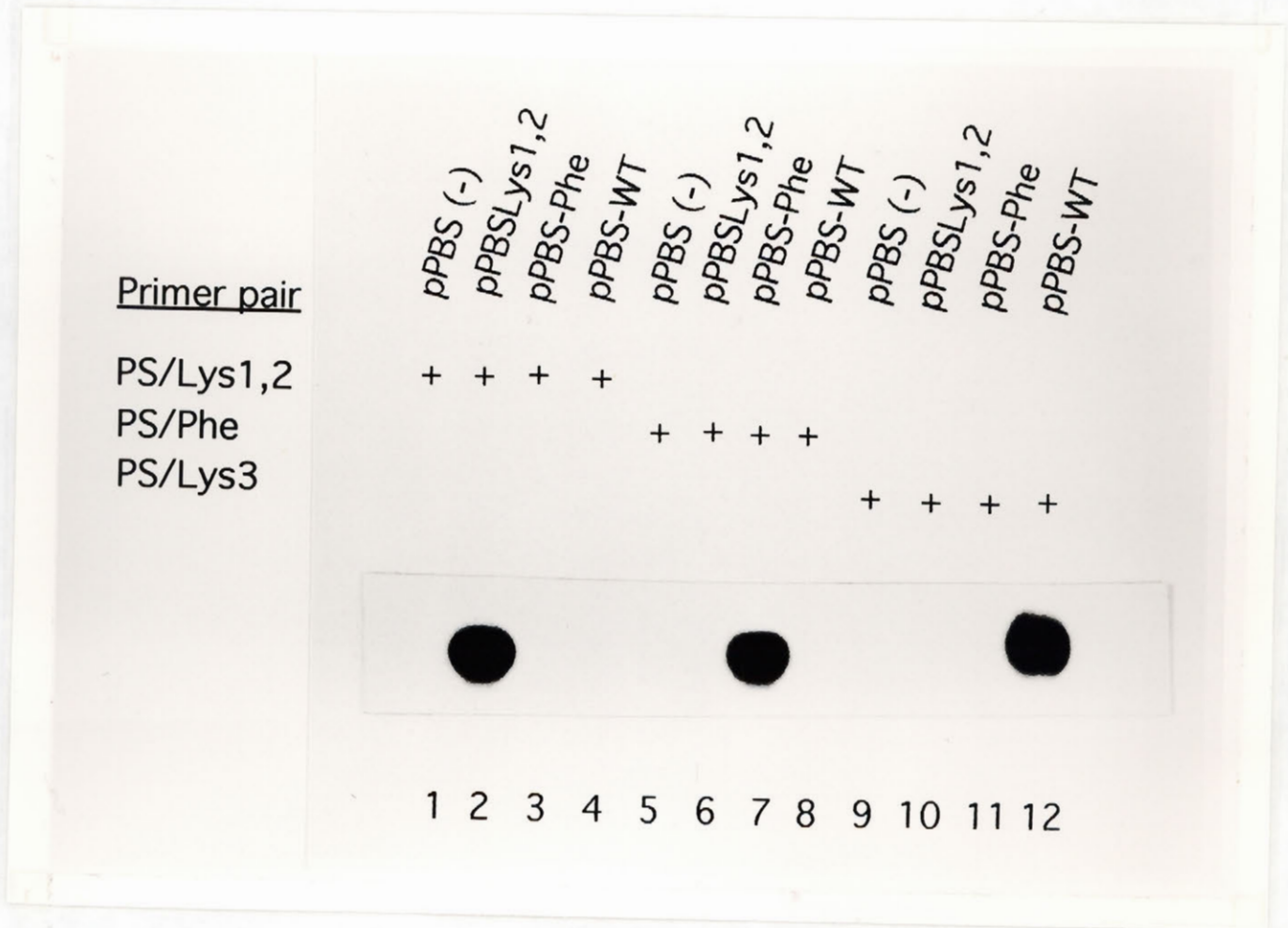

Primer pair
PS/Lys1,2
PS/Phe
PS/Lys3
pPBS (-)
pPBSLys1,2
pPBS-Phe
pPBS-WT
pPBS (-)
pPBSLys1,2
pPBS-Phe
pPBS-WT
pPBS (-)
pPBSLys1,2
pPBS-Phe
pPBS-WT
+ + + +
+ + + +
+ + + +
1 2 3 4 5 6 7 8 9 10 11 12

Fig.3 B. Analysis of PBS in reverse transcribed DNA.

Total DNA (50 μg) from infected cells at six time points was analyzed by PCR using the three sets of primers pairs described above (Figure 3A). Panel I-III show results from MT-4 cells infected by pPBS-WT (panel I), pPBS-Lys1,2 (panel II) and pPBS-Phe (panel III) viruses respectively. ß-globin served as an internal control (not shown in picture).

B

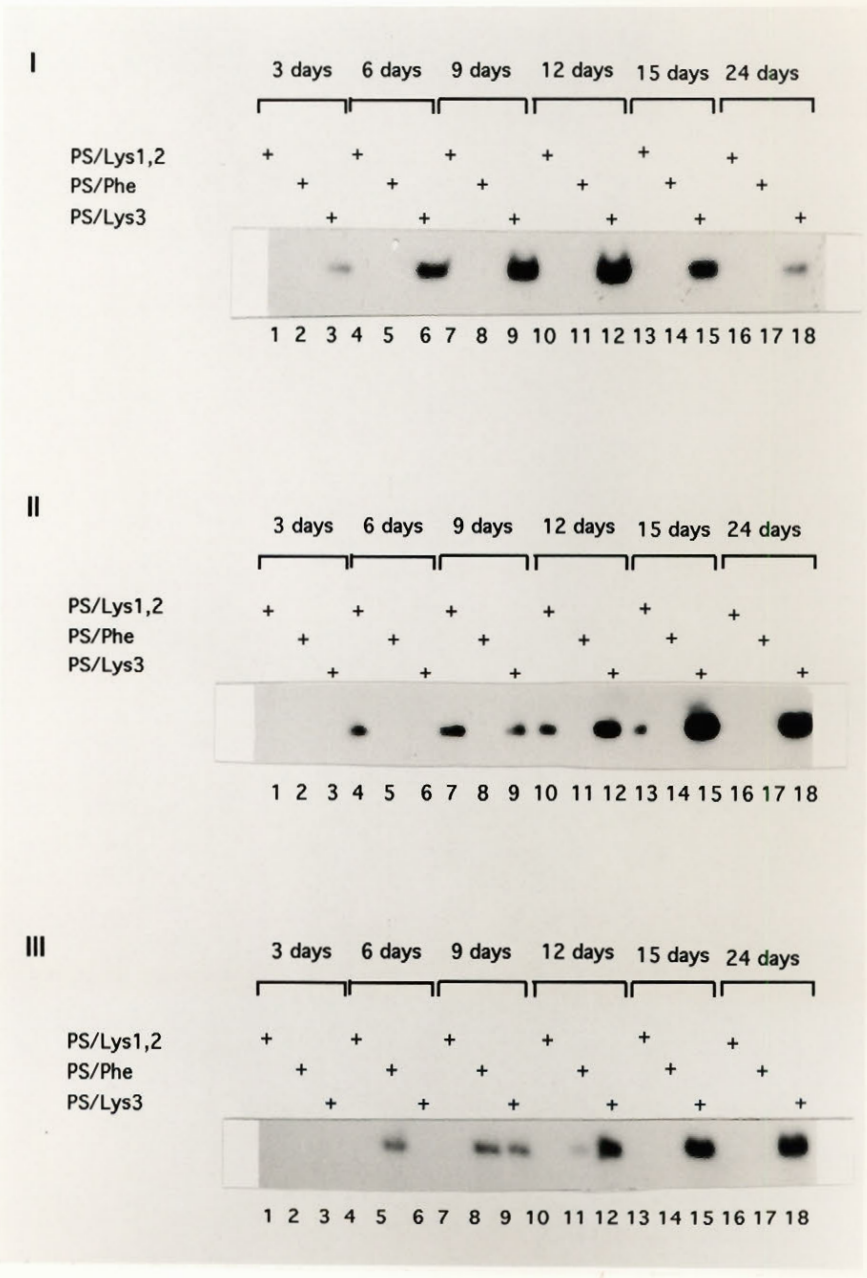

I
3 days
6 days
9 days
12 days
15 days
24 days
PS/Lys1,2
PS/Phe
PS/Lys3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
II
3 days
6 days
9 days
12 days
15 days
24 days
PS/Lys1,2
PS/Phe
PS/Lys3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
III
3 days
6 days
9 days
12 days
15 days
24 days
PS/Lys1,2
PS/Phe
PS/Lys3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Table. 1 PBS sequences detected by direct ds DNA sequencing [a]

	6 days post-infection	24 days post-infection
pPBS-WT/MT-4	TGG CGC CCG AAC AGG GAC	TGG CGC CCG AAC AGG GAC
pPBS-Lys1,2/MT-4	TGG CGC CCA ACG TGG GGC	TGG CGC CCG AAC AGG GAC
pPBS-Phe/MT-4	TGG TGC CGA AAC CCG GGA	TGG CGC CCG AAC AGG GAC

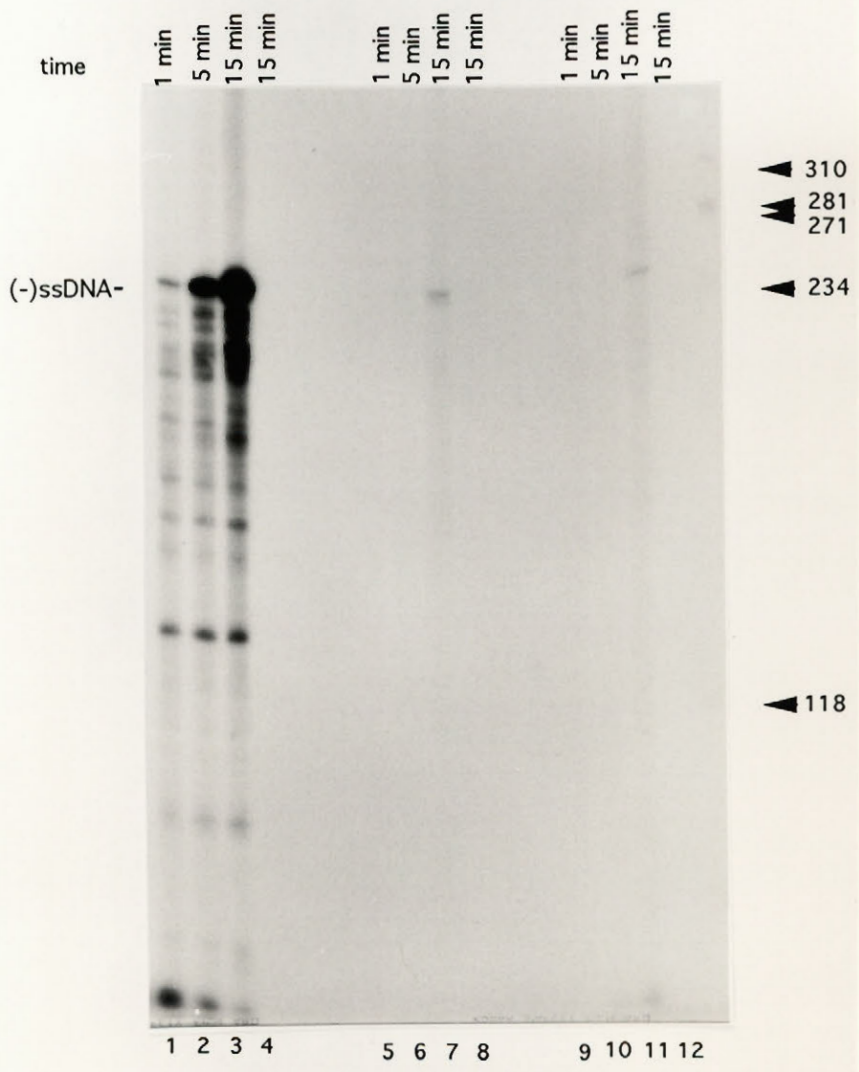
[a] Primer pair PS/PA was used to amplify a region flanking the PBS. Amplified fragments were subsequently analyzed by using the PS primer to sequence (-)DNA and the PA primer to sequence (+)DNA using a double stranded (ds) DNA cycling sequencing system (Gibco-BRL, Toronto, Canada).

4.5.3 Determination of (-)ss DNA synthesis in *in vitro* reverse transcription assay

To determine initiation efficiency by different tRNA primers, we next used a cell-free reverse transcription assay that employed synthetic RNA templates containing mutated PBS and their respective tRNA primers (from human placenta) (Arts et al., 1994). When synthetic pPBS-WT RNA template was primed in our in vitro reverse transcription assay, full-length (-)ss DNA products were detectable within 1 min after addition of RT into the reaction mixture (Fig.4, lane 1). However, when synthetic pPBS-Lys1,2 and pPBS-phe RNA templates were primed by $tRNA^{Lys1,2}$ and $tRNA^{Phe}$, respectively, it was only 15 min after reaction initiation that the full-length (-)ss DNA (249 nt) was detectable (Fig.4, lanes 7 and 11). It should be noted that none of these tRNAs were able to prime (-)ss DNA synthesis from synthetic pPBS(-) RNA template (Fig.4, lanes 4, 8, and 12), indicating that those bands (249 nt) are products from specific reaction primed by tRNA. The fast moving bands (molecular size, < 249 nt) in Fig.4 are incomplete minus strand DNA products, possibly due to pausing by RT during DNA synthesis (Huber et al., 1990; Readon et al., 1991; Klarman et al., 1993).

Figure 4. In vitro reconstituted reverse transcription assay

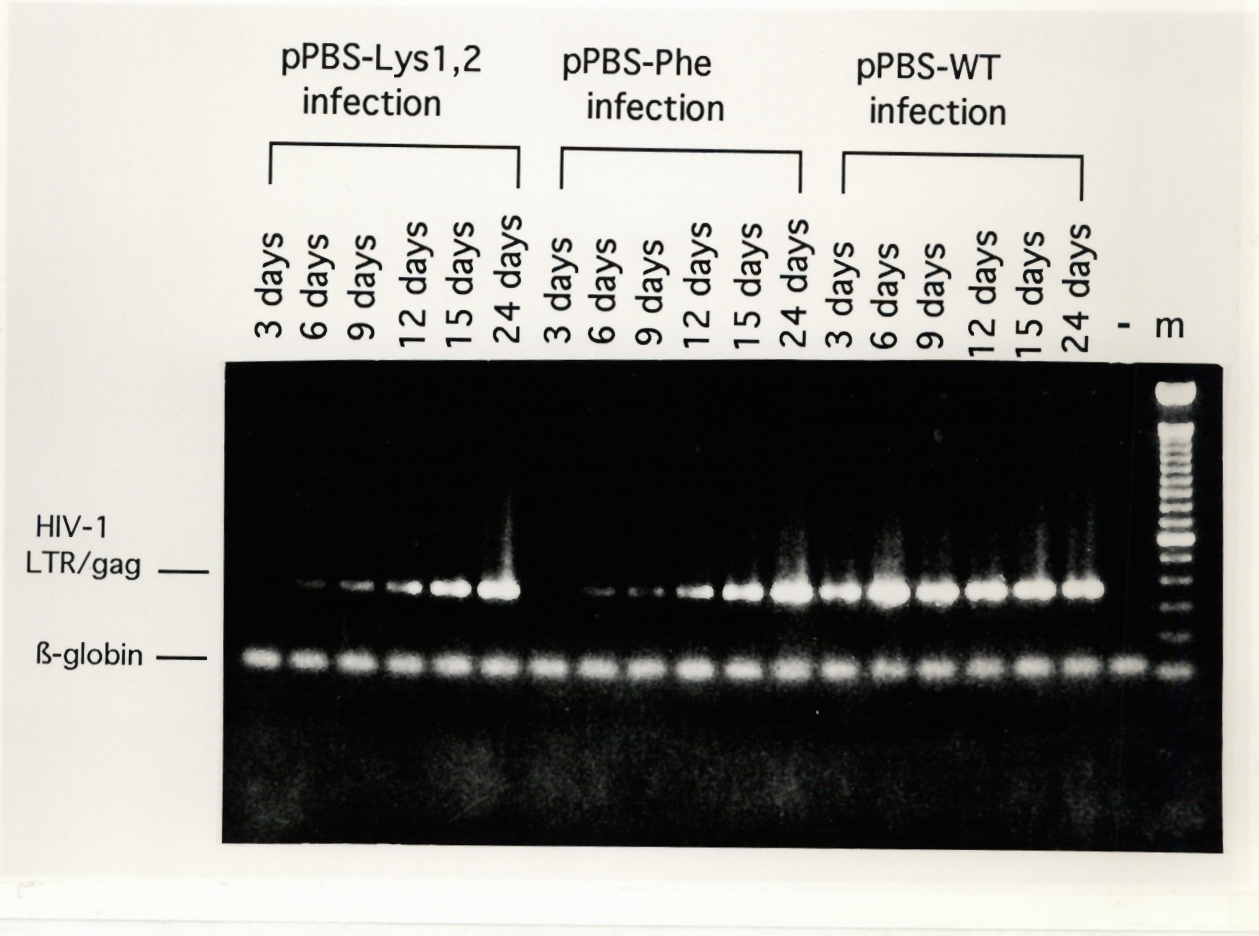
Synthetic RNA templates containing respective altered PBS sequences were primed with $tRNA^{Lys1,2}$, $tRNA^{Phe}$, and $tRNA^{Lys3}$, respectively in (-)ss DNA synthesis reaction. Lanes 1-3 represents pPBS-WT RNA template primed by $tRNA^{Lys3}$. Lane 4 represents pPBS(-) RNA template primed by $tRNA^{Lys3}$. Lanes 5-6 represent pPBS-Lys1,2. Lane 7 represents pPBS(-) RNA templates primed by $tRNA^{Lys1,2}$. Lanes 8-10 represents pPBS-Phe RNA template primed by $tRNA^{Phe}$. Lane 11 represents pPBS(-) RNA template primed by $tRNA^{Phe}$. The full-length (-)ss DNA is 249 nt. Bands with size smaller than 249 nt are incomplete minus strand DNA products, possibly due to pausing by RT during DNA synthesis (Huber et al., 1991; Klarman et al., 1993; Readon, 1991)


time
1 min
5 min
15 min
15 min
1 min
5 min
15 min
15 min
1 min
5 min
15 min
15 min
(-)ssDNA-
310
281
271
234
118
1 2 3 4
5 6 7 8
9 10 11 12

4.5.4 Alteration of HIV-1 PBS does not abolish the reverse transcription cycle

The retroviral PBS is thought to facilitate the second template switch which occurs during reverse transcription (Hu and Temin, 1990; Giboa et al., 1979; Panganiban and Fiore, 1988; Wakefiled et al., 1994). To detect full-length integrated proviral DNA by PCR, we studied high molecular weight (HMW) DNA of infected MT-4 cells using primer pair PS/PA (Fig.1), which amplifies the last region of reverse transcribed proviral DNA (Giboa et al., 1979). Fig.5 shows that full-length proviral DNA was detected throughout the course of infection by wild type as well as mutated viruses. That was true even at early time points of infections involving mutated viruses, when only mutated PBS forms were present (Fig.3B, panel II and III). These results suggest that the altered PBS sequences do not abolish the reverse transcription cycle.

Figure 5. Detection of full-length integrated proviral DNA from infected cells. HMW DNA was analyzed by PCR using primer pair PS/PA, which detects full-length proviral DNA. Fresh cells were added into parallel infection cultures to facilitate isolation of relatively intact HMW DNA (on day 12 after pPBS-WT infection and on day 18 after infections by pPBS-Lys1,2 and pPBS-Phe). Amplified products were electrophoresed on 1.5% agarose gels and detected by staining with ethidium bromide. Lane -, HMW DNA from MT-4 cells infected by heat-inactivated pPBS-WT; lane m, 100-bp DNA ladder (Gibco-BRL).


pPBS-Lys1,2 infection
pPBS-Phe infection
pPBS-WT infection
3 days
6 days
9 days
12 days
15 days
24 days
3 days
6 days
9 days
12 days
15 days
24 days
3 days
6 days
9 days
12 days
15 days
24 days
-
m
HIV-1 LTR/gag
ß-globin

4.5.5 Identification of the tRNA species in mutant viruses

Three different oligonucleotide probes, complementary to the 3'-end of $tRNALys^{1,2}$, $tRNA^{Phe}$ and $tRNA^{lys3}$, respectively, were used to detect these tRNA species in purified virus particles, using hybridization conditions and purified human placental tRNA isoacceptor species, as described (Jiang et al., 1993; Mak et al., 1993). These analyses were carried out by dot-blot hybridization; all patterns of reactivity were specific, and no cross hybridization was observed among the probes, e.g., the $tRNA^{Phe}$ specific probe did not show cross-hybridization to either $tRNA^{Lys1,2}$ or $tRNA^{Lys3}$, which were known to share more than 60% homology at the 3'-end (>10/18) and have been identified as the two most abundant tRNA species in the HIV-1 virions (Fig.6).

No significant differences in levels of $tRNA^{Lys1,2}$, $tRNA^{Phe}$ or wild type primer $tRNA^{Lys3}$ were found in each of the three mutants as compared to wild type viruses (Figure 6 A and B). Thus, the PBS does not appear to be involved in the selective incorporation of tRNA species into mature virions.

Figure 6A. Identification of tRNA species in viral particles.

Identification of tRNA species in viral particles by dot blotting using DNA oligonucleotides complementary to the 3' end of $tRNA^{Lys.1,2}$ (panel I), $tRNA^{Phe}$ (panel II), or $tRNA^{Lys.3}$ (panel III). The specificities and hybridization conditions of this assay have been described elsewhere (Jiang et al., 1993). RNA samples were obtained from purified viruses produced by COS-7 cells after transfection with pPBS constructs. Dots 1 to 4 designate viral RNA from COS-7 cells transfected by pPBS-WT, pPBS(-), pPBS-Phe, and pPBS-Lys1,2, respectively.

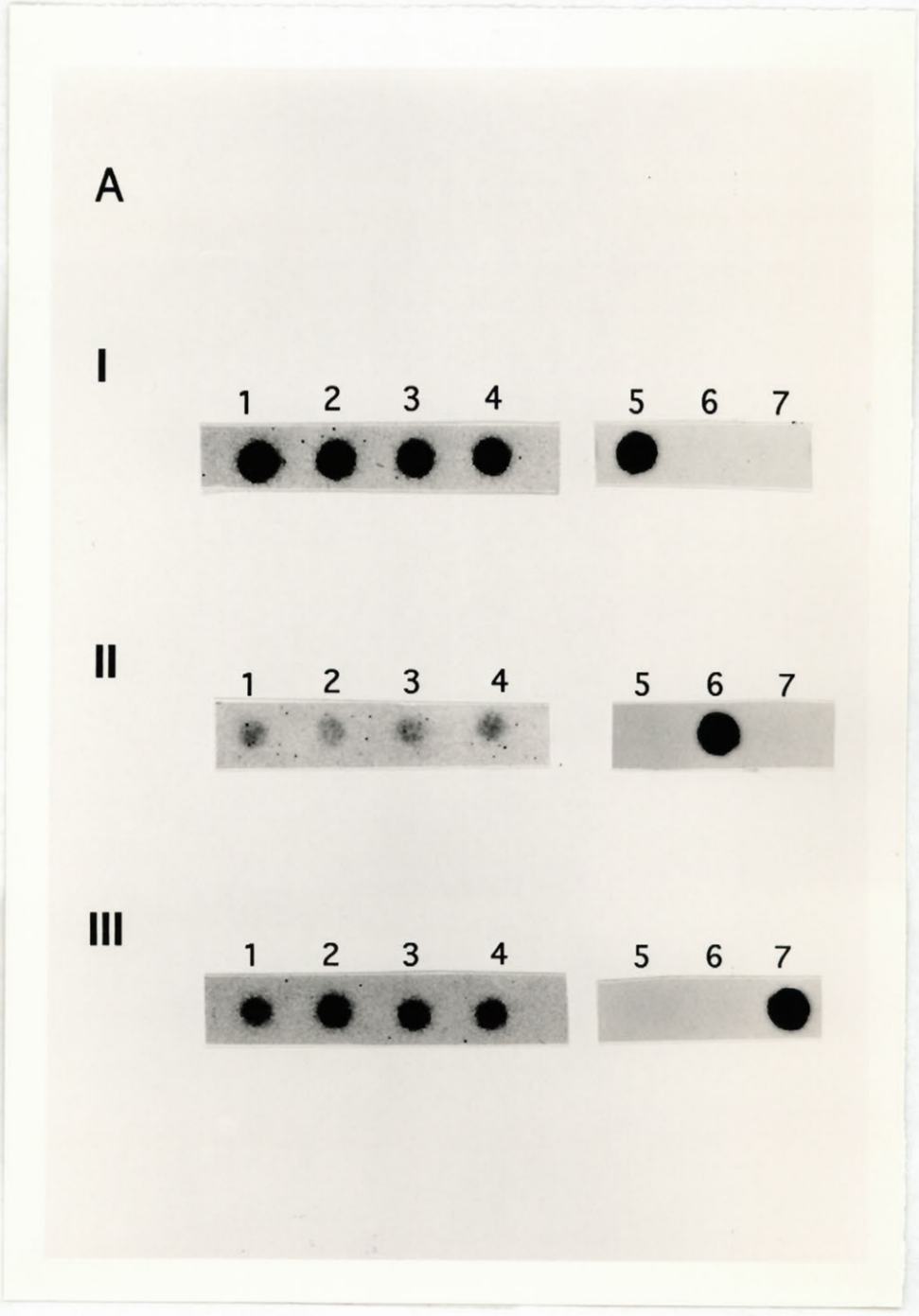

A
I
1 2 3 4
5 6 7
II
1 2 3 4
5 6 7
III
1 2 3 4
5 6 7

Figure 6B Comparison of tRNA isoacceptors packaged into viruses. Each experiment was repeated three times. The relative intensity of each dot from the hybridization in panels A to C was estimated by laser scanning in an LKB film laser scanner. Results are expressed + standard deviation. pPBS-Phe; , pPBS-Lys1,2; , pPBS(-); , pPBS-WT.

4.6 DISCUSSION

Reverse transcription of retroviral genomic RNA into proviral DNA is an early and essential step in the HIV-1 life cycle. The role of the PBS is to provide a complementary region for the binding of the specific tRNA isoacceptor species that serves as a primer for RNA-dependent DNA polymerization, and to facilitate the second template switch, allowing completion of full-length double stranded proviral DNA. During (+) strand DNA synthesis, tRNA serves as template for generation of the PBS (Giboa et al., 1979; Goff et al., 1980; Hu and Temin, 1983; Panganiban and Fiore, 1988), thus enabling identification of the specific tRNA isoacceptors used for initiation of reverse transcription.

we have generated several PBS mutants to study such multiple functional roles of PBS in HIV-1 replication. We take two reasons into account for choosing these PBS mutants: (i) The utility of tRNA as primer in retroviruses; (ii) The abundance of tRNA species in HIV-1 virions. We were unable to detect significant differences regarding tRNA isoacceptors packaged into mature virions between these mutants and wild type viruses. This appears to be consistent with previous studies in which deletion of either LTR or PBS sequences did not disrupt tRNA patterns in viruses (Jiang et al., 1993). This yields supportive evidence that viral proteins, perhaps in the form of *Gag-Pol* precursor, play an important role for specific selection of tRNA isoacceptors (Mak et al., 1993).

In our infectivity study with three different cell lines as targets, the PBS deletion mutant viruses, pPBS(-) were, not surprisingly, non-infectious, consistent with previous observations\ (Rhim et al., 1991). A novel finding of this paper is that the two PBS replacement mutants, pPBS-Lys1,2 and pPBS-Phe, were infectious, although less so than wild type viruses. However, despite a

delay in production of CA and RT in the culture supernatant, the rate of virus replication eventually reached the wild type level. Mutant viruses harvested at day 18 after infection were used to reinfect MT4 cells; we found that these viruses behaved indistinguishably from wild type. As discussed below, such shift in phenotype corresponded to a reversion to a wild- type PBS. Other workers have also reported differential results using mutated PBS sequences in a different system involving only a single round of viral replication (Lund et al., 1993).

PCR analysis at early time points of the PBS sequences found within the HMW DNA of cells infected by pPBS-Lys1,2 or pPBS-Phe showed complementarity to $tRNA^{Lys1,2}$ or $tRNA^{Phe}$, respectively. In contrast, the PBS sequences of proviral DNA from late stages of these infections were complementary to wild-type primer $tRNA^{Lys3}$, indicating that synthesis and apparent selection of the wild-type had occurred. These data suggested that during the early stages of infection, both $tRNA^{Lys1,2}$ and $tRNA^{Phe}$ can serve as primers for reverse transcription. However in the late stages of infection, it is $tRNA^{Lys3}$ that functions as primer, concomitant to massive release of viral particles into the culture. These data suggested that the replication competence of HIV-1 clones is closely related to the competence of the PBS. Our in vitro study with PBS mutated RNA templates and $tRNA^{Lys1,2}$ or $tRNA^{Phe}$ indicated that reverse transcription efficiency is very poor compared to the wild-type reaction in (-)ss DNA synthesis; this could explain why there is a lag with the two PBS mutants in virus production kinetics.

Despite the ability of $tRNA^{Lys1,2}$ and $tRNA^{Phe}$ to serve as primer, both mutated PBS forms eventually reverted to wild-type complementary to $tRNA^{Lys3}$. It is unlikely that this development was due to contamination and amplification of small quantities of wild-type forms, since our molecular

proviral clones had been repeatedly subcloned and purified, and were pure by sequencing. Also, specific PCR showed that only mutant PBS forms were present in proviral DNA at early stages of infection by mutated viruses. No reversion of the PBS(-) mutant was ever noted, as might be expected if the reversion with pPBS-Lys1,2 and pPBS-Phe mutants were due to contamination. The question concerning PBS form reversion remains open. However, it is known that the RT of HIV-1 binds preferentially to $tRNA^{Lys3}$ (Barat et al., 1989; et al., 1994; Richter-Cook et al., 1992). In addition, interaction between retroviral U5 RNA and the TΨC loop of the $tRNA^{Trp}$ may be required for efficient initiation of reverse transcription, as shown in other retroviral systems (Aiyar et al., 1992; Leis et al., 1993). Recent studies have shown that a four-nucleotide sequence in the anticodon loop of $tRNA^{Lys3}$ interacts with HIV-1 RNA genomic RNA in a region upstream from the PBS (Isel et al., 1993). The resulting loop-loop interaction between tRNA and RNA template, combined with normal PBS-tRNA binding, might give rise to significant alterations in secondary structure of the primer-template complex relative to that occurring when only the 18 nt of the viral PBS interact with tRNA (as in the case of the pPBS-Lys1,2 and pPBS-Phe mutants). The stability of additional tRNA/RNA template interactions might be dependent on particular base modifications found only in $tRNA^{Lys3}$. Such interaction could play a role in formation of RT-tRNA/RNA template transcription complexes, thereby affecting transcription efficiency. Indeed, our in vitro reverse transcription experiments indicated that initiation with $tRNA^{Lys1,2}$ and $tRNA^{Phe}$ occurred less efficiently than with tRNALys3 when (-) ss DNA synthesis was primed from a RNA template containing a PBS complementary to the respective tRNA isoacceptors. Similar results were obtained if NCp7 was added in the reverse transcription reactions.

$tRNA^{lys3}$ has been known to have extensive 3'-end homology with both $tRNA^{Lys1,2}$ (71%) and $tRNA^{phe}$ (62%) and found to be incorporated into our PBS mutants at levels similar to those found with wild types. Homology among these tRNA species implies that $tRNA^{Lys3}$ could conceivably anneal to a mutant PBS; such annealing might be further stabilized by the tRNA/RNA template interaction discussed above. Thus, $tRNA^{Lys3}$ might be able to prime reverse transcription even from a mutant PBS. Since reverse transcription with $tRNA^{Lys3}$ is more efficient than that with $tRNA^{Lys1,2}$ or $tRNA^{Phe}$, it is conceivable that mutant viruses might preferentially use a wild type primer, leading to the reverse transcription from $tRNA^{Lys3}$ of a wild-type PBS and a consequent increase in viral production. Other factors that might affect reversion of the PBS to wild type include specific interactions between HIV-1 RT and $tRNA^{Lys3}$ and the preferential incorporation of $tRNA^{Lys}$ isoacceptors into virions. Nevertheless, it is not clear whether any host cellular factors are involved in reverse transcription. Further insight will be possible once the factors that involved in the selection, incorporation and placement of primer tRNA onto the HIV-1 PBS *in vivo* are better understood(Jiang et al., 1993; Make et al., 1993; Kolsteadt and Steitz, 1992; Nagashunmugam et al., 1992; Le Grice, 1993).

CHAPTER 5

Effects of mutations in sequences flanking the primer binding site on HIV-1 replication

This chapter was adapted from an article submitted to the Journal of Virology. The authors of this paper were X. Li, C. Liang, Y. Quan, R. Chandok, M. A. Parniak, and M. A. Wainberg. This work was largely performed by X. Li under the supervision of Dr. Wainberg. Dr. C. Liang actively participated this work and is doing further characterization of the mutants. R. Chandok participated some of this work. Drs. Y. Quan and M. A. Parniak helped in devising the experiments and analyzing the results.

5.1 PREFACE TO CHAPTER 5

In Chapter 4, we have investigated the effects of altered primer binding site sequences on HIV-1 replication. We provided evidence that HIV-1 has evolved to specifically utilize $tRNA^{Lys.3}$ as replication primer for maintaining growth advantage. In this chapter, we investigated the effects of mutations in sequences that flank the primer binding site on HIV-1 replication.

5.2 Abstract

Retroviral reverse transcription is initiated from a 18 nucleotide primer binding site (PBS) of the genomic RNA, to which the host cell-derived tRNA primer is annealed. This process also involves viral genomic sequences outside of the PBS. To further investigate this subject, we constructed proviral DNA clones of HIV-1 that were selectively deleted in regard to either 1. the A-rich loop, immediately upstream of the PBS; 2. a 7 nt segment found downstream of the PBS; 3. an extended non-translated 54 nt segment located immediately downstream of the PBS. Synthesis of minus-strand (-) strong stop DNA was assessed in MT-4 cells infected with viruses derived from COS cells that had been transfected with these various constructs. We found similar levels of minus strong stop DNA were generated in cells infected with wild-type viruses or with viruses that had been deleted in the A-rich loop or 7 nt segment. In contrast, significantly lower levels of viral DNA were detected in cells infected with viruses that had been deleted on the 54 nt stretch. Furthermore, each of the first two molecular clones was able to replicat with essentially the same efficiency as the wild-type while the third displayed a significantly diminished capacity in this regard. Further investigation indicated that the 54 nt segment of 5' non-translated RNA, located immediately downstream of the PBS, also plays a role in efficient expression of viral mRNA.

5.3 INTRODUCTION

Retroviral reverse transcription begins at the primer binding site (PBS) of unspliced viral RNA, to which a host cell-derived replication tRNA primer is positioned (Coffin, 1985). The PBS of human immunodeficiency virus (HIV-1) is located approximately 180 nt from the 5' terminus of the genomic RNA and is flanked at its 5' end by a region referred to as R/U5 (Ratner et al., 1985). This R/U5 region possesses numerous functional activities, including a role in packaging of viral RNA, binding of the tat trans-activator protein, and involvement in reverse transcription and integration of proviral DNA. (Cullen 1991; Isel et al., 1993; Vicenzi et al., 1994; Miele et al., 1996). A 133 nt-uncoding/untranslated region is located downstream of the PBS and upstream of the gag initiation codon (Ratner et al., 1985). The function of this sequence, especially the 5' portion, is not well understood, even though its 3' portion is thought to be involved in packaging, splicing and dimerization of genomic RNA, translation of viral proteins (Clavel, et al., 1990; Blioz et al., 1995; Laughrea and Jette, 1994; Lever et al., 1989; Aldovini and Young, 1990; Skripkin et al., 1993; Marquet et al., 1994; Darlix et al., 1990; Kim et al., 1994).

Computer modeling in conjunction with chemical and enzymatic probing on *in vitro* synthesized 5' retroviral RNA comprising PBS and its neighboring sequences suggests that this region is highly structured (Baudin et al., 1993; Berkhout et al., 1993; Harrison et al., 1992), posing a potential barrier for primer tRNA to be annealed at the PBS site of the template. While the unfolding of both tRNA primer and RNA template is likely to be mediated by the viral nucleocapsid protein (NCp) (Khan and Giedroc, 1992; Karpel et al., 1987; Prats et al., 1988), formation of a reverse transcription initiation complex involves base pairing between the PBS and a complementary 18-nt region at

the 3' end of tRNA, as well as additional interactions between sequences that neighbor the PBS and the remainder of the tRNA primer. In avian retroviruses, the efficiency of a $tRNA^{Pro}$-PBS complex in initiation of reverse transcription was enhanced by inclusion of viral genomic sequences upstream of the PBS and the TΨC loop of $tRNA^{Pro}$ (Aiyar et al., 1992; 1994; Leis et al., 1993). Disruption of another stem-loop structure, i.e. U-IR, caused diminished reverse transcription in both avian and murine retroviruses (Cobrinik et al., 1988, 1991; Murphy and Goff, 1989). In HIV-1, a specific interaction between the A-rich loop, located in the U5 region (10 nt upstream of PBS), and the anticodon loop of $tRNA^{Lys.3}$ has been detected by foot printing (Isel et al., 1992). Work in cell-free systems has also suggested that a 6 nt sequence, located immediately downstream of the PBS, was important in specifying utilization of tRNA primer (Kohlsteadt & Steiz, 1992).

We wished to study the roles in viral infectivity of sequences that flank the PBS. Toward this end, we introduced several independent deletions into HIV-1 proviral DNA: i) a deletion of the A-rich loop (Isel et al., 1993), designated as pHIV/del-A; ii) a deletion of seven nucleotides located downstream of PBS (Kohlsteadt and Steitz, 1992), designated as pHIV/del-7; and iii) a 54 nt deletion of the 5' portion of the uncoding region located immediately downstream of the PBS, designated as pHIV/del-LD. We found deletion in either the A-rich loop (pHIV/del-A) or the 7 nt segment, immediately downstream of the PBS (pHIV/del-7), imposed little effect on reverse transcription in MT-4 cells infected with each of the two mutant viruses. In contrast, deletion of the 5' portion of the untranslated sequences (pHIV/del-LD) severely restricted generation of reverse transcribed DNA. Both the pHIV/del-A and pHIV/del-7 displayed similar replication kinetics to that of the wild type, while the pHIV/del-LD was impaired in this regard. Subsequent analysis showed that the

54 nt sequence, located downstream of the PBS, is also involved in efficient expression of viral mRNA.

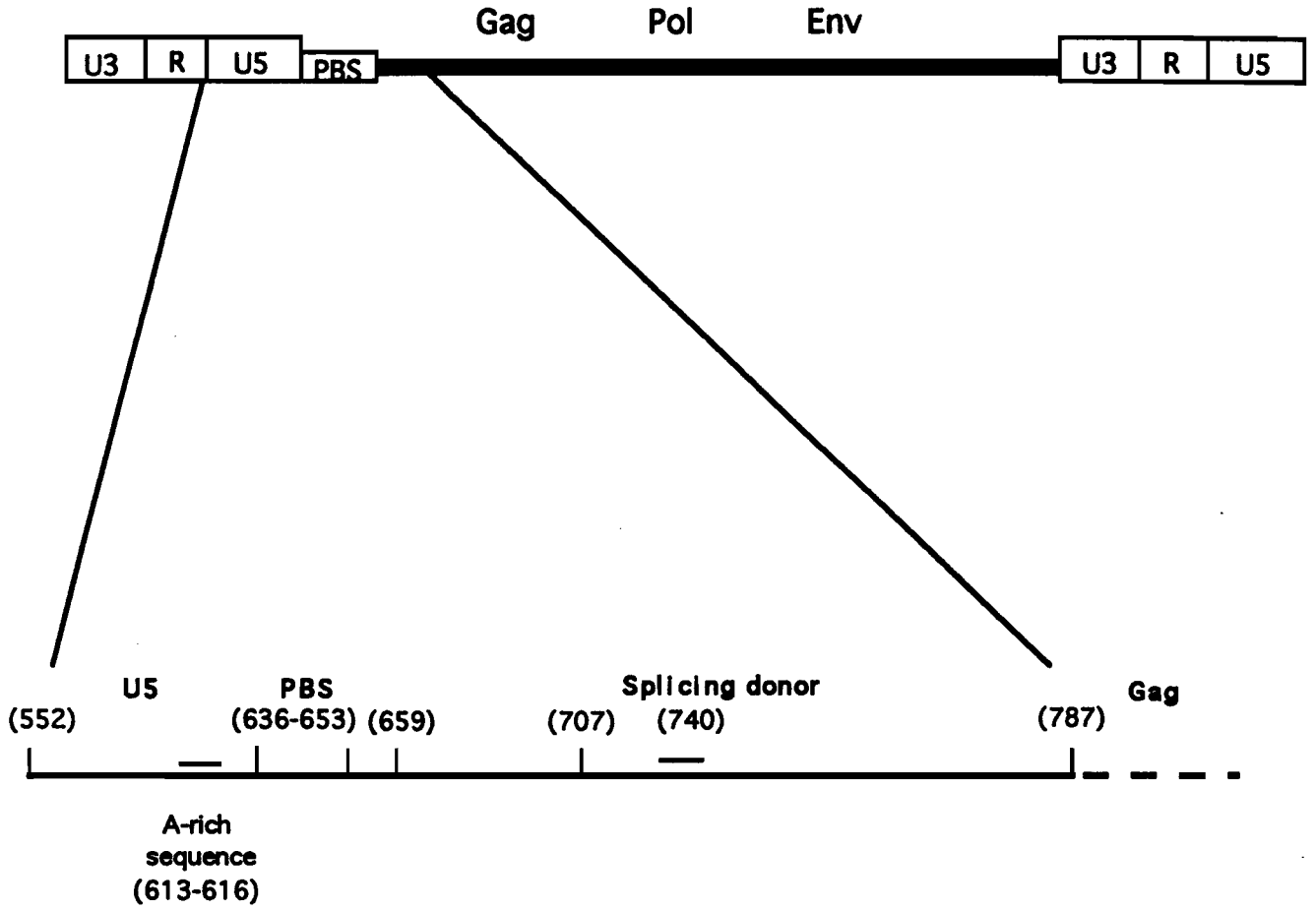
5.4 MATERIALS AND METHODS

5.4.1 Construction of molecular clones with deletion mutations in sequences surrounding the PBS of the proviral DNA

A PCR-based mega-primer mutagenesis method was used to generate deletions in the vicinity of the PBS using protocols described previously (Picard et al., 1994). The primer selected for the A-rich loop deletion, located 10 bp upstream of the PBS (i.e. nt positions 623-626), was 5'-CCCTTTTAGTCAGTGTGGTCTCTAGCAGCTGGCGCCC-3'; the primer for deleting 7 bp located downstream of the PBS (i.e. nt positions 654-660), was 5'-TGGCGCCCGAACAGGGACCTGAAAGGGAAACCAGAG-3'; the primer for deleting a 54-bp sequence immediately downstream of the PBS was 5'-TGGCGC CCGAACAGGGACCGCGCACGGCAAGAGGCG-3'. These were used as forward primers in conjunction with a backward primer (nt position 1405-1422) to specifically amplify sequences in regard to each of these deletions. The resulting amplified products were used as mega-primer with an additional primer located at the 5' terminus of the R region, PS (sequence: AGACCAGATCTGAGCCTGGGAG). Amplified fragments were then digested with Bgl II and Pst I and were inserted into a pSVK3 vector (Pharmacia Biotech, Montreal, QC). The cloned fragments were sequenced to verify that correct modifications of the viral gene sequences had been made and were introduced into HXB2D clone of infectious

HIV-1 proviral construct as described previously (Li et al, 1994). Fig.1 is a graphic description of these mutants.

Fig.1: Schematic description of deletion mutations surrounding the PBS of HIV-1 proviral DNA. pHIV/del-A represents a deletion of the A-rich loop, 10-nts upstream of the PBS; pHIV/del-7 represents a deletion of 7-nts, immediately downstream of the PBS; pHIV/del-LD represents a 54 nt-deletion downstream of the PBS. The initiation codon of the gag gene is indicated, along with relevant nucleotide positions.



pHIV/del-A: Deletion of four adenosine residues at nt position: 613-616.

pHIV/del-7: Deletion of seven nts immediately downstream of the PBS at nt position: 653-659.

pHIV/del-LD: Deletion of 54 nts immediately downstream of the PBS at nt position: 653-707.

5.4.2 Analysis of the replication ability of viral constructs

Molecular constructs containing the above mutations in the leader region, surrounding the PBS, were purified twice by $CsCl_2$ gradient ultracentrifugation. These plasmids were transfected into COS cells by standard calcium co-precipitation method (Maniatis et al., 1982). Virus-containing supernatants were harvested approximately 60-72 hr after transfection and were clarified by centrifugation at 3000 rpm for 30 min at 4°C in a Beckman bentch-top centrifuge prior to filtration with a 0.2 μ sterile membrane. Viral preparations were stored at -70°C.

For purposes of infection, the viral stock was thawed and treated with 100 U DNase I in presence of 10 mM $MgCl_2$ at 37°C for 1 hr to ensure that any contaminating plasmids from the transfection cultures had been eliminated (Li et al., 1994). Infection of MT-4 cells was performed by incubating cells at 37°C for 2 hr with virus (50ng p24), following which the cells were washed three times and incubated at 37°C with fresh medium. Culture fluids were monitored periodically for virus production by means of reverse transcriptase assay (Boulerice et al., 1990) and by p24 antigen detection kits (Abbott Laboratories, Abbott park, IL).

5.4.3 Detection of minus strong stop DNA

At various times post-infection (4-8 hr), total cellular DNA was isolated from infected cells using standard procedure (Maniatis et al., 1982), and was

subsequently analyzed by PCR using specific primers to amplify minus-strand strong-stop DNA [(-)ss DNA] (Giboa et al 1981; Zack et al 1989). PS was the forward primer located at the 5' terminus of the 'R' (nt 468-489) (Ratner et al., 1985), while the backward primer was AA55' (nt 621-604) (Ratner et al., 1985). The expected products amplified by primer pair PS/AA55' are 153 bp in length. Another set of primer pair (PS/Pst1) was selected to amplify full-length proviral DNA (Giboa et al 1981; Zack et al 1989). The sequence of Pst1 is 5' CCATTCTGCAGCTTCCTC 3' (nt position: 1522-1054)(Ratner et al., 1985). PCR assays were performed with 50 μg of sample DNA, 50 mM Tris-Cl(pH 8.0), 50 mM KCl, 2.5 mM MgCl2, 2.5 U Taq polymerase, 0.2 mM dNTPs, 10 pmols of ^{32}P-end-labeled forward primer, and 20 pmols of unlabeled backward primer. Reactions were standardized by simultaneous amplification of β-globin sequence as an internal control (Li., et al, 1994) and were run for 30 cycles of 94°C (1 min), 60°C (1 min), and 72°C (1 min). To clone the amplified products for sequencing analysis, the amplified products by primer pair (PS/Pst1) were digested with Bgl II and Pst 1 and inserted into a pSP72 cloning vector (Promega, Madison, Wisconsin). Positive clones were sequenced using a PCR-based double-stranded DNA (dsDNA) cycling sequencing system (Life Technologies, Mississauga, Ontario, Canada).

5.4.4 Analysis of viral RNA by Northern/slot Blot

Analysis of expression of viral RNA was carried out using slot blot and Northern blot procedures as described (Boulerice et al., 1990), as was transfection of COS cells with various constructs studied (Maniatis et al., 1982). The efficiency of transfection was routinely monitored by detection of viral

p24 antigen by means of immunofluorescence using monoclonal antibodies (Boulerice et al., 1990). For Northern blot, total cellular RNA from COS cells transfected with various molecular constructs was purified using a commercial RNA extraction kit (Biotecs, Houston, TX). The extracted RNA was treated with 100 U DNase I, followed by phenol-chloroform extraction and ethanol precipitation, to ensure removal of any contaminating plasmids and cellular DNA. The RNA pellets were resuspended in DEPC-treated ddH2O. RNA samples (up to 20 μg) were fractionated by 1% agarose gel containing formaldehyde as denaturant (Maniatis et al., 1982). RNA molecules were transferred to a Hybond-N nylon membrane (Amersham, Toronto, Canada) and hybridized using pBH10 viral DNA as radiolabelled probe (Nick translation system, Life Technologies, Toronto, Canada) as described (Boulerice et al., 1990).

To quantify viral RNA transcripts derived from COS cells transfected with various plasmids, total cellular RNA samples (collected at various times post-transfection) were immobilized onto nylon membranes using a slot blot apparatus, followed by UV irradiation according to manufacturer's instruction (Amersham). Hybridization was performed as described for Northern blot. The quantity of viral RNA was determined by counting excised filters corresponding each of the RNA samples after autoradiography. In some cases, viral RNA packaged in the virions (purified by sucrose gradient ultracentrifugation) was also determined using the same slot blot protocol.

5.4.5 Detection of viral proteins produced by transfected COS cells.

Expression of viral proteins in transfected COS cells was determined using a commercial ELISA kit for detection of p24 capsid antigen. Both intracellular

and extracellular p24 was determined in order to shed light on the efficiency of viral assembly.

Viral proteins were also analyzed by Western blot as described (Boulerice et., 1990). For this purpose, protein samples (standardized on the basis of viral p24) were fractionated on 12% SDS-PAGE and transferred to nitrocellulose filters (Boulerice et al., 1990). The filters were then blocked with 5% skim milk/0.05% Tween-20/PBS at 37°C for 2 hr, followed by exposure to sera from HIV-1 seropositive individuals. After extensive washing with 0.05% Tween-20/PBS, ^{125}I-labeled goat anti-human IgG (ICN) was added for further incubation for 1 hr at 37°C. The filters were washed three times, dried and exposed to a Kodak Xomat film at -70°C.

5.5 RESULTS

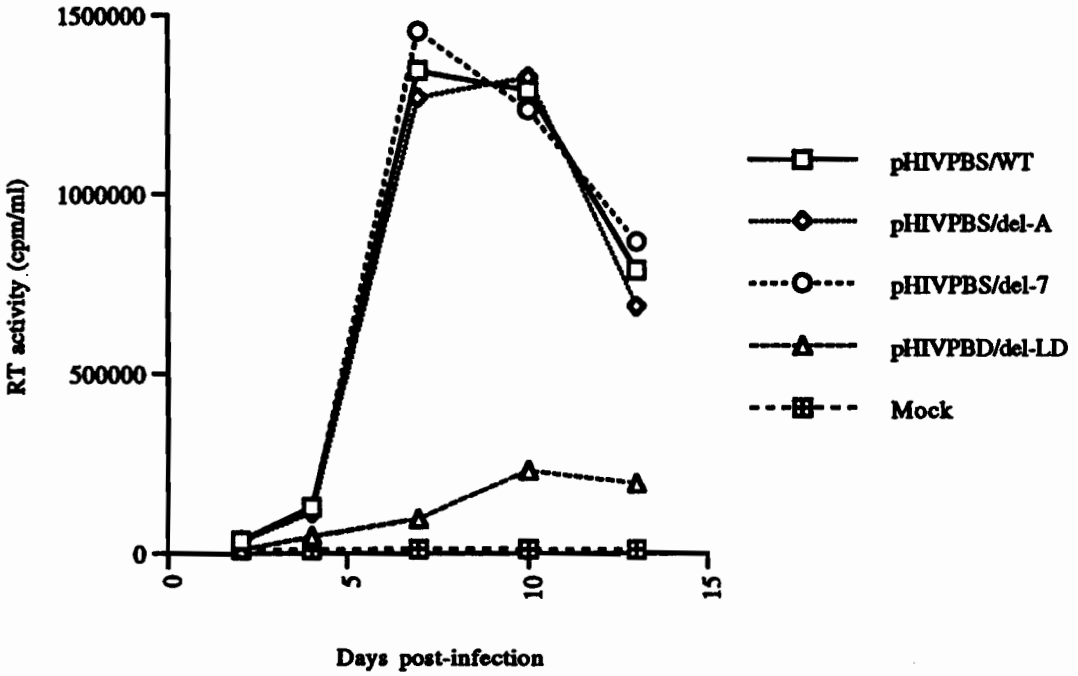
5.5.1 Replication potential of viral deletion mutants

The deletion mutations introduced into HIV-1 proviral DNA constructs (Fig.1) include a deletion of the conserved A-rich loop located 10-nt upstream of PBS (designed as pHIV/del-A), a deletion of the conserved 7-nt located downstream of PBS (designed as pHIV/del-7) (Fig.1) and an extensive deletion downstream of the PBS (pHIV/del-LD).

To investigate the replication potential of these molecular constructs, cell-free viruses (50 ng p24) from COS cells that had been appropriately transfected were used to infect MT-4 cells. Viral replication was monitored by detecting RT activities or p24 concentration in the culture supernatants (see

Materials and Methods). Fig.2 shows that wild-type virus (pHIV/WT), and each of the two deletion mutants (pHIV/del-A and pHIV/del-7) replicated efficiently, as determined by levels of RT activity in culture fluids after 3 and 7 days. In contrast, the pHIV/del-LD was significantly impeded in ability to generate viral progeny (Fig.2). Similar results were obtained on the basis of p24 determination using a wide range of viral inocula (not shown).

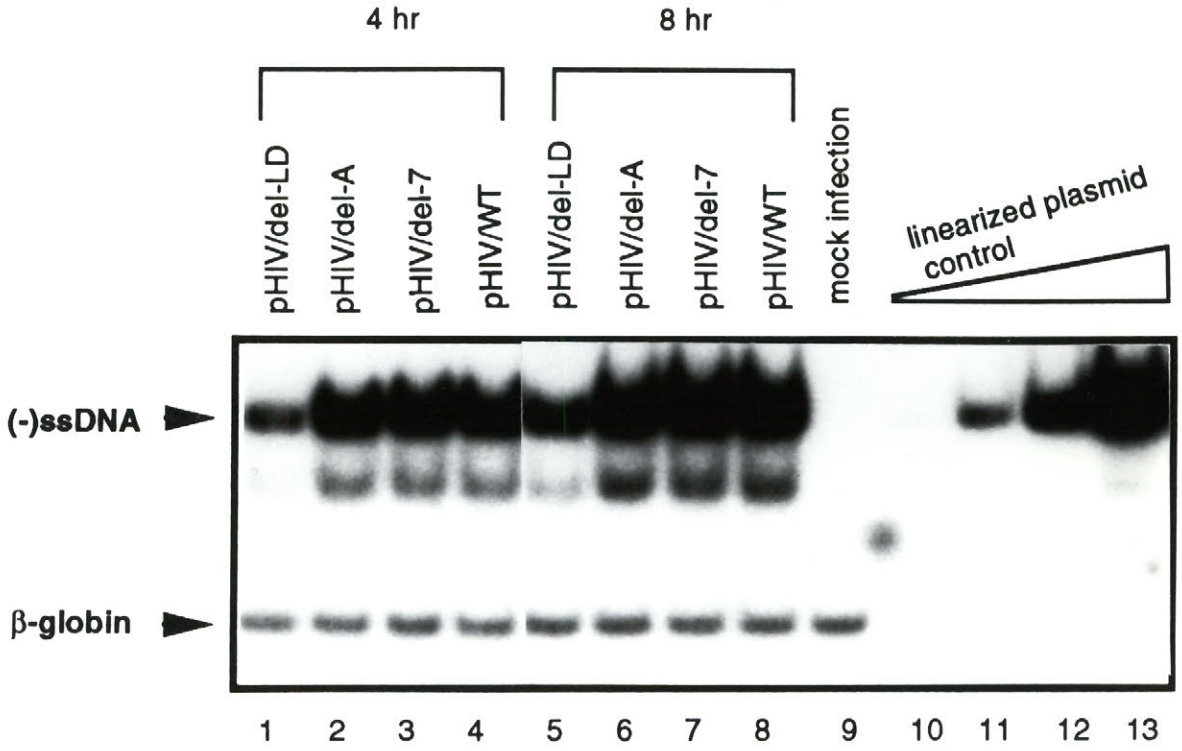
Fig.2 Viral replication capacity of various constructs. Cell-free viruses harvested from COS cells transfected with various molecular constructs (60-72 hr post-transfection) were used to infect MT-4 cells. Culture fluids were collected and monitored for reverse transcriptase activities. Decreased viral production from MT-4 cultures after one week in the case of pHIV/del-A, pHIV/del-7, and pHIV/WT was due to viral cytopathology; fresh cells were not added to these cultures.


1500000
1000000
500000
0
RT activity (cpm/ml)
0
5
10
15
Days post-infection
pHIVPBS/WT
pHIVPBS/del-A
pHIVPBS/del-7
pHIVPBD/del-LD
Mock

5.5.2 Generation of (-)ssDNA synthesis in infected cells

Similar levels of viral RNA were packaged into viruses derived from COS cells that had been transfected earlier with each of our various constructs (data not shown). We next asked whether the reduced replication potential of the pHIV/del-LD deletion mutant could be related to a defect in ability to initiate reverse transcription. Total cellular DNA was isolated at 4 and 8 hrs after infection of MT-4 cells with COS cell derived viruses, and analyzed by PCR, using selected primer pairs that specifically amplify (-)ssDNA. We found similar levels of (-)ss DNA from MT-4 cells infected by each of pHIV/del-A (Fig.3, lanes 2 and 6), pHIV/del-7 (Fig.3, lanes 3 and 7) and wild type virus (Fig.3, lanes 4/8). In contrast, MT-4 cells infected with pHIV/del-LD mutant contained significantly decreased levels of (-)ss DNA (Fig.3, lanes 1 and 4) a significant decrease in levels of synthesis were observed as compared to that of infection by the wild-type (lanes 1, 4) (approximately 10-fold decrease as quantitified by phosphor imager analysis). These results suggests that this untranslated sequence located immediately downstream of the PBS is necessary for efficient reverse transcription to occur.

Fig.3 Detection of synthesis of minus-strand strong-stop DNA [(-)ssDNA]. Viruses from COS cell cultures transfected with various molecular constructs were used to infect MT-4 cells. Total cellular DNA (approximately 50 μg) was isolated from infected cells at 4 and 8 hr after infection and subjected to PCR analysis using selected primers that specifically amplify (-)ssDNA (Zack et al., 1989). Primers amplifying β-globin sequence were used as an internal control (Zack et al., 1989). Infection by pHIV/del-LD: lanes 1, 5; pHIV/del-A: lanes 2, 6; pHIV/del-7: lanes 3, 7; pHIV/WT: lanes 4, 8; mock infection: lane 9: serially diluted HXB2D plasmids as postitive control (i.e. 10-fold dilution of plasmids in terms of copy numbers, i.e., 5×10^2; 5×10^3; 5×10^4; and 5×10^5), lane 10-14. Amplified (-)ssDNA products are shown as are β-globin products.


4 hr
8 hr
pHIV/del-LD
pHIV/del-A
pHIV/del-7
pHIV/WT
pHIV/del-LD
pHIV/del-A
pHIV/del-7
pHIV/WT
mock infection
linearized plasmid control
(-)ssDNA
β-globin
1 2 3 4 5 6 7 8 9 10 11 12 13

5.5.3 The untranslated region downstream of the PBS influences expression of viral gene transcripts

MT-4 cells infected by pHIV/del-LD contained integrated proviral DNA, even after 2-3 months (not shown). We therefore assessed whether this region could influence viral mRNA. Fig.4 depicts the results obtained of Northern blot analysis of viral RNA extracted from COS cells transfected with either mutant or wild type constructs. Levels of viral RNA transcripts expressed in COS cells transfected with pHIV/del-LD were found to be remarkably lower than those in cells transfected with pHIV/WT, although the three major bands representing unspliced, singly spliced and multiply spliced RNA were present in both cases (Fig.4). Similar patterns of viral RNA transcripts were observed from COS cells transfected with either pHIV/del-A or pHIV/del-7, as compared to that of the wild type (not shown).

These results were further confirmed by quantitative slot blot analysis. Fig.5 shows that dramatically reduced levels of RNA transcripts were present in cells transfected with pHIV/del-LD compared to the wild type (pHIV/WT), pHIV/del-A or pHIV/del-7. Differences were most pronounced at early time points after transfection. Not surprisingly, essentially equal amounts of viral RNA transcripts were detected among cells transfected with either pHIV/del-A or pHIV/del-7 as compared to the wild-type transfection throughout the whole time course (Fig.5).

To investigate protein expression and assembly in the pHIV/del-LD transfected cells, p24 detection and Western blot analyses were performed. As expected, COS cells transfected with pHIV/del-LD produced lower levels of both intracellular and extracellular p24 after 16 hr than cells transfected with

pHIV/WT (Table 1). Interestingly, transfection with pHIV/del-LD did not result in excess accumulation of intracellular p24 relative to the wild-type transfection, suggesting that viral protein assembly had proceeded normally. Viral protein profiles were essentially indistinguishable between cells transfected by mutant, or wild type constructs (Fig.6, lanes 2 and 3) (protein samples were equalized on the basis of p24 levels for purpose of comparison).

Fig.4 Northern blot for detection of viral RNA. Total cellular RNA was purified from COS cells 16 hr after transfection with either pHIV/del-LD or pHIV/WT. Lane 1: 20 μg of RNA from cells transfected with pHIV/del-LD; lane 2: 20 μg of RNA from cells transfected with pHIV/WT; Lane 3: 10 μg of RNA from cells transfected with pHIV/del-LD; lane 4: Lane 1: 10 μg of RNA from cells transfected with pHIV/WT; lane 5: 20 μg of RNA from mock-transfected COS cells. Molecular size markers are indicated.

Fig.4 Northern blot for detection of viral RNA. Total cellular RNA was purified from COS cells 16 hr after transfection with either pHIV/del-LD or pHIV/WT. Lane 1: 20 μg of RNA from cells transfected with pHIV/del-LD; lane 2: 20 μg of RNA from cells transfected with pHIV/WT; Lane 3: 10 μg of RNA from cells transfected with pHIV/del-LD; lane 4: Lane 1: 10 μg of RNA from cells transfected with pHIV/WT; lane 5: 20 μg of RNA from mock-transfected COS cells. Molecular size markers are indicated.

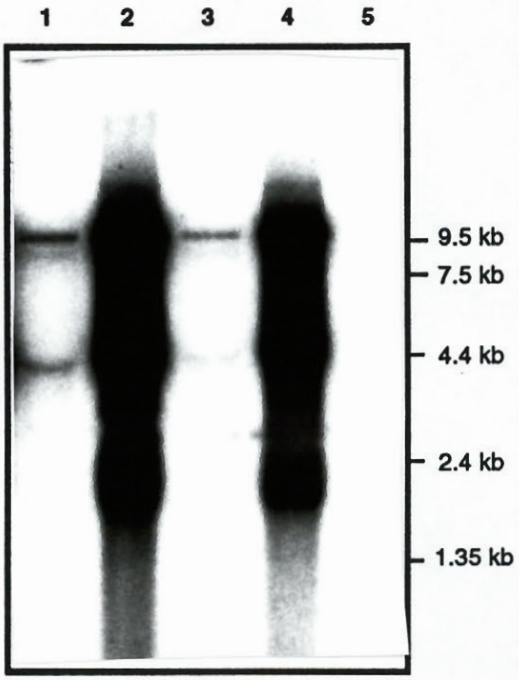

1
2
3
4
5
9.5 kb
7.5 kb
4.4 kb
2.4 kb
1.35 kb

Fig.5 Quantitative determination of viral RNA transcripts by slot blot. Total cellular RNA was harvested from COS cells and purified at 16, 24, 48, 72 hr, respectively after transfection with various molecular constructs followed by purification of total cellular RNA at various times post-transfection, i.e. 16, 24, 48, and 72 hr respectively. Relative intensities were calculated by comparison with levels of radioactivity obtained with wild-type-transfections after 72 hr defined as 100% (2478 cpm). Standard deviations (SD) are indicated by error bar (four separate experiments).

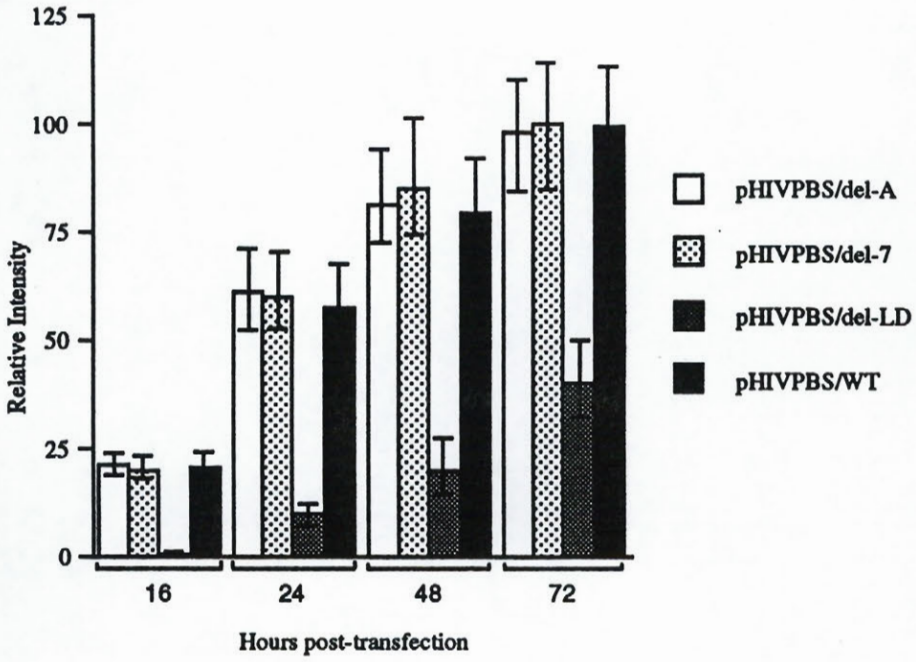
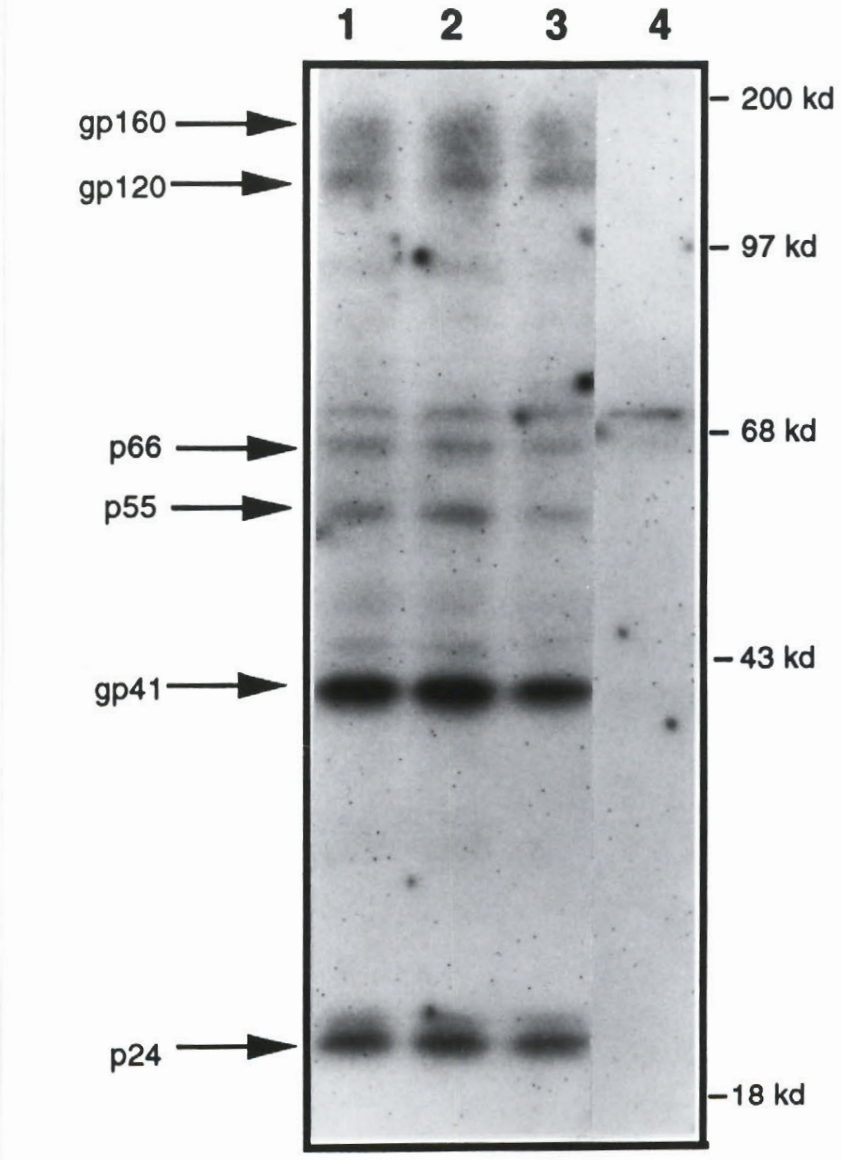

125
100
75
50
25
0
Relative Intensity
16
24
48
72
Hours post-transfection
pHIVPBS/del-A
pHIVPBS/del-7
pHIVPBS/del-LD
pHIVPBS/WT

Table 1 Determination of intracellular and extracellular p24 from COS cells transfected with either pHIV/del-LD or pHIV/WT (ng/ml)[a]

Hours post-transfection	Intracellular p24		Extracellular p24	
	pHIV/del-LD	pHIV/WT	pHIV/del-LD	pHIV/WT
16	5.22 ± 0.48	170 ± 15.2	4.49 ± 0.32	187 ± 16.8
24	17.9 ± 1.55	241 ± 22.2	18.7 ± 16.2	258 ± 25.3
48	64.8 ± 6.02	233 ± 24.5	97.0 ± 10.2	304 ± 29.8
72	62.9 ± 5.89	245 ± 23.3	145 ± 13.5	300 ± 31.2

a: COS cells were transfected with either pHIV/del-LD or pHIV/del-WT. At various time post-transfection, both intracellular and extracellular viral capsid protein (p24) was determined using Abbot ELISA kit. The data presented here were derived from 4 separate experiments. Standard deviations (SD) were also indicated.

Fig.6 Viral protein analysis by Western blot. Cellular proteins isolated from COS cells were analyzed by Western blot as described in Materials and Method. Lane 1: cellular proteins derived from COS cells transfected with pHIV/del-LD; lane 2: cellular proteins derived from COS cells transfected with pHIV/WT; lane 3: represents a positive control using proteins derived from MT-4 cells infected by HTLV-IIIb strain; lane 4: cellular protein from mock-transfected COS cells.


1
2
3
4
gp160
gp120
p66
p55
gp41
p24
200 kd
97 kd
68 kd
43 kd
18 kd

5.6 DISCUSSION

The primer binding site (PBS) of HIV-1 is situated about 180-nt from the 5' terminus of the unspliced RNA (Ratner et al., 1985). It is surrounded by the R/U5 region at its 5' border and a 133 nt untranslated sequence at its 3' end (Ratner et al., 1985). Initiation of reverse transcription takes place at PBS, where the tRNA primer is bound.

Genetic studies in the avian retroviral system suggested that the 5' viral RNA must be maintained in an appropriate configuration/orientation for efficient reverse transcription to occur (Leis et al., 1993). Indeed, sequences upstream of the PBS were found to interact with the TΨC loop of the $tRNA^{Pro}$ primer, in addition to the base pairing of the 18-nt complementary sequences between the PBS and the 3' region of the tRNA.

In the case of HIV-1, a conservative A-rich loop, located 10 nt upstream of the PBS, was shown to interact with the anticodon loop of $tRNA^{Lys.3}$ under cell-free condition (Isel et al., 1993). We now show however, that deletion of the A-loop (pHIV/del-A) did not impact on either synthesis of (-)ssDNA or viral replication. Although the A-rich loop may not be essential for viral replication, it might function, together with other sequences, to specify utilization of primer tRNA. It has been shown that the PBS (complementary to $tRNA^{Lys.3}$) can be replaced with sequences complementary to alternate tRNAs and these PBS mutants were able to replicat, although less efficiently than the wild-type. Yet, the PBS reverted back to the wild type within one month in culture (Li et al., 1994; Das et al., 1995; Wakefield et al., 1995). Most recently, one group has generated a stable HIV mutant containing a PBS complementary to $tRNA^{His}$ and the A-rich loop replaced by sequence complementary to the anti-codon loop of $tRNA^{His}$ (Wakefield et al., 1996). This virus replicated with wild-

type kinetics probably due to interactions between the tRNA anticodon loop and viral sequences upstream of the PBS. However, involvement of sequences upstream of the PBS, in addition to the A-rich loop, was inferred since substitutions of nucleotides between the A-rich loop and the PBS were observed during the late stages of infection. Therefore, these additional substitutions might have contributed to the ability of these viruses to use $tRNA^{His}$ as a replication primer and to wild type replication in tissue culture.

A previous studies suggested that a short 6 nt sequence, located immediately downstream of the PBS, might be important in specifying utilization of $tRNA^{Lys.3}$ (Kohlsteadt and Steitz, 1992). However, we have now shown that this sequence is apparently unnecessary for either synthesis of (-) ssDNA or viral replication(pHIV/del-7) (Fig.2 and 3). Others have reported that nucleotide substitutions downstream of the PBS were observed in HIV-1 revertants with original deletions within the PBS; these revertants apparently have wild type growth capability (Rhim et al., 1991). Therefore, the 7-nt sequence downstream of the PBS may not be essential for maintenance of viral RNA secondary structure, nor is this region apparently involved in interactions with $tRNA^{Lys.3}$.

We have also decided to characterize an untranslated sequence, located immediately downstream of the PBS, in the context of an infectious molecular clone. Toward this end, we generated a mutant, i.e. pHIV/del-LD, that was missing 54 nt immediately downstream of the PBS without compromising the previously identified packaging/dimerization signal and splicing donor (Lever et al., 1989; Aldovini et al., 1989; Skripkin et al., 1994; Darlix et al., 1990). This deletion resulted in drastic reductions in viral replication capacity (Fig.2). We hypothesized that the proximity of this 54 nt segment to the PBS could have played a role in limiting viral replication, due to a defect in reverse

transcription. It is also likely that a lower efficiency in RNA dimerization might contribute to the decreased levels in synthesis of (-)ss DNA (our unpublished observation obtained under cell-free conditions). Indeed, we found that the pHIV/del-LD mutant was hindered in ability to generate viral DNA (Fig.3).

Deletion of the 5' portion of the untranslated region, immediately downstream of the PBS, i.e. pHIV/del-LD, also caused a significant decrease in expression of viral RNA transcripts in infected cells. This finding was not expected, as elements located upstream of the PBS can interact with inducible or constitutive cellular transcription factors (Cullen, 1991). The diminution in levels of viral protein seen with the 54 nt deletion, is most likely due to low concentrations of viral mRNA. Therefore, the 5' portion of the untranslated sequence appears to have multiple functional roles in viral replication. Other data from our group based on work with reverse transcriptase, viral RNA, nucleocapsid protein, and $tRNA^{Lys.3}$, suggests that the defect in reverse transcription in the case of pHIV/del-LD might be due to poor hybridization between $tRNA^{Lys.3}$ and the viral RNA template (Chapter 3). Further study will be necessary to define minimal sequences within the 54 nt region responsible for regulation of reverse transcription and expression of viral mRNA.

CHAPTER 6

GENERAL DISCUSSION

The proviral genome of the human immunodeficiency virus type 1 (HIV-1) is approximately 9.5 kilobases long. The structural genes, located in the middle region, encode the *Gag*, *Pol* and *Env* polyproteins and are flanked by long terminal repeats (LTR) that are important for binding of transcription factors. Within the HIV-1 genome, open reading frames flanking the *env* gene encode several regulatory proteins including Tat, Rev, Nef, Vif, Vpr and Vpu (Cullen, 1991). The life cycle of retroviruses starts with fusion of the virus with the membrane of a susceptible cell initiated by a high-affinity interaction of the viral envelope protein with the CD4 moiety. Conversion of viral RNA to double-stranded DNA is catalyzed by the virion-encoded reverse transcriptase (RT). The reverse transcribed DNA is inserted into the cellular chromosomal DNA and becomes a permanent resident of the host cell. The virus essentially utilizes cellular transcriptional and translational machinery to generate its own progeny.

To better understand retroviral replication, particularly the mechanism of reverse transcription, it was important to investigate, using both *in vitro* and *in vivo* approaches, interactions of essential macromolecules that include nucleocapsid protein (NCp), $tRNA^{Lys.3}$, viral RNA template, and RT. These factors are believed to directly participate in reverse transcription.

We started our investigations with the expression and purification of NCp15 as a fusion protein with glutathione-S-traansferase (GST) in a bacterial over-expression system (Smith and Johnson, 1988). The native, biologically active NCp15 was readily obtained by affinity chromatography followed by thrombin cleavage of the fusion protein. Subsequent characterization of this protein with regard to its RNA binding properties indicated that NCp15 bound to HIV-1 RNA specifically, whereas the fusion protein, GST-NCp, was non-selective in the binding of RNA, with binding to all RNA transcripts, including

viral and non-viral derivatives, with equal efficiency. Importantly, the RNA binding properties of NCp15 show remarkable similarities to that of its precursor, $p55^{gag}$ (Luban and Goff, 1991), which is believed to be the key factor for incorporation of viral genomic RNA into the mature virion (Luban and Goff, 1991; Berkowitz et al, 1995). These data imply that the sequence of NCp15 within the $p55^{gag}$ precursor must be critical for selective encapsidation of viral RNA.

Another functional activity of the nucleocapsid protein (NCp) is its ability to promote reverse transcription (Prats et al., 1988). Since processing of NCp15 into four additional peptides most likely takes place inside the released, mature virion and is dependent on the viral genomic RNA (Gelderbloom et al., 1991; Henderson et al., 1992; Wondrak et al., 1993; Sheng and Erickson-Viitanen, 1994), the fully-processed nucleocapsid protein (NCp7) may be the form of NCp that is involved in reverse transcription during the new round of infection. For synthesis of minus strand DNA to occur, the tRNA primer must be annealed to the viral RNA template. However, both the viral RNA template and tRNA are highly structured, folding into several stems and loops (Rich et al., 1976; Harrison et al., 1992; Baudin et al., 1992; Berkhout et al., 1993; Litvak et al., 1993). Without being appropriately unfolded, these structures could potentially hinder the annealing process between tRNA and the viral RNA template. By virtue of its affinity to bind single strand nucleic acids (Kahn and Giedroc, 1992; Karpel et al., 1987), NCp might distabilize the secondary structure of both tRNA and the viral RNA template. Yet, essentially little is known of the mechanisms involved in the annealing processes between the primer and the template.

Since the outcome of annealing between tRNA and the viral RNA template is formation of a RNA/RNA duplex, the ability of NCp to stimulate

reverse transcription cannot be solely explained by its preferential binding to single-stranded nucleic acids, and other factors must govern the stabilization of the RNA/RNA hybrids. In an attempt to investigate the mechanism regarding NCp7-mediated formation of the tRNA/viral RNA complex in reverse transcription, we established a cell-free RT assay consisting of 5' viral RNA, $tRNA^{Lys.3}$, RT and NCp7. This system also enabled us to study primer placement. NCp was found to stimulate the generation of (-) ss DNA, consistent with previous observations (Barat et al., 1989; Prats et al., 1988). However, efficient and specific DNA synthesis was only observed at NCp7 concentrations close to the predicted NCp/RNA ratio predicated in the virion (Karpel et al., 1987), i.e., one NCp molecule covering 6 nucleotide residues. Remarkably, at these concentrations, NCp7 displayed a capability to inhibit synthesis of non-specific reverse transcribed DNA products, which were initiated due to self-priming of the RNA templates. The inhibitory effects on generation of non-specific reverse transcribed products by NCp7 is likely due to its helix-destabilizing activity. However, it is quite clear from our results that NCp7 conferred specificity to reverse transcription (Fig.2), i.e., efficient stimulation of specific reverse transcribed DNA synthesis accompanied with drastic inhibition of non-specific DNA synthesis. Whether NCp7 could inhibit generation of by-products initiated by the RNA template itself under *in vivo* physiological condition remains uncertain. It was proposed previously that the virion genomic RNA often contains nicks (Coffin, 1979; 1985; Darlix et al., 1990; Junghans, et al; 1982; Tanchou et al., 1995; Temin, 1990). These nicks might initiate synthesis of non-specific DNA products.

In sharp contrast to that of the $tRNA^{Lys.3}$-initiated reverse transcription, NCp7 was found to inhibit, in a clear dose-dependent manner, reverse transcription initiated by the 18-nt ribonucleotide primer (rPR) that

was complementary to the PBS. Further investigation using primer placement studies indicated that NCp7 was necessary for formation of the tRNA/RNA complex, whereas NCp7 was found to prevent formation of the complex between rPR and the RNA template. These results suggest that NCp7 could exert opposite effects (annealing v.s deannealing) on the two kinds of substrates, and that the remaining portion of the $tRNA^{Lys.3}$ (the bases not directly base pairing to the PBS), probably via interaction with the neighboring sequences of the PBS of the template, could be the driving force for an equilibrium that favors annealing between $tRNA^{Lys.3}$ and the viral RNA, since removal of these remaining portion of $tRNA^{Lys.3}$ (as in the case of rPR) prevented the annealing process. We also found the " A " rich loop and a 7-nt sequence, located upstream and downstream of the PBS respectively, imparted minimal effects on primer placement and synthesis of (-)ss DNA. However, an extended deletion located downstream of the PBS was found to be necessary for efficient formation of $tRNA^{Lys.3}$/RNA complex as well as (-)ss DNA synthesis.

Our studies of reverse transcription under cell-free conditions suggested that multiple site-interactions between tRNA primer and 5' viral RNA (sites more than just interaction between the complementary sequences at the PBS and the 3' end of tRNA primer) are required for efficient reverse transcription.

To further investigate functional roles of the PBS and its surrounding sequences in HIV-1 replication, we either deleted or replaced the original wild type PBS (complementary to tRNALys.3) with sequences complementary to $tRNA^{Lys.1,2}$ or $tRNA^{Phe}$. We took two reasons into account for choosing these PBS mutants: i) The utility of tRNA as primer in retroviruses, i.e., $tRNA^{Lys.1,2}$ was found to function as primer in other retroviruses, e.g., Mason-Pfizer monkey virus, whereas $tRNA^{Phe}$ has never

been found to be utilized as primer in retrovirus; ii) The abundance of tRNA species in HIV-1 virion, i.e., $tRNA^{Lys.1,2}$ is packaged into wild-type HIV-1 in amounts even greater than that of $tRNA^{Lys.3}$, whereas $tRNA^{Phe}$ is present at much lower levels (Jiang et al., 1993; Leis et al., 1993). Transfection of COS cells with these molecular constructs yielded similar levels of viral progeny that were indistinguishable with regard to viral proteins and tRNA content, suggesting PBS may not be directly involved in selective incorporation of tRNA species into the virion. This also yields supportive evidence that viral proteins may be the key determinant for encapsidation of tRNA primers (Jiang et al., 1993; Mak et al., 1993). A novel finding of this study is that virus produced after transfection by the two PBS replacement mutants, pPBS-Lys1,2 and pPBS-Phe, were infectious, although less so than the wild-type viruses. However, despite a delay in production of viral p24 [CA, capsid)] and RT, the rate of virus replication by the mutants eventually reached wild-type level. Viruses derived from long-term cultures (3 weeks after infection) were used for re-infection studies; we found that these viruses behaved indistinguishably from the wild type, suggesting there was a shift in phenotype in these viruses during subsequent rounds of infection. Molecular analysis of reverse transcribed products from cells infected by the mutated viruses indicated that $tRNA^{Lys.1,2}$ and $tRNA^{Phe}$ could function as primers for reverse transcription during the early stages of infection. However, during the subsequent rounds of infection, reversion of the mutated PBS to wild-type sequences was observed, accompanied by increased generation of viral particles in the culture fluids. These data strongly suggest that HIV-1 has a strict commitment to use $tRNA^{Lys.3}$ as a replication primer. This is in sharp contrast to its murine counterpart (MLV), which appears to be less selective in primer utilization (Le Grice, 1993; Lund et al., 1993).

The mechanisms responsible for the observed reversion are yet to be defined. Although HIV-1 RT does display, to certain extent, selectivity in its binding to $tRNA^{Lys.3}$ (Barat et al., 1989; Barat et al., 1993), data from quantitative analysis argues against the notion of specific binding between $tRNA^{Lys.3}$ and RT (Arion et al., 1993; B. Wohrl, personal communication). There is strong evidence that indicates that binding between NCp and tRNA is essentially non-specific (Mely et al., 1995). However, it has recently been suggested that formation of a competent initiation complex between tRNA and viral RNA requires not only base pairing between the PBS and the 3' end 18-nt region of tRNA, but also additional interactions between sequences neighboring the PBS and the remaining portion of the tRNA primer (Isel et al., 1993; Kohlsteadt and Steitz, 1992; Leis et al., 1993). In the avian retroviral system, additional interaction between the U5 RNA and the TΨC could play an important role to stabilize the primer/template complex, whereas in HIV-1, the " A " rich loop was suggested to interact with the anticodon region of $tRNA^{Lys.3}$. The resulting loop-loop interaction between tRNA and the RNA template, combined with normal PBS-tRNA binding, might give rise to significant alterations in secondary structure of the primer-template complex relative to that occurring when only the PBS interact with tRNA (as in the case of the pPBS-Lys.1,2 and pPBS-Phe mutants). Indeed, results from *in vitro* reverse transcription experiments indicated $tRNA^{Lys.3}$ is a more efficient primer than either $tRNA^{Lys.1.2}$ and $tRNA^{Phe}$ (Chapter 4) (Li et al., 1994). Annealing of $tRNA^{Lys.3}$ to the mutated PBS (due to partial homology) could result in regeneration of wild-type PBS (reversion of mutant virus to the wild type). Under cell-free conditions, NCp7 annealed $tRNA^{Lys.3}$ to the viral RNA template more efficiently than it did to either $tRNA^{Lys.1,2}$ or $tRNA^{Phe}$ (data not shown).

Data from Chapter 3 and 4, as well as from previous observations (Isel et al., 1993; Kohlsteadt and Steitz, 1992; Leis et al., 1993), indicated that sequences around the PBS could be important for efficient initiation of reverse transcription. However, the previously identified regions by foot-printing analysis, i.e., the " A " rich loop and the 7 nt-stretch located upstream and downstream of the PBS respectively (Isel et al., 1993; Kohlsteadt and Steitz, 1992), were found to impart minimal effects on tRNA primer placement and reverse transcription in cell-free system (Chapter 3). Therefore, it was essential to test these mutants in the context of an infectious molecular clone. In Chapter 5, we presented evidence that the two mutants, pHIV/del-A and pHIV/del-7, displayed similar replication kinetics as compared to that of the wild-type virus. In contrast, the large deletion of 54 nt located immediately downstream of the PBS resulted in a drastic reduction in viral infectivity. Quantitative PCR analysis of nascent reverse transcribed DNA from cells infected by various viruses revealed no difference with regard to the efficiency of reverse transcription following infection by pHIV/del-A, pHIV/del-7 or the wild type virus (pHIV/WT). However, a significant reduction with respect to the efficiency of reverse transcription was observed following infection of the cells by pHIV/del-LD. These results obtained from *in vivo* condition correlate reasonably well with the *in vitro*/cell-free reverse transcription studies (Chapter 3).

Overall, by using both *in vitro* and *in vivo* approaches, we investigated the functional roles of essential macromolecules that are involved in reverse transcription. We found that HIV-1 demonstrated a strict commitment for utilizing $tRNA^{Lys.3}$ as a replication primer. Alongside data from other groups, our studies indicated that such a commitment of the virus for choosing specific tRNA as replication primer may be largely based on the

mechanism by which the viral nucleocapsid protein (NCp) confers remarkable specificity to reverse transcription. This probably occurs by NCp's ability to form a competent transcription complex with $tRNA^{Lys.3}$ and viral RNA template that is recognizable by the viral enzyme (RT) to execute efficient reverse transcription. However, protein-protein interaction involving both virion- and cell-derived factors may also be important in this regard (Goff, 1990; Darlix et al., 1995; Litvak et al., 1993; Takahashi et al., 1994).

Evidence from both biochemical and genetic studies indicates that sequences outside of the PBS could play important roles to influence the efficiency of reverse transcription (Leis et al., 1993; Isel et al., 1993; Kohlsteadt and Steitz, 1992; Li et al., 1994; Das et al., 1995; Wakefield et al., 1995; Whitcome et al., 1995). We felt it was essential to investigate potential sequences that might contribute to the efficiency of reverse transcription. In HIV-1, data from cell-free systems are controversial with regard to potential regions that might be involved, e.g., while others have found the A-rich loop located upstream of the PBS and 6-nt located downstream of the PBS are involved (Isel et al., 1993; Kohlsteadt et al., 1992), we found, by using reaction conditions that are more physiological (Chapter 3), that deletion of either of the two regions imposed little effect on synthesis of (-)ssDNA. In contrast, we identified a 54-nt region downstream of the PBS that is necessary for efficient reverse transcription to occur (Chapter 3). Therefore, it was imperative to investigate the functional roles of these sequences in the context of an infectious molecular clone. To this end, we introduced several deletions into the proviral genome of HIV-1 : i) A deletion of the A-rich loop (Isel et al., 1993), designated as pHIV/del-A; ii) A deletion of seven nucleotides located downstream of PBS (Kohlsteadt and Steitz, 1992), designated as pHIV/del-7; and iii) Deletion of the 5' portion of the uncoding region located immediately downstream of the PBS, designated as

pHIV/del-LD. However, our data show on the basis of both in vitro and in vivo systems that deletion in either the A-rich loop (pHIV/del-A) or the 7 nt segment immediately downstream of the PBS (pHIV/del-7) had little effect on synthesis of (-)ssDNA or viral replication. In contrast, we identified a 54 nt-region downstream of the PBS that is necessary for both reverse transcription and viral infectivity. Further studies on this mutant virus (pHIV/del-LD) suggested that this 54-nt sequence located immediately downstream of the PBS is dispensable for encapsidation of viral genomic RNA and viral protein assembly, but is critical for the expression of viral transcripts. The near-abolition in infectivity in the case of pHIV/del-LD may be due to defects in reverse transcription possibly caused by poor hybridization of $tRNA^{Lys.3}$ and RNA template (Chapter 3). However, the mechanisms that underlie the role of the untranslated sequence downstream of the PBS on transcripts formation are not understood (Chapter 6).

Further studies will be necessary to identify minimal sequences in regulation of reverse transcription and generation of viral mRNA transcripts.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

This thesis was written by the adaptation of articles published in or submitted to refereed journals which are shown in the title page of each chapter. A summary of our contribution to original knowledge is the following:

Chapter 2. We have successfully cloned and expressed HIV-1 NCp15 in a biologically active form and investigated its RNA binding properties. The RNA binding characteristics of NCp15 were found to be strikingly similar to those described for recombinant p55^{gag} polyprotein, especially with respect to the specificity of NCp-RNA interactions. This specificity appears to be a function of NCp conformation, since the GST-NCp fusion recombinant protein shows no RNA binding specificity. This implies that the conformation of NCp sequences in the p55^{gag} polyprotein must be remarkably similar to that of mature processed 15 kDa NCp. We have also identified an additional region consisting of nucleotides 1244-1412 of the HIV-1 sequence that appears to be necessary for strong binding to NCp. Other transcripts, even those containing previously identified packaging sequences (288-344), were bound only weakly by NCp.

Chapter 3. We investigated the role of fully-processed nucleocapsid protein (NCp7) in reverse transcription by using a cell-free reverse transcription assay comprising various RNA templates, RT, tRNA$^{Lys.3}$, and NCp7. A novel finding of this work is that NCp7 confers specificity to reverse transcription by stabilizing and/or mediating the formation of the tRNA$^{Lys.3}$/RNA complex that is recognized by RT for initiation of (-)ssDNA synthesis. This is evidenced by efficient stimulation of (-)ssDNA synthesis, concomitant with the suppression of non-specific reverse transcribed DNA products. The fact that NCp7 inhibits rPR (an 18-mer ribooligonucleotid complementary to the PBS) initiated-reverse transcription in a concentration

dependent manner suggests that the remaining portion of tRNALys.3 plays an essential role in reverse transcription, presumably as a driving force for an equilibrium that favors formation of a tRNA/RNA complex mediated by NCp7. The A-rich loop and the 7 nt-sequences which were previously identified as important elements in the formation of a competent transcription primer/template complex (Isel et al., 1993; Kohlsteadt and Steitz, 1992) were found to impart relatively minor effects on reverse transcription, whereas a deletion downstream of the PBS was essential for efficient formation of the tRNA/RNA complex as well as synthesis of minus-strand DNA.

Chapter 4. We found that HIV-1 RT demonstrated remarkable specificity in using $tRNA^{Lys.3}$ (the natural primer) as a replication primer. This is evidenced by the following: i) tRNAs other than $tRNA^{Lys.3}$ can be utilized by HIV- RT as replication primers when the PBS was replaced by complementary sequences to these tRNAs during the early stages of infection. However, the PBS replacement mutants demonstrated a much lower rate of replication in tissue culture. ii) During subsequent rounds of infection, reversion of the mutated PBS to wild type sequences resulted from $tRNA^{Lys.3}$ being used as primer, and this in turn was accompanied by increased production of viral gene products. iii) Data from *in vitro* reverse transcription experiments indicated that initiation with other tRNAs, i.e., $tRNA^{Lys.1,2}$ and $tRNA^{Phe}$, occurred much less efficiently than that with $tRNA^{Lys.3}$ when (-)ssDNA was synthesized from a synthetic RNA template containing a PBS complementary to the respective tRNA isoacceptors. Ours was the first report to show that HIV-1 is able to utilize alternative tRNAs as replication primers. However, the virus has evolved to specifically choose $tRNA^{Lys.3}$ as replication primer to maintain a growth advantage.

Chapter 5. We have investigated the roles of sequences around the PBS in reverse transcription in the context of infectious molecular clones. Consistent with in vitro studies of reverse transcription, involving modified templates (Chapter 3), we found that the previously identified A-rich loop and a 7 nt-sequence, located upstream and downstream of the PBS respectively, imposed little effect on reverse transcription. This is evidenced by data from analysis of nascent reverse transcribed DNA products from cells infected with these viruses. Furthermore, these two regions appear to impose minimal influence on the whole replication cycle, as extensive characterization of these two mutants in infectivity studies which extended more than 5 months revealed non-detectable defects in viral replication as compared to that of the wild-type. However, deletion of the 54-nt downstream of the PBS greatly reduced viral infectivity by nearly 40,000 fold. In fact, production of viral progeny was gradually shut off over a certain period of time. Most importantly, prior to this study, the functional roles of this region was barely known, especially in the context of an infectious molecular clone. Here, we presented evidence that this untranslated region is crucial for viral replication, i.e., the remarkably decreased replication potential of pHIV/del-LD was caused by severe defects in both reverse transcription and expression of viral mRNA transcripts.

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