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HIV-1 Reverse Transcription Initiation: Impact of A-rich Loop Deletion and M184V Substitution and Development of Novel Antiretroviral Strategies

By

Xin Wei

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of the requirements for the degree of Doctor of Philosophy

Department of Microbiology and Immunology
McGill University, Montreal, Canada
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ABSTRACT

Reverse transcription of human immunodeficiency virus type-1 (HIV-1) is primed by cellular $tRNA^{Lys3}$, which is selectively packaged into viral particles where it is bound at its 3' terminus to a complementary sequence of viral RNA termed the primer binding site (PBS). In addition to the PBS, other regions within the viral genome also interact with $tRNA^{Lys3}$. Initiation of HIV-1 reverse transcription requires specific recognition of the viral genome, tRNA primer, and reverse transcriptase (RT). In this work, we study the important role played by the initiation complex in the initiation of HIV-1 reverse transcription. An "A-rich loop" located upstream of the PBS has been shown to interact with the anticodon loop of $tRNA^{Lys3}$ and deletion of this A-rich loop caused diminished viral replication fitness. We have now studied the mechanisms involved in the altered replication capacities of the deletion-containing viruses in the context of both wild type HIV-1 and viruses also containing the M184V substitution in RT. We found that the M184V mutation in RT compromises the ability of deletion-containing viruses to restore wild-type replication. Further biochemical study indicates that both the M184V mutation in RT and deletion of sequences upstream of PBS caused diminished viral replication fitness by compromising the efficiency of reverse transcription initiation.

Since the initiation of DNA synthesis was shown to be a highly specific process, it represents a potential target for the development of novel antiviral agents. We developed strategies for inhibition of the HIV-1 replication via interference with the $tRNA^{Lys3}$/viral RNA complex. To target primer $tRNA^{Lys3}$, we employed oligodeoxyribonucleotides (ODNs) that are complementary to different parts of the tRNA primer. To target viral RNA, we devised a $tRNA^{Lys3}$-like molecule, termed $tRNA^{Lys*}$, that

contained sequence alterations that direct initiation from a region distant from the natural PBS, designated PBS*. PBS* is involved in the formation of the natural tRNA/PBS complex and binding of $tRNA^{Lys*}$ was shown to interfere specifically with the initiation of reverse transcription. Inhibition of the synthesis of (-) strand strong-stop DNA was achieved successfully with both strategies by interfering with the formation of the initiation complex.

ABSTRACT

La transcription inverse du virus d'immunodéficience humaine type-1 (VIH-1) est amorcée par l'ARNt^{Lys3} cellulaire, qui est sélectivement encapsidé dans la particule virale où il est lié par sa région 3' terminale à une séquence complémentaire de l'ARN viral appelée site de liaison de l'amorce ("primer binding site", PBS). En plus du PBS, d'autres régions à l'intérieur du génome viral interagissent aussi avec l'ARNt^{Lys3}.
L'initiation de la transcription inverse du VIH-1 requiert une reconnaissance spécifique du génome viral, de l'amorce ARNt, et de la transcriptase inverse (ou reverse transcriptase, RT). Dans cet ouvrage, nous étudions le rôle important joué par le complexe d'initiation dans l'initiation de la transcription inverse du VIH-1. Il a été démontré qu'une boucle riche en A ("A-rich loop") située en amont du PBS interagit avec la boucle de l'anticodon de l'ARNt^{Lys3} et une délétion de cette "A-rich loop" cause une diminution de la réplication du virus. Nous avons étudié les mécanismes impliqués dans l'altération de la capacité de réplication des virus contenant cette délétion, dans le contexte du VIH-1 de type sauvage ("wild type", wt) ainsi que des virus comportant la substitution M184V dans la RT. Nous avons trouvé que la mutation M184V compromet l'abilité des virus contenant cette délétion de restaurer la réplication à un niveau comparable au type sauvage. Une étude biochimique approfondie indique que la mutation M184V dans la RT et la délétion des séquences en amont du PBS causent une diminution de la réplication virale en compromettant l'efficacité de l'initiation de la transcription inverse.

Puisqu'il a été démontré que l'initiation de la synthèse d'ADN est un processus spécifique, cela représente une cible potentielle pour le développement de nouveaux agents antiviraux. Nous avons développé des stratégies pour inhiber la réplication du VIH-1 via l'interférence avec le complexe d'ARNt^{Lys3}/ARN viral. Pour cibler l'amorce ARNt^{Lys3}, nous

avons employé des oligodeoxyribonucleotides (ODNs) qui sont complémentaires aux différentes parties de cette amorce. Pour cibler l'ARN viral, nous avons créé une molécule semblable à l'ARNt^{Lys3}, appelée ARNt^{Lys*}, qui contient des altérations dans la séquence qui redirige l'initiation à région distante du PBS, désigné PBS*. PBS* est impliqué dans la formation du complexe naturel ARNt/PBS et il a été démontré que la liaison de l'ARNt^{Lys*} interfère spécifiquement avec l'initiation de la transcription inverse. L'inhibition de la synthèse du brin (-) "strong-stop" d'ADN s'est effectuée avec succès avec les deux stratégies en interférant avec la formation du complexe d'initiation.

PREFACE

This Ph.D. thesis was written in accordance to the Guidelines for Thesis Preparation from the Faculty of Graduate Studies and Research at McGill University. These guidelines state:

"As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. These structure for the manuscript-based thesis must conform to the following: 1. Candidates have the option of including, as part of the thesis, the context of one or more papers submitted, or to be submitted, for publication...2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory...3. ...the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent."

Manuscripts are present in chapter 2, 3, respectively, as they were published, or will be published or will be submitted for publication. Connecting texts, in the form of prefaces, appear at the beginning of each chapter. The author's "contribution to original knowledge" and references cited appear at the end of the thesis.

According the continuance of works present in this Ph. D. thesis, manuscript for papers:

1. **Xin Wei, Chen Liang, Matthias Götte, and Mark A. Wainberg,** 2002. The M184V Mutation in HIV-1 Reverse Transcriptase Reduces the Restoration of Wild-Type Replication by Attenuated Viruses. AIDS. 16:2391-1398.
2. **Xin Wei, Chen Liang, Matthias Götte, and Mark A. Wainberg,** 2002. The M184V Mutation in HIV-1 Reverse Transcriptase Compromises the

Efficiency of tRNA-Primed Initiation of Viral DNA Synthesis: Consequences for Viral Replication. (In press).

are included in chapter 2.

3. **Xin Wei, Matthias Götte and Mark A. Wainberg,** 2000. Human immunodeficiency virus type-1 reverse transcription can be inhibited by oligodeoxyribonucleotides that target the tRNA primer. Nucleic Acids Research, 28: 3065-3074.
4. **Xin Wei, Elana Cherry, Balint Budai, Michael Stürzl, Mark A. Wainberg, Matthias Götte,** 2002, Inhibition of Human Immunodeficiency Virus Type-1 Reverse Transcription Using tRNA-like Molecules that Interfere with Formation of the Natural Initiation Complex. (To be submitted)

are included in chapter 3.

In chapter 2, Dr. Chen Liang provided HIV-1 A-rich loop deletion containing provirus plasmid. In chapter 3, Elana Cherry was involved in the sequencing of breakthrough viruses, Balint Budai participated in the NCp7 placement assay, and Dr. Michael Stürzl provided pNEO *in vivo* expression plasmid. All works were carried out under the supervision of Dr. Mark A. Wainberg and Dr. Matthias Götte.

Other manuscripts not included in this thesis, but with which the candidate was involved, are as follows:

1. **Wei, X., M. Detorio, K. Pantopoulos, M. A. Wainberg and M. Götte.** 2002. Mechanisms involved in inhibition of human immunodeficiency virus type-1 reverse transcriptase by metalloporphyrins. (To be submitted).
2. **McLellan, N., X. Wei, B. Marchand, M. A. Wainberg and M. Götte.** 2002. Non-radioactive detection of retroviral associated RNase H activity in a microtiter plate-based high-throughput format. Biotechniques. 2002 33:424-9.

3. **Diallo, K., X. Wei, B. Marchand, M. A. Wainberg, M. Götte.** 2002. Blockage of tRNA-primed initiation of reverse transcription provides a mechanism for the diminished fitness of HIV-1 variants containing L74V and M184V mutations. (To be submitted).

4. **Guo, X., M. Kameoka, X. Wei, B. Roques, M. Götte, C. Liang and M. A. Wainberg.** 2002. Suppression of an intrinsic strand transfer activity of HIV-1 Tat proteins by its second-exon sequences. (In press).

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LIST OF ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
bp	base pair
DDDP	DNA-dependent DNA polymerase
dNTP	2'-deoxynucleotide triphosphate
HIV	human immunodeficiency virus
LTR	long terminal repeat
M184V	methionine substitute for valine at codon 184
NC	nucleocapsid protein
nt	nucleotide
ODN	oligodeoxyribonucleotide
PAS	primer activation site
PBS	primer binding site
R	repeat region
RDDP	RNA-dependent DNA polymerase
RNase H	DNA/RNA-dependent ribonuclease
RT	reverse transcriptase
tRNA	transfer RNA
U3	3' unique region
U5	5' unique region
utRNA	unmodified tRNA/synthesized tRNA
Ψ	packaging signal
(-) ssDNA	minus-strand strong stop DNA

Chapter 1

Introduction

1.1 Retroviruses

Retroviruses comprise a large and diverse family of enveloped RNA viruses defined by their structure, composition, and most importantly, an unique replicative property, that include as essential steps reverse transcription of the virion RNA into linear double-stranded DNA and the subsequent integration of this DNA into the genome of the cell (Back et al., 1996; Boyer and Hughes, 1995; Sharma and Crumpacker, 1999). Retroviruses are broadly divided into two categories-simple and complex-distinguishable by the organization of their genomes (Cullen, 1992; Vogt, 1997; Weiss, 1996). All retroviruses contain three major genes: namely *gag*, *pol* and *env*, which encode internal virion proteins, essential enzymes like reverse transcriptase and integrase, and viral envelope protein. Simple retroviruses usually carry only this elementary information, whereas complex retroviruses code for additional regulatory non-virion proteins derived from multiply splice mRNA. Based on genetic homology, retroviruses are further subdivided into seven genera: five oncogenic genera, i.e. avian sarcoma and leucosis viral, the mammalian B-type viral group, the murine leukemia-related viral group, the human T-cell leukemia bovine leukemia viral group, the D-type group; and other two genera: lentiviruses and spumaviruses.

The discovery and study of retroviruses has had a broad impact on diverse area of biology and medicine, notably on molecular genetics, on the study of cellular growth control and carcinogenesis, and on biotechnology. It was so-called "Central Dogma" that had to be revised when the replication of retroviruses was understood. The study of retroviral oncogenesis also opened up the entire field of cellular growth control and it has lead to the discovery of proto-oncogenes. Contemporary retrovirology is largely devoted to human immunodeficiency virus (HIV). HIV belongs to the lentiviruses genus, and is the causative agent of acquired immunodeficiency syndrome (AIDS). Twenty years after the first clinical

evidence of acquired immunodeficiency syndrome (AIDS) was reported, AIDS has become the most devastating disease humankind has ever faced. Since the epidemic began, more than 60 million people have been infected with the virus. HIV/AIDS is now the leading cause of death in sub-Saharan Africa. Worldwide, it is the fourth-biggest killer. HIV poses intellectually novel and unique problems to research on all levels of analysis: transmission and pathogenesis in the patient, cell biology and genetics of infection, and molecular mechanisms of replication, of viral gene expression, and of virion assembly and release. Research in all of these areas contributes to the attainment of the most urgent goals: a protective vaccine and effective therapy for HIV infection.

1.2 Discovery and origin of HIV

Acquired immune deficiency syndrome (AIDS), characterized by a marked reduction in the number of $CD4^+$ cells and the development of infections and cancers, results from the persistent replication and spread of HIV. However, the first indication that AIDS could be caused by a retrovirus came in 1983, when Barré-Sinoussi, Chermann, Montagnier, and associates at the Pasteur Institute recovered a virus containing RT from the lymph node of a man with persistent lymphadenopathy syndrome (LAS) (Barre-Sinoussi et al., 1983). Characteristics described for this virus included some that were reported for the human T-cell leukemia virus (HTLV) (Sugamura, 1993). Further studies in 1983 by Montagnier and coworkers (Montagnier, 1984) indicated that this human retrovirus, although similar to HTLV in infecting $CD4^+$ lymphocytes, had quite distinct properties. Their virus, later called LAV (lymphadenopathy-associated virus) grew to substantial titer in $CD4^+$ cells and killed them, instead of transforming the cells in culture as does HTLV. However, these observations on LAV provided important evidence supporting the potential etiologic role of a retrovirus in AIDS. Several other laboratories were also searching for the agent responsible for this immune deficiency syndrome,

and in early 1984, Gallo and associates reported the characterization of another human retrovirus distinct from HTLV that they called HTLV-III (Gallo et al., 1983; Popovic et al., 1984; Sarngadharan et al., 1984; Schupbach et al., 1984). It was isolated from the peripheral blood mononuclear cells (PBMC) of adult and pediatric AIDS patients. Levy and coworkers (Levy et al., 1984) reported at that time the identification of retroviruses that they named AIDS-associated retroviruses (ARV). Within a short time, the three prototype viruses (LAV, HTLV-III, and ARV) were recognized as members of the same group of retroviruses, and their properties identified them as members of the *Lentiviridae* family. These AIDS viruses had many properties distinguishing them from HTLV. For all these reasons, in 1986, the International Committee in Taxonomy of Viruses recommended giving the AIDS virus a separate name, human immunodeficiency virus, or HIV (Coffin et al., 1986). Soon after the discovery of HIV-1, a separate subtype, HIV-2, was identified in West Africa (Clavel et al., 1986). Both HIV subtypes can lead to AIDS, although the pathogenic course of HIV-2 appears to be longer.

How and when this virus emerged and evolved into various sequence subtypes (or clades), including outliers (clade O), remains a mystery. The first documented evidence of HIV infection in humans can be traced to an African serum sample collected in 1959 (Nahmias et al., 1986). Several investigators favor the conclusion that HIV came from primates. A connection between Simian Immunodeficiency Virus (SIV) and HIV-2 provides the most compelling evidence, although it is not conclusive. Recently, it has been reported that a species of chimpanzee, *p. t. troglodytes*, which harbors a related SIVcpz, is the primary reservoir for HIV-1 and has been the source of at least three independent zoonotic transfers from chimpanzees to humans (Gao et al., 1999; Hahn et al., 2000).

1.3 HIV-1 virion

Under electron microscopy, the viral envelope is formed by a cell-derived lipid bilayer, into which the viral envelope glycoproteins (Env) are inserted. The envelope proteins are derived from a 160-kDa precursor, gp160, which is cleaved inside the cell (most probably by cellular enzymes in the Golgi apparatus) into a gp120 external surface envelope protein (SU) and a gp41 transmembrane protein (TM). Electron microscopic pictures and crystallization of the ectodomain of gp41 suggest that the envelope protein is organized as a trimer. The central region of the transmembrane protein binds to the external viral gp120 in a noncovalent manner, primarily at two hydrophobic regions in the amino and carboxyl termini of gp120. The virion gp120, located on the virus surface, contains the binding site(s) for the cellular receptor(s) and the major neutralizing domains. The external portion of gp41 and part of p17 have also been reported to be sensitive to neutralizing antibodies.
Within the mature virion, HIV has the characteristics of a lentivirus, with a cone-shaped core composed of the viral p24 Gag capsid protein. Inside this capsid, or nucleoid, are two identical RNA strands, with which the viral reverse transcriptase (RT) and the nucleocapsid protein are closely associated. The inner portion of the viral membrane is surrounded by a myristylated p17 core protein (MA), which provides the matrix for the viral structure and is vital for the integrity of the virion (Fig. 1-1). Closely associated with the core are the Vif and Nef proteins. Estimates of 7 to 20 molecules of Vif per virion have recently been made. Also found within the virion and most probably outside the core is the viral accessory gene product Vpr (or Vpx for HIV-2). Recent data suggest that Tat may also be located inside virions. The presence of all these viral proteins within viral particles suggests that they play a role in early events of HIV infection. Certain cytoskeletal proteins (e.g., actin, ezrin, moesin, and coflin), perhaps cleaved by the HIV protease, have also been detected

Fig. 1-1 Structural models for HIV-1 virion. SU: surface protein, TM: transmembrane protein, MA: matrix protein, PR: protease, RT: reverse transcriptase, CA: capsid, NC: nucleocapsid, IN: integrase.

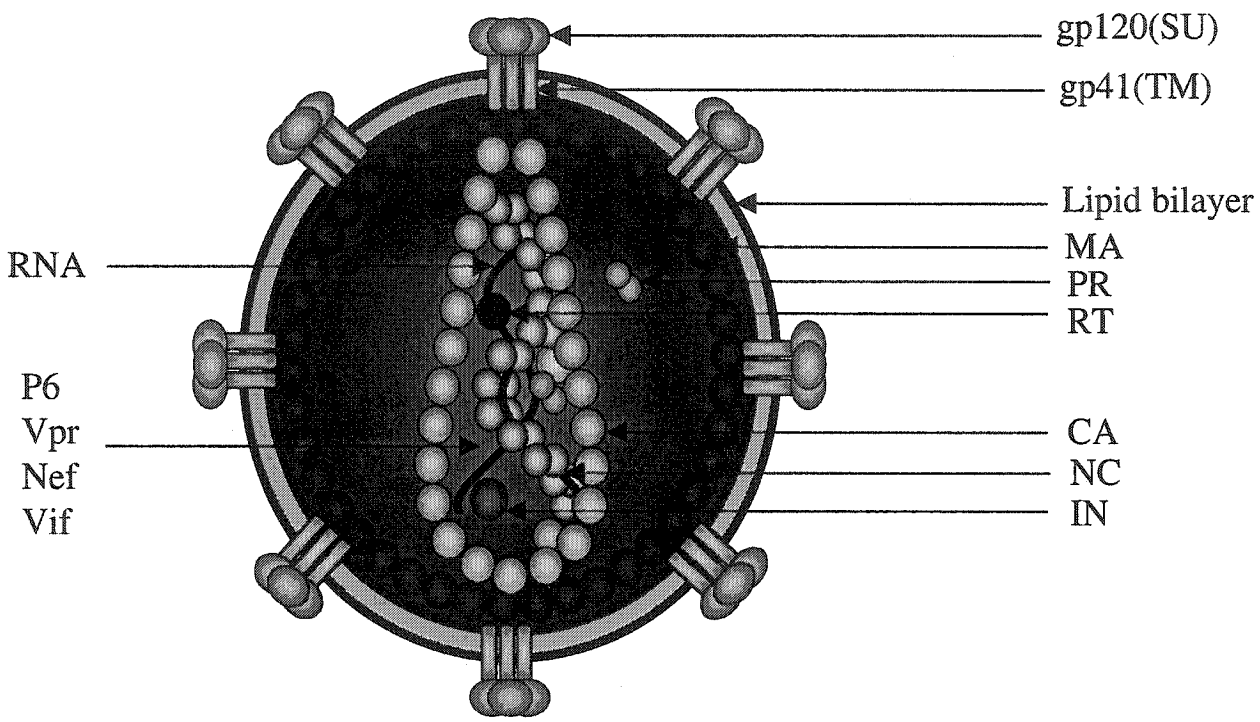

gp120(SU)
gp41(TM)
Lipid bilayer
RNA
MA
PR
RT
P6
Vpr
Nef
Vif
CA
NC
IN

Fig. 1-1

within virions; their role in infection, if any, is unknown.

1.4 HIV-1 genome and proteins

1.4.1 HIV-1 genome

HIV-1 carries two identical genomic RNA molecules that are non-covalently associated at the 5' end to form a dimer. The genomic size of HIV is about 9.8 kb, with an open reading frame coding for several viral proteins (Fig. 1-2). The primary transcript of HIV-1 is a full-length viral mRNA, and may be thought of as a large macromolecular component of the virion containing structured subdomains throughout its length. Beginning at the 5' end, several essential regions have been defined: (1) The TAR hairpin is the Tat-binding site; (2) The primer binding site (PBS) is important for initiating reverse transcription by annealing to a cellular $tRNA^{Lys3}$; (3) The packaging signal or Ψ binds NC and is critical for incorporation of genomic RNA into the virion; (4) Three dimerization site includes a "kissing loop" hairpin that facilitates incorporation of two genomic RNAs into the virion; (5) The major splice donor site is used to generate all subgenomic spliced mRNAs; (6) The Gag-Pol frameshifting region comprises a heptanucleotide slippery sequence and RNA hairpin that promote –1 ribosomal framshifting, thereby translating a fused Gag-Pol polyprotein at a frequency of ~5-10%; (7) The Rev Response Element (RRE) in the rev-binding site; (8) Splice acceptor sites are present at several downstream regions of the RNA and allow production of a relatively large number of spliced products; (9) The polyadenylation signal is used to generate the 3' end. The HIV genes in the proviral DNA are bracketed by the long terminal repeats (LTRs), identical sequences that are composed of U3, R, and U5 elements. The transcription initiation site and poly(A) addition site separate the R region from the U3 and U5 regions respectively; the initiation sites of plus- and minus- strand DNA synthesis determine the other boundaries of the U3 and U5 regions.

Fig. 1-2 Schematic illustration of HIV-1 genetic organization.

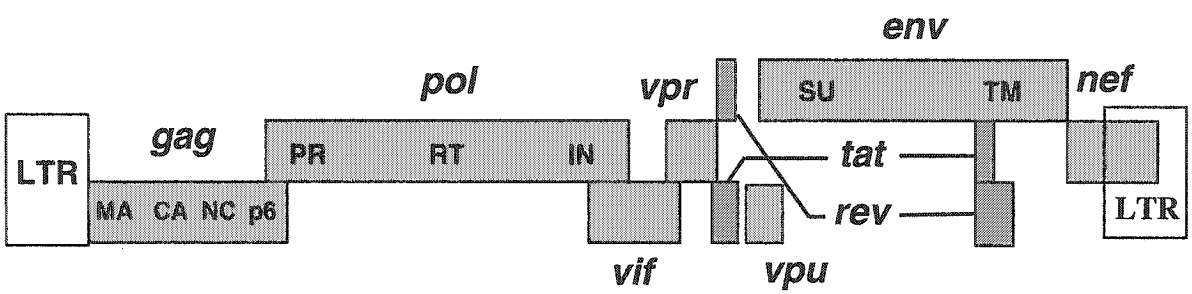

env
pol
vpr
SU
TM
nef
LTR
gag
PR
RT
IN
tat
MA CA NC p6
rev
LTR
vif
vpu

Fig. 1-2

The HIV genome consists of three major genes, *gag*, *pol* and *env*, as well as six auxiliary genes, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*. By proteolytic cleavage, the Gag precursor p55 gives rise to the smaller internal structural proteins MA, CA, and NC. The Pol precursor protein is cleaved into products consisting of the viral enzymes RT, protease, and integrase. Protease processes the Gag and Pol polyproteins; integrase is involved in virus integration. The Gag and Gag-Pol products are synthesized at a ratio of about 20:1. Splicing events producing many subgenomic mRNAs are important for the synthesis of other viral proteins. The relative amount of the unspliced to singly and multiply spliced mRNAs appears to be determined by the *rev* gene, which is itself a product of a multiply spliced mRNA. The envelope gp120 and gp41 proteins are made from a precursor protein gp160, encoded by *env*, which is a singly spliced RNA from the full-length viral mRNA. Gene products of other spliced mRNAs make up a variety of viral regulatory and accessory proteins that can affect HIV replication in various cell types, three of which (Vif, Vpr and Nef) are found in the viral particle. Two other accessory proteins, Tat and Rev, provide essential gene regulatory functions, and the last protein, Vpu, indirectly assists in assembly of the virion.

1.4.2 Gag

The Gag protein is the precursor to the internal structural protein of all retroviruses. During or after virus budding, Gag precursors are specifically cleaved to yield mature structural proteins by the viral protease. In particular, Gag has the ability to direct the budding of virus-like particles from the cell. Expression of *gag* alone leads to assembly of immature virus-like particles that bud from the plasma membrane. Because of these properties, the Gag protein has been described as a "particle-making machine". The Gag protein is also involved in packaging most of the other components of the virion, including the two copies of genomic RNA. In virion assembly, Gag proteins must interact with each other, with

components in the plasma membrane, with genomic RNA, and probably with Env proteins and with cellular proteins as well. The regions responsible for these functions are distributed in the multiple domains of Gag. After proteolysis, each domain of Gag undergoes a transition in both shape and function. Upon *de novo* infection, the mature Gag proteins play new roles in the early steps of the viral life cycle.

1.4.2.1 MA

MA is the N-terminal component of the Gag polyprotein and is important for targeting Gag and Gag-Pol precursor polyproteins to the plasma membrane prior to viral assembly. In the mature viral particle, the 132-residue MA protein lines the inner surface of the virion membrane. Two discrete features of MA are involved in membrane targeting: an N-terminal myristate group and basic residues located within the first 50 amino acids. MA proteins are trimerized in the crystal structure (Hill et al., 1996). The trimeric form is presumed to be biologically relevant because mutation of residues involved in trimerization (residues 42-77) abolishes viral assembly and because basic residues are important for membrane localization. Lysines (26, 27, 30, 32) are arranged on the putative membrane-binding surface of the trimer. The crystal structure of MA suggests that membrane binding involves the insertion of three myristate groups into the lipid bilayer located directly above the trimer and interactions between basic residues of the membrane-binding surface and phospholipid head group. In addition to targeting Gag and Gag-Pol to the membrane, MA also appears to help incorporate Env glycoproteins with long cytoplasmic tails into viral particles (Freed et al., 1995; Mammano et al., 1995). Indeed, the array of threefold symmetric holes located between matrix trimers appears to be large enough to accommodate the long cytoplasmic tails of the full-length Env (Hill et al., 1996; Massiah et al., 1996).

In addition to its function in viral assembly, MA facilitates infection of nondividing cell types, principally macrophages. Some studies have shown that a subset of phosphorylated MA proteins are associated with viral preintegration complexes and that MA contains a nuclear localization signal (NLS) that interacts with Rch1, a member of the karyopherin-α family, to facilitate rapid nuclear transport (Bukrinskaya et al., 1996; Bukrinsky et al., 1993; Gallay et al., 1996; Gallay et al., 1995b). Phosphorylation of Tyr131 was shown to mediate association with IN, thereby linking MA to the preintegration complex (Gallay et al., 1995a). However, other studies found no evidence for an MA NLS. Instead, mutation of the putative MA NLS in a macrophage-tropic HIV-1 isolate decreased infectivity in both nondividing and dividing cells and resulted in delayed proteolytic processing of the Gag polyprotein, presumably because the mutation affected association of MA with the membrane (Fouchier et al., 1997). Additional studies are needed to clarify the role of MA in infection of nondividing cells.

1.4.2.2 CA

CA is the second component of the Gag polyprotein and forms the core of the virus particle, with ~2000 molecules per virion. The 26-KD capsid protein performs essential roles both early and late in the life cycle of HIV-1. CA is initially translated as the central region of the 55-KD Gag polyprotein, where it functions in viral assembly and in packaging the cellular protein cyclophilin A (CypA). After CA has been liberated by proteolytic processing, it rearranges into the conical core structure that surrounds the viral genome at the center of the mature virus. Genetic analysis have revealed that capsid also performs essential roles as the virus enters, uncoats, and replicates in the new host cell, although these early steps in viral replication are currently poorly understood.

Capsid is composed of two distinct domains. The N-terminal domain (1-145) contributes to viral core formation and binds CypA (Luban, 1996). In

contrast, the C-terminal domain is required for capsid dimerization, Gag oligomerization and virion formation (Gamble et al., 1997). Structures of the C-terminal domain, N-terminal domain, and N-terminal domain complexed to CypA have been solved by crystallography and NMR (Gamble et al., 1996; Gamble et al., 1997; Gitti et al., 1996; Momany et al., 1996). The C-terminal domain is composed of an extended strand followed by four α-helices, with an extensive dimer interface (Gamble et al., 1997). The major homology region (MHR), a 20-amino acid sequence that is one of the most highly conserved within all retroviral Gag proteins, adopts a compact fold in which the four most conserved residues (Gln155, Gly156, Glu159 and Arg167) form a stabilizing hydrogen-bonding network. The MHR is essential for particle assembly and may have a role in incorporation of Gag-Pol precursors through interactions with Gag (Srinivasakumar et al., 1995), although not all mutants show this phenotype (Mammano et al., 1994). Biochemical experiments also suggest a possible role for the MHR in membrane affinity, perhaps reflecting exposure of hydrophobic residues (Ebbets-Reed et al., 1996). The C-terminal domain is largely responsible for dimerization. The CA subunits are seen to arrange in strips within the crystal, consistent with a plausible packing arrangement in the virion core (Gamble et al., 1996). Residues from an extended region of N-domain interact with CypA, with Ala88, Gly89 and Pro90 buried in the CypA active site groove. A short spacer peptide located between CA and NC, p2, may also influence CypA incorporation into the virion (Dorfman and Gottlinger, 1996).

1.4.2.3 NC

NC is the third component of the Gag polyprotein and coats the genomic RNA inside the virion core. It has been recognized for some time that the Gag processing site is cleaved in an ordered manner by PR in the virion and *in vitro*. Initial cleavage of Gag occurs at the amino terminus of the NC domain, producing a 15-KD intermediate, p15NC, consisting of

nucleocapsid-p1-p6 and a 39-KD intermediate consisting of matrix-capsid-p2. Later in the processing cascade, these intermediates are further cleaved to yield mature products. Thus, during virion morphogenesis, there appear to be at least four different forms of nucleocapsid: Gag, p15NC, NC-p1, and mature NC. The exact role of each NC is not known, but the presence of functional differences between them suggests that each have a particular role during virion morphogenesis.

Mature NC is 55 residues long and contains two zinc finger domains (of the CCHC type) flanked by basic amino acids. The primary function of NC is to bind specifically to the packaging signal and deliver full-length viral RNAs into the assembling virion. The packaging signal, Ψ, is not completely defined but is probably composed of three RNA hairpins located around the major splice donor site (Clever and Parslow, 1997; Laughrea et al., 1997), the first of which contains the kissing loop involved in RNA dimerization. Studies with a chimeric Gag containing NC from HIV-1 and the remainder of Gag from Moloney murine leukemia virus (Mo-MLV) demonstrate that genomic HIV-1 RNA is preferentially packaged but that additional downstream sequences, which result in packaging of spliced RNAs, may contribute (Berkowitz et al., 1995). NC is a basic protein that also binds single-stranded nucleic acids non-specifically, leading to coating of the genomic RNA that presumably protects it from nucleases and compacts it within the core. Non-specific binding also provides chaperon-like functions that enhance other nucleic acid-dependent steps in the life cycle; for example, by promoting annealing of the tRNA primer, melting of RNA secondary structures, or DNA strand exchange reaction during reverse transcription (Cameron et al., 1997; Guo et al., 1997; Huang et al., 1997a) or by stimulating integration (Carteau et al., 1997).

In addition to its function in specific RNA encapsidation, HIV-1 NC is also required for efficient virus particle assembly. Replacement of the NC fragment within Gag by polypeptides which form interprotein contacts

permitted efficient virus particle assembly and release, even when RNA was not detected in the particle; this suggests that formation of interprotein contacts by NC is essential for the normal HIV-1 assembly process (Zhang et al., 1998a).

1.4.2.4 P6

P6 comprises the C-terminal 51 amino acids of Gag and is important for incorporation of Vpr during viral assembly. Residues 32-39 and three hydrophobic residues within a highly conserved sequence motif (Leu41-X42-Ser43-Leu44-Phe45-Gly45) are important for Vpr binding (Checroune et al., 1995; Kondo and Gottlinger, 1996; Lu et al., 1995b). In Vpr, a predicted α-helical structure located near its N-terminus contains amino acids responsible for p6 binding (Cohen et al., 1996). P6 also helps mediate efficient particle release, and a region of four amino acids (Pro7-Thr8-Ala9-Pro10) has been implicated in this function (Huang et al., 1995).

1.4.3 Pol

The full-length transcript from the integrated provirus serves as the mRNA for synthesis of Gag and Gag-Pol proteins. It is suggested that the RNA molecules used for the synthesis of Gag and Gag-Pol are not the same molecules that are packaged into virions during assembly. During the replication of HIV-1, large numbers of Gag molecules must be generated to serve as precursors to the structural proteins of the virions. However, the enzymes encoded by the *pol* genes, PR, RT and IN, are, in most cases, needed in smaller numbers to carry out their catalytic functions. Groups of roughly 1500 Gag molecules, mixed with about 75 Gag-Pol molecules, direct the formation and release of viral particles. HIV-1 has developed a mechanism that permits expression of the Gag protein at high levels relative to the protein sequences encoded in the *pol* genes, while retaining coregulated expression. This linkage results from

the use of the same initiation codon in the same mRNA to express the *gag* and *pol* genes. In HIV-1, the *gag* gene is positioned at the 5' end of the viral genome, upstream of the *pol* genes. The Gag-Pol precursor is generated using a strategy, in which the termination codon that defines the 3' terminus of the gag reading frames is bypassed, allowing translation to continue into the adjacent *pol* reading frames. Bypass of the termination codon occurs by ribosomal frameshifting. Occasionally the ribosome slips backward one nucleotide (-1 frameshift, i.e., in the 5' direction) during translation of *gag*; thus the ribosome leaves the *gag* reading frame (with its down-stream termination codon) and shifts into an overlapping portion of the *pol* reading frame.

The three Pol proteins, PR, RT and IN, provide essential enzymatic functions in the HIV-1 infectious cycle. All of them represent important targets for antiviral therapy. Understanding their structure and function has become the basis for the design and development of antiviral inhibitors.

1.4.3.1 PR

As the core virion is assembled to include the Gag and Gag-Pol polyproteins, the Vif, Vpr and Nef proteins, and genomic RNA, and as the membrane coat containing gp120 and gp41 surrounds the particle, the virus buds from the membrane surface and is released. The immature particles formed are non-infectious. The Gag and Gag-Pol polyproteins must be cleaved by PR, and conformational rearrangements must occur within the particle, to produce mature infectious viruses. Some of these "maturation" events may occur simultaneously with assembly and budding (Kaplan et al., 1994); the precise timing is not clear.

Sequence alignments revealed that there are similarities between PR and the aspartic proteinase family of enzymes. All aspartic proteinases share the feature that two aspartic acid residues, each placed in the highly conserved motif Asp-Thr/Ser/Gly, coordinate a water molecule used to

hydrolyze the target peptide bond. However, HIV-1 PR has only a single Asp-Thr-Gly motif and is too small to represent both domains of this cellular counterpart. HIV-1 PR functions as a dimer of two identical subunits. PR has been a prime target for drug design, and the crystal structure of many PR inhibitor complexes have been solved, including both peptidomimetic and nonpeptide inhibitors (Wlodawer and Erickson, 1993). The enzyme active site is formed at the dimer interface, with each 99-residue monomer contributing a catalytically essential aspartic acid. The PR dimer contains flexible flaps that close down on the active site upon substrate binding. Amino acid side chains surrounding the cleavage site bind within the hydrophobic pocket of PR.

PR cleaves at several polyprotein sites to produce the final MA, CA, NC and p6 proteins from Gag, and PR, RT, and IN proteins from Pol. The sequences of cleavage sites in the polyprotein precursors are diverse but some generalizations can be made. By convention, the peptide bond that is cleaved is referred to as the scissile bond. The most important determinant is the four amino acids flanking either side of the scissile bond of the substrate that are recognized by flaps of PR (Ringe, 1994; Wlodawer and Erickson, 1993). Furthermore, cleavage efficiencies can vary substantially among sites, thereby influencing the order of appearance of different processed proteins (Dunn et al., 1994). The p2 spacer peptide located between CA and NC may also help control relative cleavage rates and infectivity (Krausslich et al., 1995; Pettit et al., 1994), and processing of NC and p6 may be further influenced by RNA binding to NC (Sheng et al., 1997). The final stoichiometries are determined largely by the amount of Gag-Pol produced by ribosomal frameshifting and incorporated into the virion. Because assembly and maturation must be highly coordinated, factors that influence PR activity can have dramatic effects on virus production. PR activity initially depends on the concentration of Gag-Pol and the rate of autoprocessing, which may be influenced by adjacent p6 sequences (Zybarth and Carter, 1995). Since Gag-Pol is normally

expressed as a minor product relative to Gag, it was hypothesized that an excess of Gag may limit intracellular dimerization, and thus activation, of PR. Overexpression of PR can lead to aberrant rates of processing and decreased infectivity (Luukkonen et al., 1995). Premature activation and processing by PR would preclude proper assembly and would be fatal to the virus. Premature activation of PR has been observed during acute HIV infection and this appears to result in a pool of processed products that are excluded from the assembly pathway.

1.4.3.2 RT

Before the viral genome can be integrated into the host chromosome, it must first be reverse transcribed into duplex DNA. RT catalyzes both RNA-dependent and DNA-dependent DNA polymerization reactions and contains an RNase H domain that cleaves the RNA portion of RNA-DNA hybrids generated during the reaction.

RT has been a major target for drug design, and crystal structures of unliganded RT, a RT-DNA complex, and RT-inhibitor complexes have been solved (Esnouf et al., 1995; Hsiou et al., 1996; Jacobo-Molina et al., 1993; Kohlstaedt et al., 1992; Ren et al., 1995; Rodgers et al., 1995). RT is a heterodimer containing a 560-residue subunit (p66) and a 440-residue (p51) both derived from the Pol polyprotein. The p51 subunit is derived from p66 by a protease-mediated cleavage of the p66 subunit between F440 and Y441 and lacks the RNase H domain. Each subunit contains a polymerase domain composed of four subdomains called finger, palm, thumb, and connection, and p66 contains an additional RNase H domain. Even though their amino acid sequences are identical, the polymerase subdomains are arranged differently in the two subunits, with p66 forming a large active-site cleft that is required to bind nucleic acid primer/template substrates and p51 forming an inactive closed structure (Wang et al., 1994). The p66 polymerase active site contains a catalytic YXDD motif, a highly conserved structure in all polymerases involving

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. The RNase H active site is defined by three acidic residues within the C-terminal domain of p66.

During the course of reverse transcription, several conformationally distinct substrates are encountered and must be accommodated by the enzyme. HIV-1 RT must utilize RNA/RNA, DNA/RNA, and RNA/DNA, as well as DNA/DNA template/primer combinations in order to complete reverse transcription. In addition, the RNase H activity of RT is required for both highly specific cleavage events and non-specific degradation of the viral genome. The cocrystals of HIV-1 RT with duplex DNA showed that the 3'-OH group of the primer strand is positioned close to the active site for a nucleophilic attack on the incoming nucleoside triphosphate (Jacobo-Molina et al., 1993). The DNA in this complex has primer and template strands clamped between the palm, thumb, and finger subdomains of p66 and is bent. Portions of the DNA near the active site adopt an A-form geometry, whereas the DNA near the RNase H site is B-like in form. The physical distance between polymerase and RNase H active sites is 15-18 bases. The A- to B-form transition is accompanied by an overall bend of about 40°. Other biochemical studies indicate that RT can bind to its various primer/template combinations with the same orientation. Assuming the scenario that the polymerase active site interacts specifically with the 3'-end of the primer, the RNase H active site is always located over the template strand in the duplex region. However, the exact position depends on the type of complexed nucleic acid combination.

Two classes of RT inhibitors are in clinical use: nucleoside analogs such as AZT and ddI that are presumed to bind to the polymerase active site and non-nucleoside inhibitors such as nevirapine. The structures of several non-nucleoside inhibitor-RT complexes show a common

hydrophobic binding site near to, but distinct from, the polymerase active site that rearranges to fit the particular drug and lock RT into an inactive conformation (Kohlstaedt et al., 1992; Ren et al., 1995). Mutations that confer resistance to nucleoside or non-nucleoside inhibitors map to different parts of RT, including regions in and around the active site and the DNA-binding cleft, suggesting that some mutations directly alter the drug-binding site while others have more indirect effects (Kohlstaedt et al., 1992; Tantillo et al., 1994). Structures of the unliganded RT show substantial variability in the position of the p66 thumb subdomain (Esnouf et al., 1995; Hsiou et al., 1996; Rodgers et al., 1995), an indication that large-scale conformational rearrangements occur upon nucleic acid or drug binding. Such conformational changes may be important during reverse transcription, for example, to allow translocation of RT along the nucleic acid or to correctly position the RNase H and polymerase active site.

1.4.3.3 IN

Following reverse transcription, IN catalyses a series of reactions to integrate the viral genome into a host chromosome. Linear viral DNA contains at its termini long terminal repeat (LTR) sequences. The viral IN specifically recognizes the ends of these LTRs. Integration occurs in two well-characterised catalytic steps, referred to as end processing and joining, respectively. The IN recognition sequence within the LTR is relative short. Cross-linking and substitution of bases in the LTR of HIV-1 have demonstrated that specific interactions between IN and the terminal LTR sequences are required for the end-processing and joining reactions (Esposito and Craigie, 1998; Heuer and Brown, 1997; Heuer and Brown, 1998).

IN is active as an oligomer, probably a tetramer (Rice et al., 1996), and the 288-residue monomer can be divided into three domains whose structure have been determined. Within the N-terminal domain (residue 1-

55) of IN is a putative zinc finger of the HHCC type. Amino acid substitutions in any or all of the HHCC residues in HIV-1 IN nearly or completely abolish the end-processing and joining reactions *in vitro* and *in vivo*, respectively (Engelman and Craigie, 1992; Engelman et al., 1995). Recently a solution structure of the N-terminal domain was determined and revealed a dimeric structure having an HHCC zinc-binding motif that coordinates zinc. The folds of the N-termini are similar to those of other DNA binding proteins in having a helix-turn-helix structural motif (Cai et al., 1997). The central core domain (residue 50-212) is thought to be the catalytic domain of the IN, and contains a D, D(35), E motif (Katzman and Sudol, 1998). This triad for HIV-1 IN is D64, D116, and E152. This motif is conserved among integrases, is crucial for the processing and joining reactions, and is proposed to bind the active site metal ion (Rice et al., 1996). The isolated catalytic domain cannot perform processing or joining reactions but can perform an apparent reverse reaction, termed disintegration, indicating that it contains the catalytic site for polynucleotidyl transfer. The crystal structure of the catalytic core, coordinating a divalent cation, Mg^{2+}, shows a dimeric structure, with each monomer containing a five-stranded β-sheet and six α-helices similar to other polynucelotidyl transfer enzymes (Dyda et al., 1994). Asp64 and Asp116 of the D, D(35), E motif are clearly seen in the structure, but Glu152 is located on a disordered loop. The two active sites in the dimer are too far apart to permit five base pair staggered cleavage of the target DNA, suggesting either that a very large conformational change occurs during catalysis or, more likely, that IN functions as a tetramer or other oligomeric form during some steps of the reaction (Cai et al., 1997; Rice et al., 1996). The C-terminal domain (residues 220-270) is the least highly conserved of the three domains. It has non-specific DNA-binding activity and forms a dimer of parallel monomers, as shown by NMR. The structure of each monomer consists of a five-stranded β barrel strikingly similar to a SH3 domain, with a saddle-shaped groove that might accommodate

double-stranded DNA and containing Lys264, an important DNA-binding residue (Eijkelenboom et al., 1995; Lodi et al., 1995).

1.4.4 Env

In the infected cell, the envelope glycoproteins are synthesized as an approximately 845- to 870- amino acid precursor in the rough endoplamic reticulum. Asparagine-linked, high-mannose sugar chains are added to form the gp160 glycoprotein, which assembles into oligomers (Chan et al., 1997; Earl et al., 1990; Earl et al., 1991; Leonard et al., 1990; Lu et al., 1995a; Pinter et al., 1989; Weiss et al., 1990; Weissenhorn et al., 1997). The preponderance of evidence suggests that these oligomeric complexes are trimers (Earl et al., 1990; Earl et al., 1991; Lu et al., 1995a; Pinter et al., 1989; Weiss et al., 1990; Chan et al., 1997; Weissenhorn et al., 1997). The gp160 trimers are transported to the Golgi apparatus, where cleavage by a cellular protease generates the mature envelope glycoprotein: gp120, the surface envelope glycoprotein, and gp41, the transmembrane glycoprotein (Allan et al., 1985; Veronese et al., 1985). The gp41 glycoprotein has an ectodomain that is largely responsible for trimerization (Earl and Moss, 1993; Shugars et al., 1996), a membrane-spanning anchor, and a long cytoplasmic tail. Most of the surface-exposed elements of the mature, oligomeric envelope glycoprotein complex are contained on the gp120 glycoprotein. Selected, presumably well-exposed, carbohydrates on the gp120 glycoprotein are modified in the Golgi apparatus by the addition of complex sugars (Leonard et al., 1990). The gp120 and gp41 glycoproteins are maintained in the assembled trimer by noncovalent, somewhat labile, interactions between the gp41 ectodomain and discontinuous structures composed of N- and C-terminal gp120 sequences (Helseth et al., 1991). When they reach the infected cell surface, a fraction of these envelope glycoprotein complexes are incorporated into budding virus particles. A large number of the complexes disassemble, releasing gp120 and exposing the previously

buried gp41 ectodomain. These events contribute to the formation of defective virions, which predominate in any retroviral preparation (Tsai et al., 1996).

1.4.4.1 SU(gp120)

Virus entry is initiated by the binding of the gp120 glycoprotein, located on the viral membrane surface, to specific cell surface receptors. The major receptor for HIV-1 is CD4, an immunoglobulin (Ig)-like protein expressed on the surface of a subset of T cells and primary macrophages. Multiple approaches have yielded insight into the structural basis for CD4 binding by the HIV-1 gp120 glycoproteins. Early comparisons of gp120 sequences revealed the existence of five variable (V1 through V5) regions interspersed with five conserved regions (C1 through C5) (Starcich et al., 1986). Intramolecular disulfide bonds in the gp120 glycoprotein result in the incorporation of the first four variable regions into large looplike structures (Leonard et al., 1990). Antibody binding studies and deletion mutagenesis have indicated that the major variable loops are well exposed on the surface of the gp120 glycoprotein (Moore et al., 1994; Pollard et al., 1992; Wyatt et al., 1993). The more conserved regions fold into a gp120 core, which has been recently crystallized in a complex with fragments of CD4 and a neutralizing antibody (Kwong et al., 1998; Wyatt et al., 1998). The gp120 core is composed of two domains, an inner and an outer domain, and a β sheet (the "bridge sheet") that does not properly belong to either domain. The domain names reflect the likely orientation of gp120 in the assembled envelope glycoprotein trimer: the inner domain faces the trimer axis and, presumably gp41, whereas the outer domain is mostly exposed on the surface of the trimer. Elements of both domains and the bridging sheet contribute to CD4 binding.

CD4 binds in a recessed pocket on gp120, making extensive contacts over ~800Å^2 of the gp120 surface. Two cavities are evident in the gp120-CD4 interface. A shallow cavity is filled with water molecules, and a deep

cavity extends roughly 10Å into the interior of gp120. The opening of this deep cavity is occupied by phenylalanine-43 of CD4, which has been shown by mutagenic analysis to be critical for gp120 binding (Arthos et al., 1989; Ashkenazi et al., 1990; Brodsky et al., 1990; Choe and Sodroski, 1992; Peterson and Seed, 1988; Ryu et al., 1990; Wu et al., 1997). Most of the gp120 residues previously identified as important for CD4 binding (Cordonnier et al., 1989a; Cordonnier et al., 1989b; Kowalski et al., 1987; Lasky et al., 1987; Olshevsky et al., 1990) surround the opening of the deep cavity and contribute to interactions with Phe43 of CD4. In addition, Asp368 of gp120 forms a salt bridge with Arg59 of CD4, also shown by mutagenesis to be important for gp120 binding (Arthos et al., 1989; Ashkenazi et al., 1990; Brodsky et al., 1990; Choe and Sodroski, 1992; Peterson and Seed, 1988; Ryu et al., 1990; Wu et al., 1997). In addition, main-chain atoms on gp120 and CD4 form hydrogen bonds bridging the two proteins.

The gp120-CD4 interaction is not sufficient for HIV-1 entry. A major function of CD4 binding is to induce conformational changes in the gp120 glycoprotein that contributes to the formation or exposure of the binding site for the chemokine receptors and subsequent viral entry (Bandres et al., 1998; Hill et al., 1997; Lapham et al., 1996; Sattentau and Moore, 1991; Sattentau et al., 1993; Thali et al., 1993; Trkola et al., 1996; Wu et al., 1996). The variable V3 loop of gp120 is an important determinant of viral tropism. It becomes exposed upon CD4 binding and presumably interacts with the cognate coreceptor (Clapham and Weiss, 1997). However, the V3 loop is probably not the sole determinant of coreceptor specificity, because HIV-1 isolates that use the same coreceptor can have highly variable V3 sequences (Choe et al., 1996; Cocchi et al., 1996; Oravecz et al., 1996). Other, conserved gp120 structures also appear to play an important role in chemokine receptor binding. Antibodies that recognize conserved, discontinuous gp120 epitopes that are more exposed after CD4 binding are potent inhibitors of gp120-CCR5 interaction

(Bandres et al., 1998; Hill et al., 1997; Lapham et al., 1996; Trkola et al., 1996; Wu et al., 1996). Determinants for virus specificity are located in each of the extracellular regions of the coreceptors, and the signaling functions of these receptors are apparently not important for viral infection.

1.4.4.2 TM (gp41)

The primary function of gp41, a 345-amino acid protein located in the viral membrane, is to mediate fusion between the viral and cellular membranes following receptor binding. An N-terminal hydrophobic glycine-rich "fusion" peptide has been predicted to initiate fusion, and a transmembrane region is important both for fusion and for anchoring Env in the viral membrane (Hernandez et al., 1996). Two crystal structures of the core region (TM_{core}) have been reported (Chan et al., 1997; Weissenhorn et al., 1997). In the larger of the two structures, residues 30-79 and 113-154 of gp41 were fused to a 31-residue trimeric coiled-coil from GCN4 in place of the N-terminal fusion peptide(Weissenhorn et al., 1997). TM_{core} lacks residues 80-112 of gp41.

It is likely that the interaction of the gp120-CD4 complex with the appropriate chemokine receptor promotes additional conformational changes in the envelope glycoportein complex. By analogy with the influenza hemagglutinin, it has been suggested that the HIV-1 gp41 ectodomain undergoes major conformational changes during virus entry (Bullough et al., 1994; Carr and Kim, 1993; Weissenhorn et al., 1996). TM_{core} forms a trimer containing a central parallel α-helical coiled-coil (residues 1-77) and an outer antiparallel α-helical layer (residues 117-154) (Weissenhorn et al., 1997). The structure of TM_{core} probably does not represent the native gp41 structure but rather a structure formed during the fusion reaction, as suggested by the following. First, mutations at the interface between the outer and central helical layers (including Ile62) specifically block membrane fusion. Second, TM_{core} is extremely

thermostable, a feature predicted for the fusion-active protein and not the native protein (Chan et al., 1997; Weissenhorn et al., 1997). Third, the structures of TM_{core} and a low pH fusion-active form of the influenza virus HA_2 protein are strikingly similar (Chan et al., 1997; Weissenhorn et al., 1997). Fourth, the estimated distance between the C-terminus of TM_{core} and the viral lipid bilayer cannot be spanned by the 18 C-terminal extracellular residues missing from the structure. However, the distance is consistent with a conformation in which the fusion peptides and the transmembrane regions are located at the same end of the central rod structure when viral and cell membranes are brought together (Weissenhorn et al., 1997). The structure helps to explain how two peptides known to inhibit fusion may act. A peptide from the C-terminus may bind to the central trimer, disrupting the structure of the N-terminal region, whereas a peptide from the N-terminus may either compete with folding of the central trimer or bind to the C-terminal region and prevent association with the central core (Chan et al., 1997; Weissenhorn et al., 1997). Emerging rules for the design of the coiled-coil may aid in the development of new fusion inhibitors.

1.4.5 Accessory proteins

HIV-1 manipulates fundamental host cell processes in sophisticated ways to achieve optimum efficiency. In addition to the three usual replication genes *gag*, *pol* and *env*, the HIV-1 genome also encodes six accessory/regulatory proteins that are not found in other classes of retroviruses. Each of these proteins acts as a molecular connector between two macromolecules to recruit cellular metabolic pathways for the purpose of efficient viral replication and *in vivo* pathogenesis. Detailed analysis of the interplay between these viral proteins and normal cellular activities has provided new insights into central questions of virology and host cell biology.

1.4.5.1 Tat

The HIV-1 promoter is located in the 5' LTR and contains a number of regulatory elements important for RNA polymerase II (RNAP II) transcription. Despite the existence of several cellular transcription factors (Jones and Peterlin, 1994), transcription complexes initiated at the HIV-1 promoter are rather inefficient at elongation and require an ~14-KD viral protein Tat to enhance the processivity of transcribing polymerases. HIV-1 Tat is a small nuclear protein of 101 amino acids, and the domain structure of Tat is typical of many transcriptional activators and includes an activation domain and a nucleic acid (in this case, RNA) binding domain. Tat function is dependent on a bulged RNA stem-loop structure, TAR (Tat activation region), which is present at the 5' terminus of all viral mRNAs. Although HIV-1 transcription is mediated by cellular RNA polymerase II, Tat acts mostly at the level of transcriptional elongation rather than at initiation itself. A cellular protein kinase complex called Cdk9, a cyclin-dependent kinase, was identified that specifically binds to the activation domain of Tat and can phosphorylate the C-terminal domain (CTD) of RNAP II (Herrmann and Rice, 1995) - a step that had already been implicated in regulation of transcriptional elongation (Reines et al., 1996). A cyclin-related partner, cyclin T (CycT), confers substrate specificity on the kinase(Wei et al., 1998). CycT binds the activation domain of Tat both on its own and in the context of a Cdk9-CycT complex (Cdk9 does not bind Tat on its own). Once the first 57-nucleotides of nascent viral RNA are synthesized, the TAR structure is formed. Following binding to TAR, Tat brings the CycT - Cdk9 complex close to the RNAP II complex. Since the nascent RNA exit channel is located adjacent to CTD, Cdk9 is then positioned close to the CTD of RNAP II and can thus hyperphosphorylate this critical domain (Garber et al., 1998). In addition to its role in transcription, Tat might also regulate a number of other viral activities, one of which is reverse transcription. Although Tat has not been shown to be present within virus particles, it is possible that

the levels of Tat within virions may be too low to be detected by currently available techniques. For example, it is conceivable that as few as two molecules of Tat may be bound to the two copies of viral genomic RNA within each virion. Hence, Tat can conceivably be part of the reverse transcription complex and directly participate in RT reactions following infection of target cells. A recombinant form of Tat was shown to strongly inhibit reverse transcription in cell-free assays (Kameoka et al., 2002; Kameoka et al., 2001). The elongation, and not the initiation stage of reverse transcription was suppressed. Several studies have shown that the TAR binding activity of Tat is not required in order for Tat to exert its effects on reverse transcription (Harrich et al., 2000; Ulich et al., 1999). The biological importance of the suppressor activity of Tat in reverse transcription still needs further verification.

1.4.5.2 Rev

HIV-1 uses alternative splicing of its full-length transcript to generate the array of mRNAs required for efficient expression of all viral genes. HIV-1 RNAs that contain functional introns must be selectively exported from the nucleus. HIV-1 has evolved a sequence-specific nuclear RNA export factor, termed Rev, that is able to bind to a *cis*-acting RNA target [the Rev response element (RRE)] present in all unspliced viral transcripts and targets them for nuclear export (Malim et al., 1989; Pollard and Malim, 1998). The ~116 aa Rev protein consists of an N-terminal domain that mediates RRE-binding, Rev-Rev multimerization as well as nuclear localization, and a C-terminal leucine-rich domain that contains a nuclear export signal (NES) that allows Rev to shuttle between the nucleus and cytoplasm (Meyer and Malim, 1994). NES interacts with a nucleoporin-like protein (hRip/Rab) located at the nuclear pore (Bogerd et al., 1995; Fritz et al., 1995; Stutz et al., 1995). The interaction with hRip/Rab may be bridged by CRM1, a nuclear export receptor that is important for Rev export (Ullman et al., 1997). Rev binding to the RRE is believed to target

the attached mRNA to the nuclear export machinery. The RRE contains several hairpins and binds several Rev monomers, nucleated by the interaction of a single monomer with a high-affinity site, hairpin IIB (Zemmel et al., 1996). Oligomeric binding is important for Rev function, presumably because it increases the concentration of NES sites on a single mRNA.

1.4.5.3 Vpu

The other auxiliary proteins of HIV-1 are conserved in some or all of the animal lentiviruses (Tat, Rev, and Vif) or at least in all primate immunodeficiency viruses (Nef and Vpr). However, Vpu appears unique to HIV-1 and the closely related SIV_{cpz} isolates. Newly synthesized Env glycoproteins (gp160), which are later cleaved into gp120 and gp41, are sometimes held in the endoplasmic reticulum through interactions with newly synthesized CD4 molecules. Vpu promotes degradation of CD4 in these complexes, thus allowing Env transport to the cell surface for assembly into viral particles.

Vpu is an 81-residue oligomeric integral membrane protein with an N-terminal 24-residue hydrophobic membrane-spanning domain and a C-terminal cytoplasmic tail (Cohen et al., 1996; Lamb and Pinto, 1997). Amino acids important for receptor binding and degradation have been mapped to the C-terminal region of Vpu and to putative α-helices in the cytoplasmic tail of CD4 (Tiganos et al., 1997). Vpu, via its cytoplasmic tail, binds to CD4 molecules that have been retained in the ER and also recruits a cellular factor termed h-βTrCP to the ER membrane. The h-βTrCP protein then recruits a cellular targeting factor for ubiquitin-mediated proteolysis termed Skp1. This recruitment targets CD4 for degradation, most probably by the proteasome, while Vpu is apparently recycled (Margottin et al., 1998). Vpu can also downregulate cell surface expression of MHC class I proteins, which may protect infected cells from recognition and killing by cytotoxic T lymphocytes (Kerkau et al., 1997).

In addition to its role in CD4 degradation, Vpu can also stimulate virion release. Vpu appears to facilitate virion release both by promoting the budding of virions from plasma, as opposed to intracytoplasmic, membrane structures and by facilitating the release of budding virions from the membrane (Klimkait et al., 1990; Lamb and Pinto, 1997). In contrast to Vpu-mediated CD4 degradation, its effect on particle release requires the N-terminal hydrophobic transmembrane domain. This effect appears to be relatively non-specific, in that Vpu has also been reported to facilitate the budding of unrelated retroviruses (Cohen et al., 1996; Lamb and Pinto, 1997).

1.4.5.4 Nef

Nef is the largest auxiliary protein, at ~266 aa in length. Nevertheless, Nef was initially found to exert a quite modest effect on the rate of HIV-1 replication in culture, with some early reports even suggesting that Nef was a negative factor. However, more recent research has demonstrated that Nef exerts at least three distinct activities in infected cells: down-regulation of cell surface CD4, down-regulation of cell surface MHC I, enhanced virion infectivity.

The Nef protein is posttranslationally modified by myristylation of its N-terminus, and this modification targets Nef to the inner surface of the plasma membrane and to the trans-Golgi network (TGN). In contrast to the effects of Env and Vpu on CD4 en route to the plasma membrane, Nef acts to remove CD4 that is already on the cell surface by accelerating endocytosis through clathrin-coated pits (Mangasarian and Trono, 1997). New evidence suggests that endocytosis occurs through interactions between Nef and a protein complex, the AP-2 adaptor complex, that recruits transmembrane proteins to clathrin-coated pits (Greenberg et al., 1997; Piguet et al., 1998). By downregulating CD4, Nef may enhance Env incorporation into virions, promote particle release, and possibly affect $CD4^+$ T-cell signaling pathways (Mangasarian and Trono, 1997). In addition to CD4, Nef also induces the specific down-regulation of cell-

surface MHC I receptors, albeit with somewhat lower efficiency (Le Gall et al., 1998). Because MHC I is required to present viral particle epitopes to cytotoxic T lymphocytes (CTL), down-regulation of cell surface MHC I could inhibit the CTL-mediated lysis of HIV-1-infected cells (Collins et al., 1998). Analysis of the Nef protein demonstrates that CD4 and MHC I down-regulation can be, at least in part, mutationally segregated (Greenberg et al., 1998). At present, the simplest interpretation of the available data is that Nef connects both CD4 and MHC I to the intracellular protein sorting machinery by binding to CD4 or MHC I on the one hand and either AP-1 or AP-2 on the other. Regardless of whether AP-1 or AP-2 is recruited, which may largely depend on whether recruitment occurs at the TGN or at the plasma membrane, these target proteins are then sorted into clathrin-coated vesicles that deliver them for degradation in lysosomes.

1.4.5.5 Vif

Vif is ~192 aa in length and is expressed at high levels in the cytoplasm of infected cells. A substantial fraction of Vif is membrane associated and colocalizes with the HIV-1 Gag protein, a property that is likely to be important for its biological activity. Vif protein is important for the production of highly infectious mature virions. Vif mutant viruses show markedly reduced levels of viral DNA synthesis and produce highly unstable replication intermediates (Cohen et al., 1996; Simon and Malim, 1996), suggesting that Vif functions before or during DNA synthesis. It is intriguing that Vif mutants show defects in infectivity only when produced in certain cell types, designated non-permissive or semipermissive, but not when produced in permissive cells. It is possible that permissive cells produce a factor or factors that compensate for a lack of Vif or that expression of Vif in permissive cells blocks an inhibitor of viral infectivity (Cohen et al., 1996). Vif activity may be regulated by posttranslational modification because mutation of one of these serine

phosphorylation sites (Ser144) causes a defect in viral infectivity (Yang et al., 1996).

Although no cellular target for Vif is as yet known, some data demonstrate that such a target must exist (Simon et al., 1998). It has been estimated that 7 to 100 molecules of Vif are packaged into the virion (Camaur and Trono, 1996; Fouchier et al., 1996; Karczewski and Strebel, 1996; Liu et al., 1995), suggesting that Vif may function directly within the particle. Incorporation of Vif is probably nonspecific because there is no apparent requirement for any viral protein or RNA and, like Nef, Vif can be incorporated into Mo-MLV particles (Camaur and Trono, 1996).

1.4.5.6 Vpr

The Vpr protein is a late HIV-1 gene product of ~96 aa in length that is packaged into the virion nucleocapsid in molar amounts equivalent to those of the Gag protein. Vpr may therefore be viewed as a virion structural protein. Packaging into virions is mediated by the p6 protein, located at the very C-terminus of the p55 Gag precursor, and also requires specific sequences located toward the center of Vpr (Paxton et al., 1993). Following fusion and entry, the virus is "uncoated" in the cytoplasm and nucleoprotein complexes [(often termed preintegration complexes (PICs)] are rapidly transported to the host cell nucleus, mediated by Vpr protein (Cohen et al., 1996). Vpr becomes associated with the PICs through an interaction with the C-terminal region of MA. Vpr does not contain a canonical karyophilic NLS but instead contains two important putative N-terminal amphipathic α-helices (Emerman, 1996). This unusual NLS localizes Vpr to the nuclear pores rather than to the interior of the nucleus and does not use an importin-dependent pathway (Emerman, 1996; Gallay et al., 1996). A search for potential cellular target proteins for Vpr has demonstrated that Vpr can directly interact with a subset of nucleoporins, thus potentially explaining this localization. In addition, Vpr can also bind to Imp α, the adaptor protein that normally mediates the interaction

of basic NLS sequences with Imp β nuclear import factor (Mattaj and Englmeier, 1998). While the reason for the interaction of Vpr with Imp α is uncertain, it has been proposed that it could enhance the affinity of Imp α for basic NLSs present on HIV-1 PICs, and thereby enhance PIC import into the nucleus (Popov et al., 1998). In any case, the interaction of Vpr with nucleoporins and Imp α appears likely to be critical for its role in HIV-1 PIC nuclear import and, hence, in enhancing HIV-1 replication in macrophages and other nondividing cells.

In addition to its nuclear uptake function, Vpr can also induce G2 cell cycle arrest prior to nuclear envelope breakdown and chromosomal condensation, and sustained expression can reportedly kill T cells by apoptosis (Emerman, 1996). An interesting rationale for the Vpr-mediated arrest of cells in G2 is provided by the observation that the HIV-1 LTR promoter is more active in G2-arrested cells (Goh et al., 1998). In addition to roles in nuclear localization and cell cycle arrest, Vpr can also influence mutation rates during viral DNA synthesis and has been proposed to form an ion channel (Lamb and Pinto, 1997).

1.5 HIV-1 life cycle

HIV-1 binds to target cells initially through high-affinity interactions of its gp120 envelope glycoprotein with surface CD4 receptors. This interaction triggers conformational changes in gp120 which promote engagement of the HIV coreceptor. These events, in turn, activate the gp41 envelope protein to mediate fusion of viral and cellular membranes. Fusion leads to "microinjection" of the HIV-1 capsid component of the virion. Once inside the cell, the HIV capsid undergoes "uncoating". Uncoating is followed by reverse transcription of the viral RNA genome which cultimates in formation of the preintegration complex (PIC). The PIC must traverse from the plasma membrane to the nuclear envelope. The proviral DNA is transported into the nucleus and integrated into cellular DNA. Transcription of the provirus by cellular RNA polymerase II

generates spliced and unspliced mRNA and progeny RNA genomes. Viral mRNA is translated in the cytoplasm. The viral proteins and progeny RNA are assembled at the cell periphery, and the viral particles are released by a process of budding followed by proteolytic cleavage of virion polyproteins into infectious virus (Fig. 1-3).

1.5.1 HIV-1 attachment and entry:

The attachment of HIV-1 involves the formation of a stable complex between the gp120 of the virus and CD4. This interaction is highly specific and is conserved among all primate lentiviruses. The CD4 molecule belongs to the immunoglobulin superfamily and it consists of an extracellular region containing four immunoglobulin-like domains (D1-D4), a membrane-spanning region and a charged cytoplasmic domain. Immunological and molecular analyses confirmed that HIV-1 binds to the first immunoglobulin domain of CD4. Some of the amino acids on gp120 that form the CD4 binding site are variable; however, for these residues, the peptide backbone rather than their side chains are involved in contacting CD4 (Kwong et al., 1998).

CD4 expression appears to be the principal correlate for virus attachment to T cells, probably mediating most of the initial association between gp120 and the host cell. This interaction induces co-receptor binding, leading to the formation of a gp120-CD4-coreceptor complex, and the initiation of the fusion process (Kwong et al., 1998; Wyatt and Sodroski, 1998). Over 14 different seven transmembrane (7TM) receptors have been identified as potential co-receptors for HIV and SIV by their capacity to support infection of $CD4^+$ cell lines *in vitro*. These receptors are members of (or closely related to) the chemokine receptor family. CCR5 and CXCR4 are the major co-receptors and all HIV-1 isolates can use one or both. CCR5 is used primarily during transmission and the asymptomatic phase of infection, whereas CXCR4 is generally used at later stages in the infection (Berger, 1997). The phenotypic switch from CCR5 use (R5

virus) to CCR5/CXCR4 (R5X4) or CXCR4 use (X4) predicts progression to AIDS (Berger, 1997), but the dynamics of this switch are not understood. *In vitro*, R5 viruses infect primary clusters of both lymphocytes and macrophages, while X4 isolates also infect T-cell lines. The capacity of X4 strains to infect macrophages is controversial (Stent et al., 1997); however, some studies have shown that primary X4 isolates infect at least some populations of macrophages (Simmons et al., 1996). In addition to CCR5 and CXCR4, approximately a dozen other coreceptors have been identified through use of *in vitro* assays. In general, these alternative coreceptors are used by only a subset of HIV or SIV strains, and they tend to support virus infection less efficiently than the major coreceptors. The basis for the tropism for either CCR5 or CXCR4 residues in the ability of gp120 to interact directly with these receptors. The co-receptor binding site on gp120 is not usually fully exposed until CD4 is bound. The variable V1/V2 loops are probably the main cover for the co-receptor binding site and these loops become repositioned when CD4 is bound (Kwong et al., 1998).

HIV viral particles interact with a range of other cell surface receptors via interactions that involve gp120. These interactions do not actively support HIV entry but aid attachment of HIV virions to cell surfaces that contain suboptimal levels of CD4 or co-receptors, e.g. on macrophages and astrocytes. Some of these interactions are mediated by the sugar groups on the envelope glycoprotein associating with other sugars or with receptors that contain lectin-like domains on the cell surface, e.g. the

Fig. 1-3 Schematic overview of major steps in the HIV-1 life cycle.

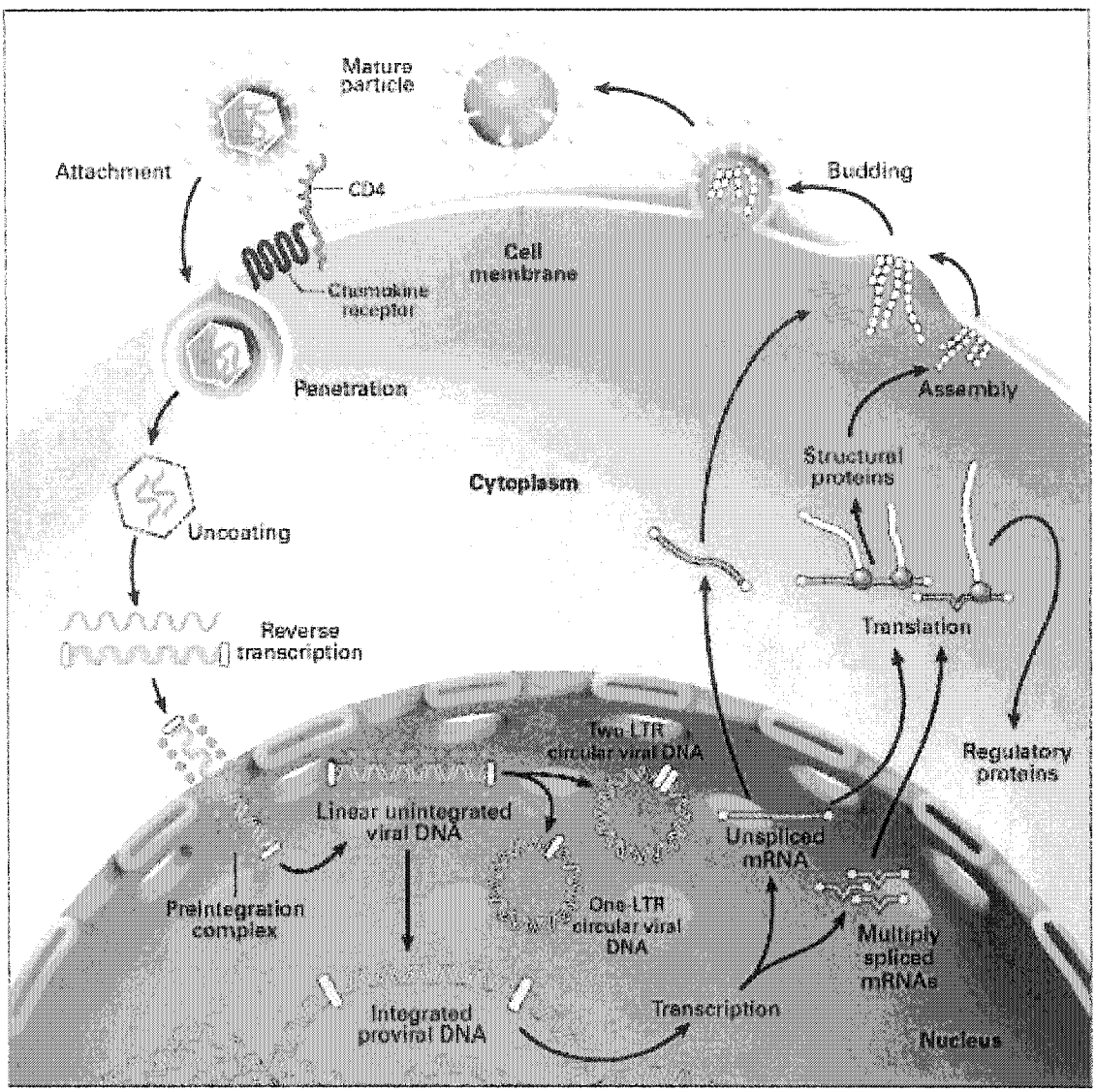

Mature particle
Attachment
CD4
Chemokine receptor
Cell membrane
Budding
Penetration
Assembly
Structural proteins
Cytoplasm
Uncoating
Reverse transcription
Translation
Regulatory proteins
Two LTR circular viral DNA
Linear unintegrated viral DNA
Unspliced mRNA
Preintegration complex
One-LTR circular viral DNA
Multiply spliced mRNAs
Integrated proviral DNA
Transcription
Nucleus

Fig. 1-3

mannose specific macrophage endocytosis receptor (Larkin et al., 1989) and DC-SIGN (Geijtenbeek et al., 2000). HIV envelope gp120 also binds the glycolipid, galactocerebroside (gal-C) and its sulphated derivate, sulphatide (Fantini et al., 1993; Harouse et al., 1991). These molecules are expressed on neuronal and glial cells in the brain, colon epithelial cell lines (Fantini et al., 1993) and importantly also on macrophages (Seddiki et al., 1994). Both DC-SIGN and Gal-C bind gp120 with a high affinity, similar to the binding affinity of monomeric gp120 for CD4. Although these receptors may aid HIV attachment, fusion will not occur until sufficient CD4 and co-receptor molecules are recruited to trigger formation of a fusion pore.

It is likely that the interaction of the gp120-CD4 complex with the appropriate chemokine receptor promotes additional conformational changes in the envelope glycoprotein complex. The proposed result of these changes is the insertion of the hydrophobic gp41 N-terminus (the "fusion peptide") into the membrane of the target cell. Coreceptor binding leads to the formation of a triple-stranded coiled-coil that enables the hydrophobic fusion peptide at the N-terminus of gp41 to insert into the target cell membrane, making gp41 an integral component of both the viral and cellular membranes. The triple-stranded coiled-coil then bends back on itself, forming a six helix bundle in which the gp41 fusion peptide and transmembrane domain are at the same end (Chan et al., 1997; Weissenhorn et al., 1997). A recent study by Melikyan et al. has shown that it is the transition of gp41 from the coiled coil to the six helix bundle that is the proximal cause of membrane fusion (Melikyan et al., 2000). Clustering of a certain number of CD4 and coreceptor molecules is presumed to be necessary for the efficient fusion of viral and host cell membranes. It was recently proposed that about 5 CCR5 molecules seem required to form a fusion pore (Kuhmann et al., 2000) and three CD4 binding events are needed to efficiently activate HIV-1 Env trimers. Whether these are the actual numbers of receptors required for fusion and

entry remains to be confirmed. In *in vitro* experiments using cell lines, the HIV-1 Env-mediated fusion event may be facilitated by the high expression of chemokine and CD4 molecules on the cell surface. In primary cells, the lower number of chemokine receptors ($CD4^+$ T cells) or CD4 molecules (macrophage) may be a limiting factor in fusion pore formation (Doms, 2000). Therefore, existence of an active mechanism for receptor clustering may be of particular importance. Lipid rafts and caveolae are plasma membrane domains that are enriched in cholesterol and sphingolipid. Both lipid rafts and caveolae are docking sites for specific proteins involved in signal transduction. The role of lipid rafts could also be to increase the local concentration and rearrangement of the HIV-1 fusion pore components (Dimitrov, 2000).

Once the virion has fused with the target cell, the viral conical core formed by viral capsid protein (CA) is released into the host cell cytoplasm, while the envelope glycoproteins are left on the surface of the cell membrane. In order to replicate, the conical core must dissemble properly resulting in the release of viral nucleic acids into cytoplasm. Based on electron microscopic analysis of acutely infected cells, it is generally thought that the conical core does not persist for long following fusion of viral and cellular membranes (Grewe et al., 1990). The available biochemical data are also consistent with rapid disassembly of the core, as CA was undetectable in reverse transcription complexes isolated from infected cells (Fassati and Goff, 2001). However, in some studies, cores that disassembled too rapidly were also defective for reverse transcription in cells. A model that may reconcile this paradox is that upon delivery into the cytoplasm, the CA shell targets the reverse transcription complex to a specific intracellular compartment that is conducive to successful infection. In this model, HIV-1 cores that disassemble too rapidly are not transported. However, following delivery to this compartment, the CA shell must also dissociate in order for the viral ribonucleoprotein complex to initiate reverse transcription. Emerging

evidence suggests that the target cell cytoskeleton facilitates early steps of HIV-1 infection (Bukrinskaya et al., 1998).

1.5.2 Reverse transcription

1.5.2.1 Reverse transcription

Reverse transcription is a central step in the life cycle of the HIV-1, during which the single-stranded genomic RNA is converted into a double-stranded DNA that is later integrated into the host chromosome. The process is catalyzed by reverse transcriptase (RT) protein and is mainly carried out in the cytoplasm soon after viral penetration into the cell. RT is a multifunctional enzyme with both RNA-dependent (RDDP) and DNA-dependent DNA polymerase (DDDP) activities, as well as a ribonuclease H (RNase H) activity that specifically degrades the RNA strand of RNA/DNA hybrids (Gilboa et al., 1979; Telesnitsky and Goff, 1997). All of these activities are essential for reverse transcription.

The process of reverse transcription may be brokendown into 5 discrete steps, as depicted in Figure (Fig. 1-4).

1. The synthesis of minus-strand strong-stop DNA [(-) ssDNA]. The initiation of reverse transcription requires a cellular tRNA, $tRNA^{Lys3}$, as a primer, which the virion selectively incorporates during its assembly in its cell of origin. The primer tRNA is annealed to an 18-base sequence near the 5' end of the viral RNA genome termed the primer binding site (PBS), and is used to prime the reverse transcriptase catalyzed synthesis of minus-strand DNA, the first step in reverse transcription. Synthesis of minus-strand DNA proceeds toward the 5' end of the viral genome to generate (-) ssDNA, the first discrete product during reverse transcription. Concomitant with DNA synthesis, the RNA strand of the newly formed RNA/DNA hybrid is degraded by the RT-associated RNase H activity. First cuts are seen directly upstream of the PBS, whereas the initially

Fig. 1-4 The current model of HIV-1 reverse transcription. (Modified from Götte et al. 1999).

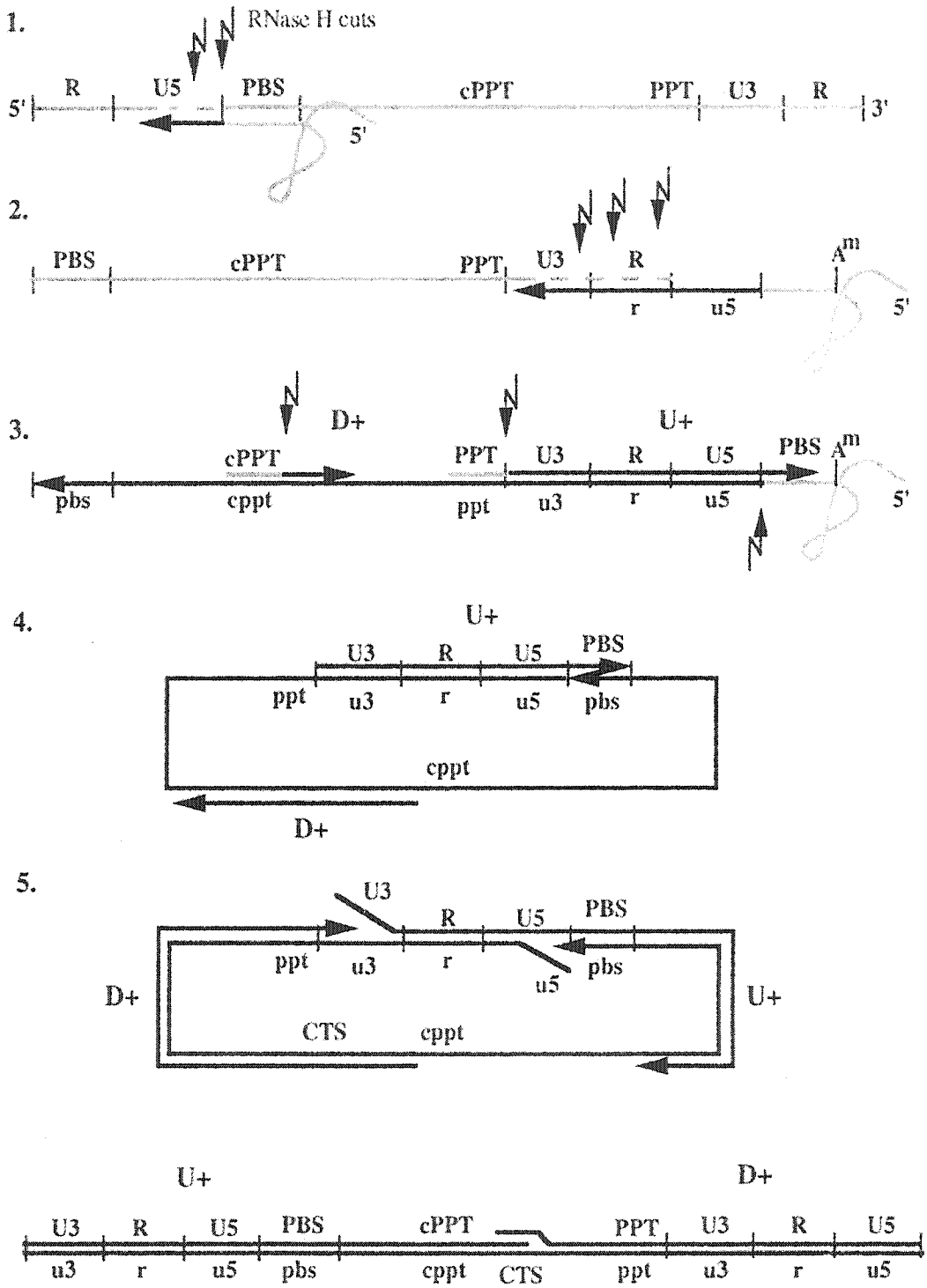

1.
RNase H cuts
5'
R
U5
PBS
cPPT
PPT
U3
R
3'
5'
2.
PBS
cPPT
PPT
U3
R
r
u5
A^m
5'
3.
D+
U+
cPPT
PPT
U3
R
U5
PBS
A^m
pbs
cppt
ppt
u3
r
u5
5'
4.
U+
U3
R
U5
PBS
ppt
u3
r
u5
pbs
cppt
D+
5.
U3
R
U5
PBS
ppt
u3
r
u5
pbs
D+
U+
CTS
cppt
U+
D+
U3
R
U5
PBS
cPPT
PPT
U3
R
U5
u3
r
u5
pbs
cppt
CTS
ppt
u3
r
u5

Fig. 1-4

bound PBS sequence itself, which forms an RNA/RNA homoduplex with tRNA primer, remains resistant to RNase H degradation.

2. The first strand transfer. The nascent (-) DNA must undergo a template switch or strand transfer to continue the synthesis of minus-strand DNA. Because of the complementarity of the newly transcribed R sequences of (-) ssDNA and those of the 3' end of the viral genome, (-) ssDNA anneals to this region on the same (intrastrand switch) or on the other strand (interstrand switch) of viral RNA. RNase H degradation of the 5' end of the RNA genome releases the (-) ssDNA, thereby facilitating its translocation to the 3' end of genome.

3. Synthesis of plus-strand strong-stop DNA [(+) ssDNA]. As synthesis of minus-strand DNA proceeds, RNase H digests away the template RNA genome. However, the digestion is incomplete, because there are purine-rich stretches of the RNA template that are highly resistant to RNase H digestion. These regions are located close to the 3' end as well as in the center of the viral genome and are known as the polypurine tracts (3'PPT) and the central PPT (cPPT), respectively, and function as RNA primers for plus-strand DNA synthesis. As the minus-strand DNA synthesis continues to the 5' end of the genome, the synthesis of the two plus-strand DNA segments will be initiated by these PPT primers using the newly transcribed minus-strand DNA as a template. The two distinct plus-strand DNA segments thus formed are called downstream (D+) and upstream segments (U+) and are initiated by the cPPT and 3'PPT, respectively. Plus-strand DNA synthesis continues into the first 18 nucleotides of the $tRNA^{Lys3}$ to generate so-called plus-strand strong stop DNA [(+) ssDNA]. The signal for termination of (+) ssDNA synthesis lies within the tRNA primer. This base is a methylated adenosine located 19 residues upstream from the 3' end of the tRNA. Mutation of this base disrupts reverse transcription and inhibits replication of HIV-1 (Renda et al., 2001). The partial copy of $tRNA^{Lys3}$ regenerates the PBS at the 3' terminus of the (+) ssDNA. The remaining portion of $tRNA^{Lys3}$ is cleaved by RNase H, which

facilitates the subsequent second strand transfer. RNase H also cleaves the PPTs after they have primed plus-strand DNA synthesis.

4. The second strand transfer. The second strand transfer is facilitated by the newly synthesized PBS of the 3' end of (+) ssDNA and a complementary minus-strand DNA copy that is formed when minus-strand DNA synthesis advances into the PBS region. So, during reverse transcription, the PBS not only provides an anchor for the tRNA primer at the initiation of the reaction, but also makes the second strand transfer possible. This second strand transfer is presumably an intramolecular event that involves formation of a circular intermediate.

5. Generation of a double-stranded blunt-ended DNA. HIV-1 RT strand displacement activity is required to break up the U3-R-U5 duplex formed during U+ DNA synthesis, which allows continuation of the synthesis of the minus-strand and of D+ DNA. The synthesis of U+ DNA requires the displacement of about 100nt of the D+ DNA sequence and continues until RT encounters the central termination sequence (CTS). As a result, the final product of HIV-1 reverse transcription is a double-stranded blunt-ended DNA, with a long terminal repeat (LTR) composed of U3, R, and U5 at the 3' and 5' ends, and also with a plus-strand overlap in its center, which is usually referred to as the central DNA flap.

1.5.2.2 Primer $tRNA^{Lys3}$

Retroviruses use cellular tRNAs as primers for reverse transcription during their replication cycle. Different retroviruses use different cellular tRNAs as primer (Mak and Kleiman, 1997). $tRNA^{Trp}$ is the primer for all avian retroviruses and $tRNA^{Pro}$ is a common primer for murine retroviruses. There are three major $tRNA^{Lys}$ isoacceptors in mammalian cells (Raba et al., 1979). $tRNA^{Lys1,2}$, representing two $tRNA^{Lys}$ isoacceptors differing by one base pair in the anticodon stem, is also the primer tRNA for several retroviruses, including Mason-Pfizer monkey virus (MPMV) and human foamy virus (HFV). $tRNA^{Lys3}$ serves as the

primer for mouse mammary tumor virus (Peters and Glover, 1980; Waters, 1978) and the lentiviruses, such as equine infectious anemia virus (EIAV), feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV), and HIV-1 and HIV-2 (Leis, 1993).

Primer tRNAs are selectively packaged into retroviruses during their assembly, i.e., their concentration in the low molecular weight RNA population increases in the virus over that found in the cytoplasm. $tRNA^{Lys3}$ increases from 5%~6% to 50%~60% in HIV-1 (Mak et al., 1994). In HIV-1, estimates of approximately 20 molecules of $tRNA^{Lys}$/virion have been reported, with about 8 molecules $tRNA^{Lys3}$ and 12 molecules $tRNA^{Lys1,2}$ per virion, reflecting cytoplasmic ratios (Huang et al., 1994b). So, $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ are packaged into HIV-1 with equal efficiencies. The function of $tRNA^{Lys1,2}$ in the HIV-1 life cycle is unknown.

$tRNA^{Lys}$ isoacceptors are selectively packaged into HIV-1 independently of Gag and Gag-Pol processing, and independently of genomic RNA incorporation (Mak et al., 1994). Gag-Pol is required for $tRNA^{Lys}$ packaging, and particles composed of Gag alone do not package $tRNA^{Lys}$ (Mak et al., 1994). RT sequences within Gag-Pol play an important role in interacting with $tRNA^{Lys}$. Selective packaging of tRNA primer does not occur in RT-negative ASV, RSV, MuLV, or HIV-1 (Levin et al., 1984; Mak et al., 1994; Peters and Glover, 1980; Sawyer and Hanafusa, 1979). *In vivo* studies have indicated an important role of the RT thumb subdomain within Gag-Pol in the viral packaging of $tRNA^{Lys3}$ (Khorchid et al., 2000). The RT thumb domain also seems important for the *in vitro* interaction between mature RT p66/p51 and $tRNA^{Lys3}$ sequences. It has been shown that a 127-amino acid cyanogen bromide fragment of RT (amino acids 230-357, containing all of the thumb subdomains flanked by small regions from the palm and connection subdomains) was cross-linked to a synthetic $tRNA^{Lys3}$ thiolated at base 36 in the anticodon loop (Mishima and Steitz, 1995). Another group found an interaction between

the 3' terminus of purified bovine liver $tRNA^{Lys3}$ and an RT peptide whose amino terminus contains the conserved amino acid sequence 241VQPI244, which is found at the junction of the palm and thumb subdomains of RT (Dufour et al., 1999). Arts et al. also found evidence for an *in vitro* interaction between the anticodon loop of $tRNA^{Lys3}$ and a small crevice in the p66 thumb domain of mature RT, and mutations at K249 and R307 in the thumb subdomain disrupted this interaction (Arts et al., 1998). However, these same mutations had no effect upon $tRNA^{Lys3}$ packaging *in vivo* (Khorchid et al., 2000). These *in vitro* experiments support the *in vivo* finding that the thumb region of RT Gag-Pol is important during $tRNA^{Lys}$ packaging, but whether it interacts with the anticodon arm or the 3' terminus of $tRNA^{Lys3}$ is not clear. However, quantitative binding assays have also demonstrated that RT can bind to nonincorporated forms of tRNA, with the same efficiency as to $tRNA^{Lys3}$ (Thrall et al., 1996). Thus the enzyme may recognize the gross L-shaped tRNA structure rather than specific structural features of its cognate primer. The different conclusions reached by these studies could be due to the use of a synthetic $tRNA^{Lys3}$ versus fully modified natural $tRNA^{Lys3}$. However, it is consistent that specificity for the selective incorporation of the three $tRNA^{Lys}$ isoacceptors may be mediated by the RT region of Gag-Pol precursor proteins or Gag / Gag-Pol complex (Huang et al., 1998a; Huang et al., 1997a; Mak et al., 1994; Mak et al., 1997).

Recently, it has been reported that lysyl-tRNA synthetase (LysRS) plays an important role in the $tRNA^{Lys}$ packaging. LysRS is the enzyme that amino-acylates $tRNA^{Lys}$ and is a $tRNA^{Lys}$-binding protein. Because LysRS is selectively packaged into the viral particle (Cen et al., 2001), it may be the signal that targets the $tRNA^{Lys}$ isoacceptors for incorporation into HIV-1. Although nucleocapsid protein sequences have also been reported to play an important role in retroviral processes such as primer tRNA annealing, genomic RNA packaging and dimerization, and minus strand strong stop DNA strand transfer (Darlix et al., 1995; Guo et al., 1997),

there is currently no evidence from mutational analysis that they play a direct role in $tRNA^{Lys}$ packaging.

1.5.2.3 HIV-1 5' untranslated leader RNA

Reverse transcription of retroviral genomes is primed by a cellular $tRNA^{Lys3}$ molecule that binds to the PBS that is located in the 5' untranslated leader region of the viral RNA genome. Extensive secondary structure in the 5' untranslated leader region of the HIV-1 genome has been suggested by electron microscopy, replication studies with mutant viruses, and biochemical RNase probing studies (Baudin et al., 1993; Clever et al., 1995; Das et al., 1997b; Harrison and Lever, 1992; Hoglund et al., 1997; McBride and Panganiban, 1996). These results, combined with phylogenetic analyses and computer assisted structure prediction, led to a model of the secondary RNA structure of the complete leader region of the HIV-1 genome (Berkhout, 1996) (Fig. 1-5). Several RNA secondary structures in the leader RNA have been reported to regulate important viral replication steps of HIV-1; examples are transcriptional transactivation by Tat, mRNA polyadenylation, and dimerization of the viral RNA genome. According to this model, the PBS is predicted to be part of an extended RNA structure. This structure consists of a small U5-PBS hairpin that contains part of the PBS, and a large stem region formed by sequences of the upstream U5 region and the downstream leader region, the U5-leader stem. Stabilization of the hairpin was found to inhibit reverse transcription because of reduced tRNA primer annealing. Destabilization of the U5-PBS hairpin did not affect tRNA binding, and initiation of reverse transcription was in fact slightly activated (Beerens et al., 2000b). The thermodynamic stability of the U5-PBS hairpin apparently has to stay within narrow limits for efficient HIV-1 replication. Additional base pairing interactions between RNA sequences in the U5 region and the tRNA primer have been suggested to stimulate primer

Fig. 1-5 The secondary structure of HIV-1 5' untranslated leader sequence.

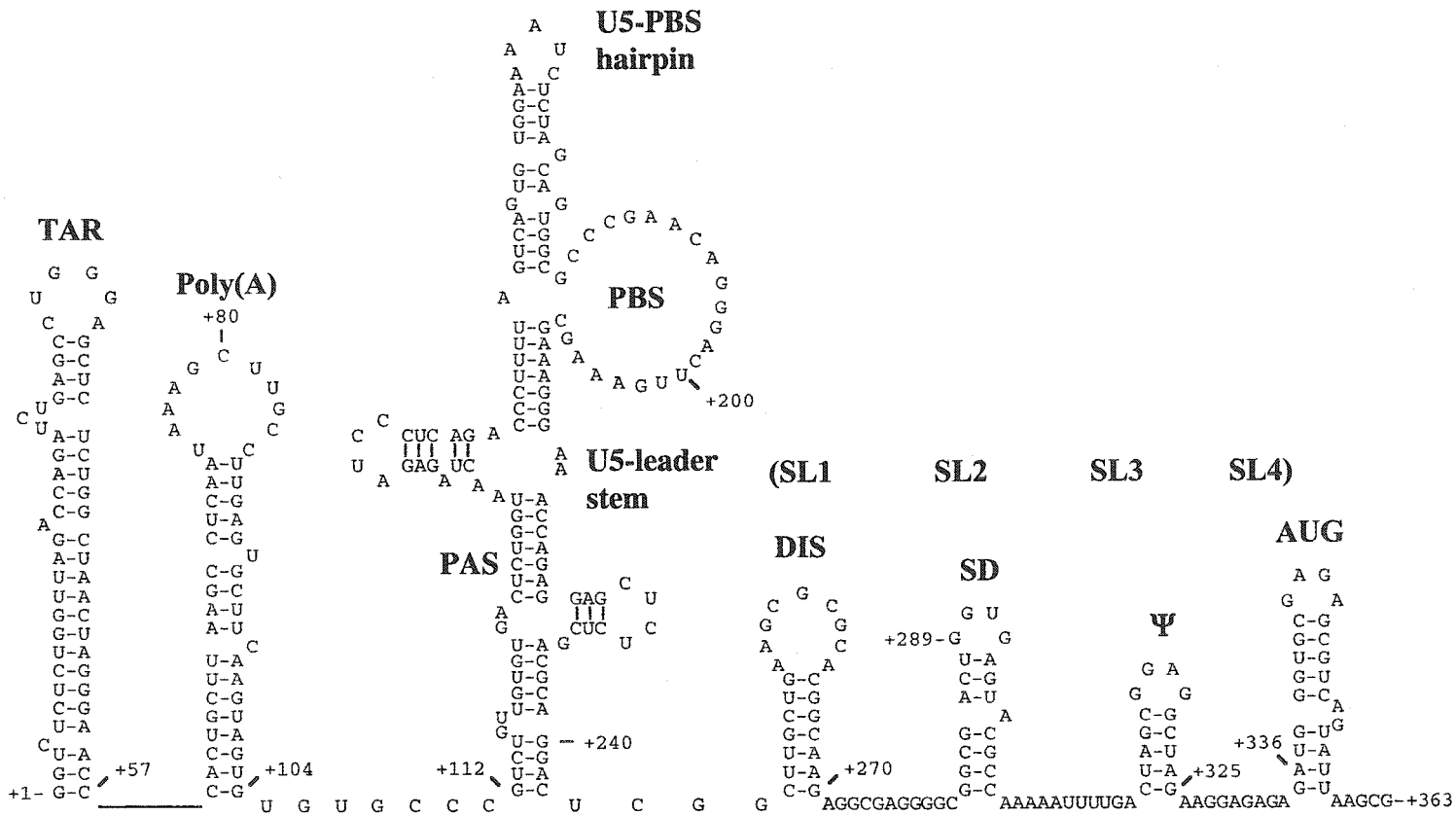

TAR
Poly(A)
U5-PBS
hairpin
PBS
U5-leader
stem
PAS
(SL1
SL2
SL3
SL4)
DIS
SD
Ψ
AUG
+1
+57
+80
+104
+112
+200
+240
+270
+289
+325
+336
+363
U G U G C C C U C G G
AGGCGAGGGGC
AAAAAUUUUGA
AAGGAGAGA
AAGCG

Fig. 1-5

annealing onto the PBS. The interaction between the tRNA anticodon loop and the A-rich loop of the U5-PBS may be affected by mutation of the U5-PBS hairpin. These combined results suggest that the U5-PBS hairpin is involved in both the proper annealing of the tRNA primer onto the viral genome and the initiation of reverse transcription (Beerens et al., 2000b). Further study of the HIV-1 U5-leader stem using detailed mutation analysis suggested that the U5 region contains a motif that is critical for $tRNA^{Lys3}$-mediated initiation of reverse transcription but not for reactions that are initiated by a DNA primer. As a result, Berkhout et al. identified an eight-nucleotide sequence in the U5 region that is not involved in tRNA annealing but that is important for initiation of reverse transcription (Beerens et al., 2001). This U5 motif, therefore, was termed the primer activation signal (PAS). It is proposed that this PAS sequence base pairs with the TΨC arm of the tRNA molecule, similar to the additional interaction proposed for the RSV genome and the corresponding $tRNA^{Trp}$ primer, thereby triggering initiation of HIV-1 reverse transcription (Aiyar et al., 1992).

The importance of the PAS element was supported by the following evidence. Deletion of the PAS motif results in a 10-fold inhibition (Beerens et al., 2001). By simultaneous alteration of the PAS and PBS motifs to accommodate another tRNA molecule, the identity of the priming tRNA species could be modulated (Beerens and Berkhout, 2002a). Furthermore, the PAS motif is absolutely conserved among all HIV-1 isolates. The PAS-TΨC arm interaction does not require additional melting of the tRNA because $tRNA^{Lys3}$-PBS annealing will open both the acceptor and TΨC stem. However, the PAS sequence in the HIV-1 genome is occluded by base pairing in the U5 leader stem, as was demonstrated by biochemical probing experiments and, thus, needs to be unwound for interaction with the tRNA primer.

The presence of the PAS enhancer motif that is initially repressed by base pairing provides a unique mechanism for positive and negative regulation

of HIV-1 reverse transcription (Beerens and Berkhout, 2002b; Beerens et al., 2001). Although binding of $tRNA^{Lys3}$ to the PBS can occur relatively early, *e.g.* in virus-producing cells, primer activation will require a structural rearrangement of the viral RNA/ $tRNA^{Lys3}$ complex to establish the PAS-TΨC arm interaction. It is possible that viral NC protein, which acts as an RNA chaperone (Rein et al., 1998), can mediate this conformational change. Because NC is only released from the Gag precursor protein during maturation of virion particles, this mechanism will ensure precise timing for activation of reverse transcription. This mechanism may also preclude premature reverse transcription in the virus-producing cell such that the viral RNA genome is copied only after it is appropriately dimerized and packaged into virions. This mechanism would rigidly limit reverse transcription to the correct template and, hence, protect the host cell from potentially deleterious unrestricted reverse transcription (Dhellin et al., 1997).

1.5.2.4 Reverse transcription initiation complex

Initiation of HIV-1 reverse transcription requires specific recognition of the viral genome, $tRNA^{Lys3}$, and reverse transcriptase. The specificity of this ternary complex is mediated by intricate interaction between HIV-1 RNA and $tRNA^{Lys3}$.

Recent studies have proposed that, in addition to the PBS, other regions within the viral RNA genome interact with $tRNA^{Lys3}$. A stretch of adenosine residues that form an A-rich loop in the HIV-1 secondary structure have been shown to interact with the anticodon loop of $tRNA^{Lys3}$ (Isel et al., 1995; Isel et al., 1993). This A-loop/anticodon interaction is supported by *in vitro* structure probing, mutagenesis studies, and genetic studies. Mutation of the A-rich loop of HIV-1 RNA has been shown to disrupt interaction with the anticodon loop. Deletion of the A-rich loop caused diminished synthesis of DNA and reduced viral infectivity (Liang et al., 1997a). *In vivo*, a $tRNA^{Lys3}$ with a mutation in the anticodon loop

does not prime reverse transcription (Huang et al., 1996). Interestingly, the A-rich loop can also play a role in determining the identity of the primer tRNA used in HIV-1. Transfection of HIV-1 proviral DNAs with PBS sequences altered so that they were complementary to tRNAs other than $tRNA^{Lys3}$ yielded viruses that eventually reverted back to the $tRNA^{Lys3}$ PBS. Alternative primer tRNAs were stably maintained only if the HIV-1 genome was mutated both in the PBS and A-rich loop to complement the alternate tRNA (Kang and Morrow, 1999; Kang et al., 1997; Kang et al., 1999; Wakefield et al., 1996; Zhang et al., 1996; Zhang et al., 1998b; Zhang et al., 1998c). It had been shown that the post-translational modification of $tRNA^{Lys3}$ was necessary for the interaction between the anticodon loop and A-rich loop. In particular, the sulphur moiety of nucleotide S34 is crucial for the stability of this interaction (Isel et al., 1993).

Based on chemical and enzymatic probing, Isel *et al.* revealed an unexpectedly complex and compact pseudoknot-like secondary structure for the HIV-1 RNA/ $tRNA^{Lys3}$ initiation complex (Isel et al., 1995) that included additional base-pairing between the tRNA and the HIV-1 RNA beyond the 18 nucleotides of PBS and the A-loop/anticodon loop interaction (Fig. 1-6). They showed that the 3' strand of the anticodon stem and the 5' part of the variable loop of $tRNA^{Lys3}$ were also involved in base-pairing with regions 12 to 39 nucleotides upstream of the PBS. The core of the binary complex is a complex junction formed by two single-stranded sequences of $tRNA^{Lys3}$, an intramolecular viral helix, an intramolecular tRNA helix, and two intermolecular helices formed by the template/primer interaction. In order to form this complex, the tRNA and HIV-1 RNA must be significantly unfolded from their native structures. The D stem and loop is the only piece of secondary structure that is conserved from the tRNA clover-leaf structure in this model. These complex interactions between $tRNA^{Lys3}$ and HIV-1 RNA, and the intra-molecular rearrangements did not depend on the presence of upstream and

downstream viral sequences. A short RNA template, encompassing nucleotides 123-217 of the HIV-1 Mal genome, was able, together with the primer tRNA, to adopt the same structure as longer viral RNA templates (Isel et al., 1998).

The specificity of the initiation of reverse transcription appears directly linked to virus-specific interactions between the primer tRNA and genomic RNA in addition to the general PBS-tRNA interaction. *In vitro*, extension of natural $tRNA^{Lys3}$ is much more efficient than that of a synthetic $tRNA^{Lys3}$ lacking post-translational modifications and of an 18mer oligoribonucleotide complementary to the PBS (Arts et al., 1996a; Isel et al., 1996; Lanchy et al., 1996a). The HIV-1 RNA/$tRNA^{Lys3}$ complex is efficiently extended by HIV-1 RT, but not by heterologous RTs, including those of other lentiviruses that have the same natural primer (Arts et al., 1996b; Isel et al., 1996). Conversely, HIV-1 RT is unable to efficiently extend $tRNA^{Lys1,2}$, $tRNA^{Trp}$, $tRNA^{Pro}$, $tRNA^{Phe}$, and $tRNA^{Ile}$ annealed to HIV-1 RNAs whose PBSs are mutated to be complementary to the 3' end of these primers (Li et al., 1994; Oude Essink et al., 1996). Likewise, mutating the PBS does not suffice to stably change the primer usage of HIV-1 in cell culture (Das et al., 1995; Li et al., 1994; Wakefield et al., 1995). As mentioned above, complementarity between the anticodon loop of the tRNA and the A-rich loop located upstream of the PBS is required for efficient replication of HIV-1. The fact that the RNA/$tRNA^{Lys3}$ complex is recognized efficiently only by HIV-1 RT suggests that some of the structural features of this complex are unique to HIV-1.

However, some controversy remains concerning this model. The additional interactions between the tRNA and viral RNA cause the structure to be topologically knotted (Elgavish et al., 1999). This would make DNA elongation from the tRNA primer by reverse transcription difficult to accomplish without breaking and unwinding the tRNA with a topoisomerase, whose existence has not been shown. There is also no

Fig. 1-6 The secondary structure of HIV-1 RNA and $tRNA^{Lys3}$ primer initiation complex. (Modified from Isel et al. 1995).

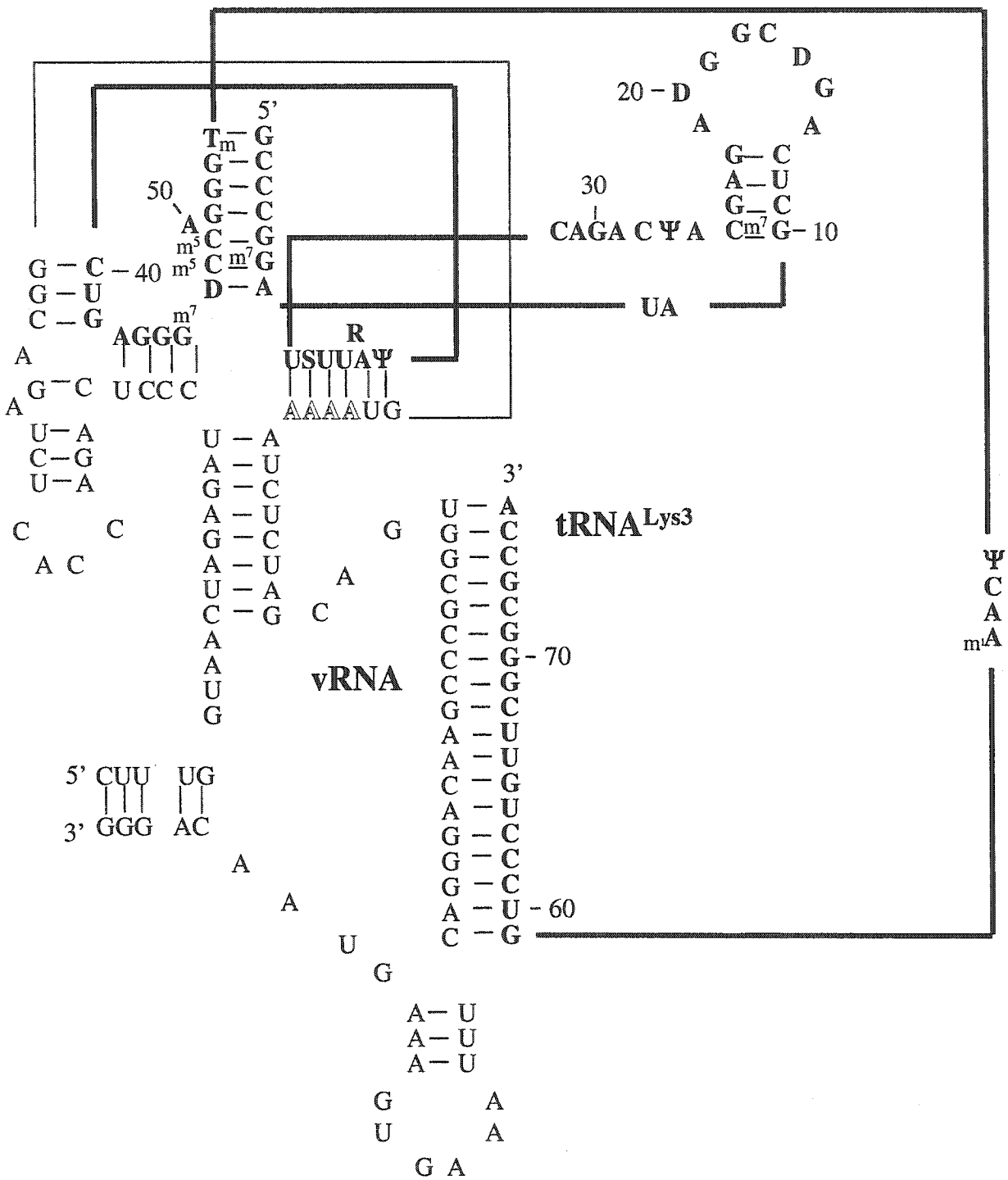

tRNA Lys3
vRNA
5'
3'
20
30
40
50
60
70
R
UA
CAGA C Ψ A
AGGG
USUUAΨ
AAAAUG
5' CUU UG
3' GGG AC
Ψ
C
A
mA

Fig. 1-6

interaction between the 5' portion of TΨC arm and the viral genomic RNA to be postulated in this model. This region would be freed when the 3' portion of the TΨC anneals to the PBS sequence, and such an interaction has been proposed to occur in HIV-1 (Beerens et al., 2001).
The three-dimensional structure of the viral RNA/$tRNA^{Lys3}$ complex was further studied (Isel et al., 1999), using chemical and enzymatic footprinting, which suggested that the secondary structure of the RNA/$tRNA^{Lys3}$ complex was unaffected by RT binding and that the tertiary structure underwent no dramatic change. The extended interactions between these molecules are not directly recognized by RT. Rather, they favor RT binding by preventing steric clashes between the nucleic acids and the polymerase and inducing a viral RNA/ $tRNA^{Lys3}$ conformation which fits perfectly into the nucleic acid binding cleft of RT. The 3' adenosine of $tRNA^{Lys3}$ is in the polymerase catalytic site and C196 on the template, which is located 18nt downstream from the initiation site of reverse transcription, is close to the catalytic residues of the RNase H domain of HIV-1 RT. The interaction between the RNase H domain of HIV-1 RT and the RNA/ $tRNA^{Lys3}$ complex extends to stem-loop 8 of the template. Recognition of the 3' end of $tRNA^{Lys3}$ and of the first template nucleotides by RT is favored by a kink in the template strand promoted by the short junctions present in the established secondary structure (Isel et al., 1999).

1.5.2. 5 Initiation and elongation of reverse transcription

In HIV-1, the initiation stage of synthesis of (-) strand DNA, primed by $tRNA^{Lys3}$, can be distinguished from subsequent strand elongation in regard to both the binding and kinetic properties of reverse transcriptase (Isel et al., 1996; Lanchy et al., 1996a; Lanchy et al., 1996b).
The first steps of initiation are characterized by frequent pausing of RT at position +1, +3, and +5 (Liang et al., 1998) or solely at positions +3 and +5 (Isel et al., 1996), depending on the concentrations of dNTPs used.

This is not an unexpected finding because HIV-1 RT possesses both RDDP and DDDP activities. During reverse transcription, the enzyme is bound to either RNA-DNA or DNA-DNA hybrids during RNA-dependent DNA polymerization and DNA-dependent DNA polymerization, respectively, except at the initiation of synthesis of (-) ssDNA, when enzyme is bound to an RNA-RNA hybrid. After initiation takes place, the role of primer is effectively replaced by the newly made DNA, from which further extension will occur. Therefore the initiation of (-) ssDNA synthesis is a distinct stage of reverse transcription. Because the RT enzyme was originally bound to an RNA-RNA helix, it may require a conformational rearrangement to switch its binding mode from an RNA-RNA duplex to a DNA-RNA hybrid during synthesis of (-) ssDNA. Consequently, reverse transcription primed by the DNA primer processes without pausing at the +1 position (Liang et al., 1998).

Pausing at the +1 position indicates that this conformational rearrangement may occur as soon as the first dC has been added. Direct evidence for this comes from studies of the relationship between the RNase H cleavage site and the polymerization active site. Generally, a constant distance of 18nt must exist between the RNase H cleavage and the nascent primer 3' terminus. However, a distance of 19nt instead of 18nt was observed between these sites after incorporation of the first dC at the 3' end of primer $tRNA^{Lys3}$ (Götte et al., 1995). Because the spatial relationship between the functional RNase H cleavage site and the polymerization active site serves as an indication of RT conformation, the 19nt distance suggests a novel conformation for RT after incorporation of the first nt. Further supporting this notion, mutagenesis studies of the HIV-1 RT palm subdomain have shown that RNA and DNA primers may be differentially recognized by RT, i.e. RT may be associated with RNA *versus* DNA primers in different conformations (Ghosh et al., 1997).

It was also demonstrated that the synthesis of (-) ssDNA paused at the +3 position, regardless whether $tRNA^{Lys3}$ or a DNA primer was employed.

This suggests that the secondary structure of the complex formed between the viral RNA template and the primer must play a role in the specification of this pause site. The binding of $tRNA^{Lys3}$ primer to the PBS results in a complex with altered secondary structure in the region of the PBS, in which the three nt (GAC) at the 5' end of the PBS were looped out and the eight nt at the 5' end of the PBS bind to other complementary sequences to form a stable stem structure (Isel et al., 1995; Isel et al., 1993). Therefore, when extension from the primer reaches the +3 stage (dG), the stem structure has to be disrupted before the fourth nucleotide (dC) can be added, resulting in pausing at the +3 nt position. Another proof comes from mutagenesis assays in which this stem structure was disrupted. As a consequence, pausing no longer occurred at the +3 position. A similar secondary structure is probably formed when the DNA primer is bound to the PBS, such that pausing at the +3 stage also occurs in this circumstance.

Even though the interaction between the anticodon loop of $tRNA^{Lys3}$ and the A-rich loop is located 12-17 nucleotides upstream of the PBS, probing of the viral RNA revealed that this interaction progressively unwinds during the addition of the third to the sixth nucleotide to $tRNA^{Lys3}$ (Lanchy et al., 2000). The primer $tRNA^{Lys3}$ can form a binary complex with HIV-1 RT in which the anticodon loop is in close contact with the polymerase. A possible role for the interaction between the anticodon loop of natural $tRNA^{Lys3}$ and A-rich loop of viral RNA may be to prevent interactions of RT with the anticodon of $tRNA^{Lys3}$. Such an interaction may impair efficient initiation and/or switching to elongation of reverse transcription by blocking RT on the tRNA. The progressive unwinding of the anticodon of $tRNA^{Lys3}$/A-rich loop interaction during the addition of the third to the sixth nucleotides might explain the finding that most annealed $tRNA^{Lys3}$ in extracellular viral particles is extended by two nucleotides (Huang et al., 1997b). The unwinding results suggest that the intermolecular interactions might be stabilized in the viral particles and

might block reverse transcription when low concentrations of nucleotides are available.

The initiation and elongation phases strongly differ by their polymerization rate and by the dissociation rate of RT from the primer-template complex (Lanchy et al., 1996a; Lanchy et al., 1998). Once the sixth DNA residue has been incorporated, the enzyme switches from an initially slow rate of DNA synthesis to a faster and more processive mode of polymerization. RT dissociated approximately ~200 times faster from the initiation than from the elongation complex. Nucleotide incorporation in reduced by a ~50-fold factor during initiation of reverse transcription, compared with elongation. As a consequence, processivity of HIV-1 RT in the initiation complex is close to unity, while it increases by four orders of magnitude during elongation. The processivity change is reminiscent of the transition for initiation to elongation of reverse transcription. Transition to an elongation complex after addition of six nucleotides is also facilitated by extended primer-template interactions. The mechanism by which the extended HIV-1 RNA/ $tRNA^{Lys3}$ interactions facilitate transition from initiation to elongation remains unclear. Since RT dissociates during this transition, these interactions may favor the re-binding of HIV-1 RT to the +3 to +5 products by preventing a steric clash between RT and $tRNA^{Lys3}$ (Jager et al., 1994; Kohlstaedt and Steitz, 1992). Probing and footprinting data indicate that RT does not directly recognize the extended primer-template interaction. Instead, these interactions favor binding of the homologous RT by preventing steric clashes and ensuring proper orientation of the template domains that directly interact with RT (Isel et al., 1999). Since the chemical reaction is the same in initiation and elongation of reverse transcription, inhibition of the latter but not of the former step by Mn^{2+} further suggests that distinct conformations of RT are involved in these processes (Isel et al., 1996). Thus not only the RNase H active site (Götte et al., 1995) but also the

polymerization site of HIV-1 RT is able to distinguish between RNA-RNA and DNA-RNA hybrids.

HIV-1 reverse transcription is also affected by a number of viral proteins including Nef (Aiken and Trono, 1995; Schwartz et al., 1995), Tat (Harrich et al., 1997; Huang et al., 1994a; Kameoka et al., 2001)and Vif (Courcoul et al., 1995; Sova and Volsky, 1993), Vpr (Stark and Hay, 1998), matrix protein (MA) (Bukrinskaya et al., 1998; Kiernan et al., 1998; Yu et al., 1992), nucleocapsid protein (Barat et al., 1989) and integrase (Tsurutani et al., 2000; Wu et al., 1999). Nef and Vif are believed to affect virion morphology, so that, in their absence, a defect in steps between entry and uncoating leads to inefficient proviral DNA synthesis. Vpr may play a role in reverse transcription by inhibiting $tRNA^{Lys3}$ aminoacylation by LysRS so as to increase the amount of $tRNA^{Lys3}$ available to act as a primer. NCp7 is a nucleic acid chaperone that has several functions. As discussed above, it plays a role in improved annealing of the $tRNA^{Lys3}$ to the RNA template and increased efficiency of both reverse transcription initiation and the first-strand transfer reaction. Both Tat and IN play distinct roles in reverse transcription which, while less well defined, are required for efficient reverse transcription initiation.

Finally, initiation and elongation of reverse transcription are distinct process as that may be selectively inhibited. One may use this observation as the conceptual basis for the development of agents that would specifically block initiation of reverse transcription. Such agents might not affect DNA synthesis by cellular polymerases, and, since one of the components of the initiation complex is not allowed to vary ($tRNA^{Lys3}$), the emergence of resistant strains may be limited.

1.5.3 Viral DNA entry into the nucleus

In contrast to oncoviruses that require mitosis to replicate, HIV is able to infect non-dividing cells, such as macrophages. Non-dividing cells of the

monocyte/macrophage lineage are thought to be among the first targets of HIV infection (Connor and Ho, 1994; Fenyo et al., 1989) and are likely to contribute significantly to HIV persistence (Innocenti et al., 1992; Meltzer et al., 1990) and the complication of AIDS (Ho et al., 1994). This important characteristic for the physiopathology of HIV infection relies on a specific process that ensures efficient import of the viral DNA through the envelope of the interphase nucleus, prior to integration. This process depends on the ability of the HIV-1 preintegration complex (PIC) to interact with the cellular nuclear import machinery. Components of the PIC include the double-stranded DNA version of the viral genome as well as the reverse transcriptase, matrix, integrase, and Vpr proteins (Bukrinsky et al., 1993; Gallay et al., 1997; Heinzinger et al., 1994; von Schwedler et al., 1994). Selected host proteins are incorporated into the PIC including barrier to auto-integration factor and HMG I (Y) (Farnet and Bushman, 1997). Prior to integration, the viral PIC must traverse from the plasma membrane to the nuclear envelope, a distance which may approach 20 μm. Preliminary evidence indicates that this transit involves the dynamic association of the HIV PIC with microtubules (Hope, 1997). Real time fluorescence microscopy has revealed that the sub-viral particle slides down these microtubules in an energy-dependent fashion delivering the PIC to the vicinity of the nuclear envelope.

Nuclear transport of macromolecules occurs through the nuclear pore complexes (NPC) and is controlled by the nuclear localization signals (NLS). These pores serve as the conduits for bi-directional transport of macromolecules that are critically required for maintenance of normal cellular physiology (Nigg, 1997). While the precise factors governing directionality of transport remain poorly understood, it is thought that the steep gradient of RanGTP/RanGDP (high ratio in the nucleus and low ratio in the cytoplasm) plays a key role (Nachury and Weis, 1999). The most common type of NLS is a short stretch of basic amino acids that introduces an overall net positive charge crucial for the nuclear targeting

properties of these sequences (Dingwall and Laskey, 1991). The nuclear import signals are recognized by a cellular protein, Imp α, which, in turn, binds to a second protein, Imp β. This trimeric complex then engages the nucleoporin components of NPCs and progressively moves across the pore in a series of sequential binding and release steps that require energy (Gorlich et al., 1996; Nigg, 1997).

While integrase is clearly essential for the introduction of retroviral cDNA into the host chromosome, this viral protein also appears to play a role in nuclear uptake of the PIC. Recent studies have revealed a conserved 13-amino acid nonbasic type NLS buried within the catalytic domain of integrase that plays a key role in the nuclear accumulation of the viral PIC (Bouyac-Bertoia et al., 2001). Mutation of this NLS revealed that the import function of integrase could be selectively disrupted while preserving overall catalytic activity. Most important, it was also demonstrated that the integrase import appears to be essential for productive infection of both nondividing and dividing cells. This observation may change the dogma that nuclear import is selectively required for nondividing cell infections, and that NLS-deficient viruses, like oncoretroviruses, can exploit nuclear envelope breakdown to bypass the NPC. This implies that all retroviruses may enter via active import via the NPC, but perhaps at different times of the cell cycle.

IN is not the only HIV-1 PIC protein to have been ascribed postentry nuclear import function. The first was MA, where a short stretch of basic amino acids was described as being NLS, and selectively important for infection of nondividing cells (Bukrinsky et al., 1993; Gallay et al., 1995a; Nadler et al., 1997). The other PIC protein to be identified as a facilitator of PIC import was Vpr. Vpr contributes to the HIV-1 nuclear import by a distinct mechanism: while MA and IN provide their NLSs for interaction with Imp α, Vpr regulates this process by increasing the affinity of the Imp α-NLS interaction (Popov et al., 1998). Despite lacking any identifiable classical import signal, recent studies have

indicated that Vpr contains at least two novel import signals and a CRM1-dependent NES (Sherman et al., 2001). The presence of these various import and export signals probably ensures representation of Vpr in two different cellular compartments, where it performs critical functions in the viral life cycle. Finally, in recent studies, it has been shown that Vpr alters the structure of the nuclear lamina in a manner that leads to the formation of nuclear hernia that intermittently rupture (de Noronha et al., 2001). These ruptures in the nuclear envelope may provide a freely accessible portal for uptake of the large HIV PIC uptake in selected situations.

Additional factors contribute to nuclear import of the viral PIC, including a central DNA flap. This element corresponds to a triple-stranded intermediate created during reverse transcription. Early studies suggested that mutations in the central PPT interfered with HIV replication at a step after reverse transcription (Charneau et al., 1994). Subsequent studies have revealed that the central DNA flap acts as an important signal for the PIC (Zennou et al., 2000). The mechanism by which a nucleic acid structure alters transport through the NPC remains unclear; however, there is precedence for such control in the case of RNA export (Hamm and Mattaj, 1990).

In summary, the ability of HIV to establish productive infections in non-dividing cells has led to exploitation of HIV as a gene transfer vehicle permitting stable expression of various target genes in non-replicating cells of various tissues. The currently available data suggest that the HIV integrase play a leading role in nuclear import of the PIC, with the DNA flap, MA, and possibly Vpr proteins serving as supporting cast for this process. It will be important to determine how the integrase protein mediated nuclear uptake, and to look for the potential to disrupt this pathway in a virus-specific manner.

1.5.4 Integration

Integration is an obligatory replication step for all retroviruses. Linear DNA contains at its termini long terminal repeats (LTR) sequences. The ends of these LTRs are specifically recognized by the viral integrase (IN). Once the PIC associates with the host chromosome, viral IN catalyzes the insertion of the viral sequences into the host DNA. Integrated viral DNA is termed the provirus.

Integration occurs in two well-characterized catalytic steps, referred to as end processing and joining, respectively. Analysis of viral DNA isolated from infected cells shows that the preparation for DNA integration begins in the cytoplasm. As soon as viral DNA synthesis is completed, the linear duplex product undergoes end processing which involves removal of a dinucleotide, adjacent to a highly conserved CA dinucleotide, from the 3' strand of the U3 and U5 viral DNA LTRs in a reaction involving a water molecule or other nucleophile (Engelman et al., 1991; Vink et al., 1994). This exposes a 3' hydroxyl group, whose oxygen is used as an attacking nucleophile on the target DNA during the joining reaction, in which the viral DNA is inserted into the cellular (Engelman et al., 1991; Vink et al., 1994). Once nuclear entry is gained, the processed viral DNA ends are inserted into the host cell's genome via a second catalytic event termed joining. The joining reaction must be coordinated *in vivo*. It comprises a concerted cleavage and ligation reaction in which staggered phosphates in the duplex target DNA backbone are attacked by the two 3' OH ends of the processed viral DNA. These viral DNA ends are inserted into opposite strands of the target DNA. The resulting single-stranded gaps in the target that flank the 5' ends of the viral DNA must be filled in, and the two-nucleotide 5' over-hang of viral DNA removed, to restore the continuity of the host DNA. The result is a short, direct duplication of the target sequence on either side of the integrated viral DNA whose length is characteristic of the virus; for HIV-1, it is 5bp. *In vivo*, the gap repair

machinery of the host cell is presumed to be responsible for these last steps.

The IN recognition sequence within the LTRs is relatively short. In the case of HIV-1, IN recognizes 20bp (Sherman and Fyfe, 1990). Cross-linking and substitution of bases in the LTR of HIV-1 have demonstrated that specific interactions between IN and the terminal LTR sequences are required for the end-processing and joining reactions (Esposito and Craigie, 1998; Heuer and Brown, 1997; Heuer and Brown, 1998). Hotspots for joining have been observed *in vitro* (Fitzgerald et al., 1992; Kitamura et al., 1992; Shih et al., 1988). The choice of target site appears to be influenced by structural features in the DNA determined by chromatin organization and DNA-binding proteins, as well as by components of the nucleoprotein complex and the viral integrase itself (Craigie et al., 1990; Leavitt et al., 1992; Pryciak et al., 1992; Pryciak and Varmus, 1992). In general, a bend of slightly unwound DNA appears to be a perfect target for joining (Muller and Varmus, 1994).

Certain nuclear proteins, such as the HMG class of DNA binding proteins and Ini-1 (Integrase interactor 1), can enhance concerted integration of viral DNA ends *in vitro*, suggesting that such proteins might play a role as accessory factors in viral DNA integration *in vivo* (Aiyar et al., 1996; Farnet and Bushman, 1997). The cellular protein HMG I(Y) was subsequently found to be associated with PIC isolated from HIV-1 infected cells. The involvement of HMG I(Y) in integration appears to depend on interaction with DNA and not the integrase (Farnet and Bushman, 1997). The idea that cellular proteins contribute to HIV-1 DNA integration was first suggested by the isolation of a specific protein that binds to integrase, called Ini-1, that is a human homologue of the yeast SNF5 transcription factor (Kalpana et al., 1994). Ini-1 promotes the joining of viral DNA ends to a target DNA *in vitro*. However, the relevance of this interaction to the integration of viral DNA *in vivo* has not yet been established.

1.5.5 Transcription and translation

In the retroviral life cycle, proviral DNA is the template for transcription. Transcription is mediated by the host-cell RNA polymerase II, which synthesizes cellular mRNAs and some small nuclear RNA (snRNAs). HIV-1 contains a large array of *cis*-acting elements in its proviral DNA that are characteristic of cellular sequences, and most of these *cis*-acting elements, including the promoter and multiple enhancer sequences, lie in the U3 region of the 5' end LTR of the proviral DNA. With their help, transcription initiation from the LTR promoter is well controlled in different cell types or at different times, which likely determines whether a provirus is quiescent or actively replicating. For example, it has been suggested that NF-κB proteins are important for HIV transcription. The two NF-κB-binding sites in the HIV LTR are conserved in all HIV-1 isolates. The role of NF-κB binding to these sites in controlling transcription from LTR in both monocytes and T cells in conjunction with NF-AT has been well defined (Alcami et al., 1995; Grilli et al., 1993; Moses et al., 1994; Nabel and Baltimore, 1987). Activation of the expression of latent HIV proviruses in both T-cell and monocyte cell lines by cytokines such as TNF-α and IL-1 is correlated with induction of nuclear NF-κB (Duh et al., 1989; Osborn et al., 1989). It is worth noting that HIV proviruses containing deletions or mutations in the NF-κB-binding site still replicate both in primary T cells and in T-cell lines in culture (Leonard et al., 1989). The multiple remaining transcription-factor-binding sites (such as the Sp1-binding site) apparently compensate for the loss of NF-κB-binding site in supporting HIV replication (Jones et al., 1986).

As a complex retrovirus, HIV-1 also encodes a *trans*-activating viral protein, Tat. Tat is an atypical transcriptional activator. Optimal Tat activity requires a composite responsive element consisting of DNA and RNA. Thus functionally within cells, RNA-bound Tat protein interacts

with transcription complexes formed at the TATAA element (Berkhout and Jeang, 1992) and with upstream DNA-bound Sp1 (Berkhout et al., 1990; Chun et al., 1998; Jeang et al., 1993). HIV-1 Tat protein acts by binding to the TAR RNA element, a 59-base stem-loop structure located at the 5' ends of all nascent HIV-1 transcripts (Berkhout et al., 1989). In the presence of Tat, activated transcription increases up to several thousand folds over its basal potency. How a Tat bound to TAR RNA mechanistically effects this amplification has been an area of active investigation.

Several possible steps at which Tat can function include: (1) anti-termination of RNAP II-directed transcription, (2) increased processivity of transcribing the RNAP II complex, (3) augmented formation of transcriptionally competent complexes at the promoter, and (4) egress or clearance of initiated complexes from the promoter. It is also possible that, like other activators, Tat could influence several discrete steps of transcription. An important breakthrough was the identification of the TAR-Tat-Cyclin (Cyc) T1-Cyclin-dependent kinase 9 (CDK9) complex, in which CDK9 can hyperphosphorylate the carboxyl-terminus domain (CTD) of the RNAP II complex (Garber and Jones, 1999). Upon CTD phosphorylation, RNAP II changes its conformation and, as a consequence, dissociates from negative elongation factors (N-ELF). Tat may also enhance transcription through interactions with other components within the RNAP II complex. For instance, Tat may recruit other kinases to phosphorylate CTD (Zhu et al., 1997); Tat may regulate the dephosphorylation of CTD by inhibiting the CTD phosphatase activity of FCP1 (Marshall et al., 1998). It is also possible that Tat may stimulate re-initiation from HIV-1 LTR. Another important role of Tat in transcription relates to the regulation of gene expression from proviral DNA which becomes integrated into host chromosomal DNA and is protected by histone proteins in nucleosome structures. Tat is able to increase the accessibility of the RNAP II complex to HIV-1 LTR by

acetylation of histone proteins through Tat-associated histone acetyltransferase (TAH) (Benkirane et al., 1998; Hottiger and Nabel, 1998; Marzio et al., 1998; Weissman et al., 1998).

Following transcription of the provirus, retroviral RNA transcripts are subject to the same processing events as cellular RNAs, including cap addition at the 5' end, cleavage and polyadenylation at the 3' end, and splicing to form subgenomic-sized RNA molecules. The cap addition and 3' end processing precede splicing events; thus, all viral RNAs are capped and polyadenylated. Full-length retroviral RNA transcripts serve two functions: They encode the *gag* and *pol* gene product, and they are packaged into progeny virion particles as genomic RNA. Subgenomic-sized RNA molecules provide mRNAs for the remainder of the viral gene product.

1.5.6 Assembly and budding

The production of an infectious HIV-1 is not simply a matter of putting together a multitude of proteins and nucleic acid. Rather, it is a complex progression of many molecular interactions and rearrangements involving an unknown number of intermediate structures.

1.5.6.1 Packaging and formation of the viral particle

Fortunately for studies of assembly, not all of the ever-changing virion components are required for the formation of a particle. As it turns out, the Gag protein alone is sufficient for forming virus-like particles (Swanstrom, 1997). Thus, the Gag protein has been described as a "particle-making machine".

Gag proteins contain three small, modular domains that work together to produce a virus particle. The 32 amino acid membrane-binding (M) domain of HIV-1 can direct heterologous proteins to the plasma membrane and hence contains all of the target information. The most powerful region of interaction among Gag proteins is the interaction (I) domain, which is

contained within the NC sequence and interacts non-specifically with RNA. I domain enables the tight packing of Gag proteins that results in particles of the proper density. The final step in budding results in the separation of the viral and cellular membranes. This late function is mediated in some way by the late (L) domain, which resides in the p6 sequence.

The M domain of Gag protein is located at the amino termini and is contained entirely within the MA sequence. During budding, the MA sequence of Gag is closely associated with the membrane and thus is in close proximity to the cytoplasmic domain of the viral glycoprotein. Thus, the MA sequence within Gag protein may play a part in Env packaging, even though the Env glycoproteins themselves are not needed for budding (Dorfman et al., 1994). Interaction between HIV-1 Gag and Env stabilizes the Env protein on the surface of the cell where it is otherwise rapidly internalized (Lee et al., 1997). It has been long known that the viral glycoproteins are sorted to the appropriate cell surface when expressed in polarized cells in the absence of other viral components, and this suggests that they may have a critical role in determining the site of assembly. The gp41 protein is responsible for redirecting Gag to the basolateral membrane.

Although the CA sequence is not required for budding, it is essential for infectivity. After capsid has been liberated by proteolytic processing, it rearranges into the conical core structure that surrounds the viral genome at the center of the mature virus. Extensive structural analyses of purified CA have been performed, including NMR spectroscopy of the N-terminus, and X-ray crystallography of dimeric CA proteins, CypA bound to the N-terminal fragment, and the C-terminal fragment alone (Gamble et al., 1997; von Schwedler et al., 1998). Several observations suggest that the N-terminal capsid β-hairpin form after proteolytic cleavage at the MA-CA junction. The functional capsid refolding results in the creation of a new CA-CA interface (N-terminal interface) in the mature capsid core. The N-

terminal domain interface further cooperates with the C-terminal dimer interface to mediate higher order capsid protein assembly. Repetition of these two capsid dimer interfaces would create a strip of capsid molecules that could then create the core of HIV-1 (Gross et al., 1998). Cyclophilin A is a cellular protein that is incorporated into HIV-1 particles at a ratio of 1:10 (relative to CA) and is important for virus replication. It binds to a flexible loop in CA and is incorporated into the particle via its interaction with the CA domain of Gag. Based on analysis of individual steps in viral replication, it has been suggested that CypA play a role in viral entry. CypA may also destabilize the spherical protein shell during viral maturation and facilitate the formation of the cone shaped core. Recently, it has been identified that another cellular protein, HP68, is associate with Gag after translation and promotes its assembly into immature capsids. HP68 appears to act as a molecular chaperone during capsid assembly, promoting a conformational change that may be important for immature capsid integrity or maturation (Zimmerman et al., 2002).

HIV-1 full length (unspliced) RNA is less than 1% of the total mRNA in an infected cell (Berkowitz et al., 1996; Swanstrom, 1997), yet it is almost exclusively packaged into virions even in a gross excess of cellular mRNAs. Recognition of the HIV-1 genome occurs by means of interactions between the NC sequence within the I domain and a 120-nucleotide region of the unspliced viral RNA known as the Ψ-site, which is located between the 5' long terminal repeat and the gag initiation codon. Extensive site-directed mutagenesis, chemical modification, nuclease accessibility mapping, and free energy computational studies indicate that the HIV-1 Ψ-site contains four stem-loop structures, denoted SL1 through SL4. Although mutagenesis experiments indicate that all four of these structures are important for efficient encapsidation, SL3 is of particular interest because its sequence is highly conserved among different strains of HIV-1 despite heterogeneity at adjacent positions, and

because linkage of SL3 to heterologous RNAs is sufficient to direct their recognition and packaging into virus-like particles(De Guzman et al., 1998). The two zinc knuckle domains within NC participate directly in genome recognition and encapsidation.

Host and viral proteins, other than NC, have also been implicated in specific RNA packaging. The HIV-1 p2 domain may also contribute to specific recognition of the HIV-1 packaging signal (Kaye and Lever, 1998), Furthermore, human staufen (hStau), a double-stranded RNA binding protein important in mRNA transport, was found to be associated with viral genomic RNA, and is incorporated into HIV-1 virions. Over-expression of hStau enhances its virion incorporation level, as well as encapsidation of HIV-1 genomic RNA, indicating that it may participate in retroviral genome selection and packaging as well (Mouland et al., 2000).

In HIV-1 particles, full length genomic RNAs are found in homodimeric form. *In vitro* studies have demonstrated a "kissing-loop" dimerization model (Clever et al., 1996; Laughrea and Jette, 1994; Mujeeb et al., 1998; Skripkin et al., 1994). The dimerization site (DIS) is mapped to the six-base palindromic sequence in the loop region of SL1. The dimerization is initiated by base pairing of the DIS loop between two RNA molecules, and then the two DIS stems are melted and reannealed to form a stable intermolecular duplex. The overlapping of the packaging and dimerizaition signals suggests that these two processes are likely to be intricately associated. However, more convincing evidence is still needed to clarify the nature of dimer formation in virions, as well as the relationship between encapsidation and dimerization.

1.5.6.2 Proteolytic processing

Proteolytic processing at specific sites in the Gag and Gag-Pol (and sometimes Env) precursors by the viral PR is an essential step in the viral life cycle.

HIV-1 PR is a dimer of two identical subunits. There is a long cleft between the subunits where the substrate polypeptide binds; on the floor of the cleft are the catalytically important aspartic acids. A comparison of the structures of the HIV-1 PR with and without a bound inhibitor shows that PR undergoes significant structural changes with binding (Miller et al., 1989).

Premature activation and processing by PR would preclude proper assembly and would be fatal to the virus. Under normal circumstances, however, retroviruses are able to delay the appearance of PR activity. How this activity is controlled is not yet clear. It has been shown that the expression of Gag-Pol alone results in complete intracellular cleavage of the precursor protein and is cytotoxic. This observation suggests that the ratio of Gag-Pol to Gag is critical for proper control of PR activity. Since Gag-Pol is normally expressed as a minor product relative to Gag, it was hypothesized that an excess of Gag may limit intracellular dimerization, and thus activation, of PR. More recently, Gatlin et al suggested that inhibition of PR activity was dependent upon the context of PR expression. Sequences capable of mediating this inhibition were localized to capsid. A mechanism through which Gag regulates PR activity was proposed whereby the disproportionate synthesis of Gag inhibited the activation of PR in the cytoplasm (Gatlin et al., 1998).

1.5.6.3 Maturation and budding

The least understood step in budding for all envelope viruses is the final step during which the fully assembled particle separates from the host cell. Immediately prior to this event, the particle is attached to the cell by means of a short stalk. The contents of the stalk are unknown but must account for the length and diameter of this narrow structure. Although virus-cell separation is usually described as a "pinching-off" step, it requires the mixing of lipids in the opposing membranes of the stalk in a manner directly analogous to the fusion event that occurs when the virus

enters the cell. The point of separation appears to take place at the base of the stalk (in other words, closer to the cell than to the virus). The release of particles requires the L domain sequence contained within Gag. When L is nonfunctional, virus particles accumulate at the cell surface but fail to pinch-off.

The first L domain to be identified was that of HIV. It is located near the beginning of the p6 sequence, and its critical amino acids include the proline-rich sequence P-T-A-P (Gottlinger et al., 1991; Huang et al., 1995). Although there are multiple lines of evidence proving that p6 contains a late budding function, this activity appears to be influenced by PR and/or to be cell-type specific. More recently it has been found that mutants lacking p6 are released in an anomalous manner that creates particles of extremely large size (Garnier et al., 1998).

The mechanism by which L domains mediate particle release is unknown, but it is very likely to involve direct interactions with cellular proteins. *In vitro* and *in vivo* studies of HIV indicate that the unprocessed Gag protein interacts with polymerized actin (F-actin), but the domain of Gag involved in binding remains unknown. Another study has shown that actin, cofilin, ezrin, and moesin (all involved in regulation of actin assembly) are incorporated into HIV virions, suggesting that the actin cytoskeleton could be involved in virion release (Ott et al., 1996).

In addition to the L domain, other viral proteins are known to influence the efficiency of HIV release from the cell surface. Vpu enhances particle production by Gag proteins from retroviruses, both closely and distantly related to HIV, and, in its absence, budding structures accumulate at the cell surface (Schwartz et al., 1996). The only other protein identified as an enhancer of particle release from different retrovirus is HIV-2 envelope protein. One likely explanation is that the amphipathic α-helices, contained in the glycoprotein cytoplasmic domain of HIV-2 and also in Vpu, interact with lipid bilayers, and form membrane channels of pores which destabilize membranes and favor virus budding. Recent studies by

independent groups implicate the cellular protein ubiquitin in a late step in budding (Patnaik et al., 2000; Schubert et al., 2000; Strack et al., 2000). Ubiquitin is a small highly conserved 76 aa protein well known for its role in protein degradation by the proteasome. A ubiquitin ligase is pulled to the site of budding by interaction with the late domain, and there it conjugates ubiquitin to Gag and/or to other cellular protein. Recent studies established a putative causal link between budding and ubiquitin, by showing that depletion of the intracellular pool of free ubiquitin inhibits budding. However, the role of ubiquitin in retrovirus budding is still unknown.

1.6 HIV-1 inhibitors and the M184V mutation in HIV-1 RT

Currently, several anti-retroviral drugs are approved for clinical use, and they are either RT or PR inhibitors. The introduction of combinational anti-retroviral therapy (highly active anti-retroviral therapy, HAART) in 1995-1996 changed the prognosis of HIV-infection. HIV-related morbidity and mortality rates in patients with advanced HIV infection have significantly declined. However, current therapy is not able to eradicate the virus, only suppress it; therefore, long-term use of the drugs is required to keep viral load under control. As a consequence, resistance develops in a significant portion of patients. Furthermore, various side effects have been observed associated with the therapy. These side effects include hypersensitivity, mitochondrial toxicity, lypodistrophy syndrome, insulin resistance and cardiovascular disorders. Further drug development is necessary to design new compounds that have efficacy similar or greater to the currently used drugs in the management of HIV infection and that are potent against the resistant viruses but do not exhibit unwanted metabolic side effects.

1.6.1 HIV inhibitors and drug resistance

The first drug used in HIV therapy was a nucleoside analog RT inhibitor (NRTI), 3'-azido-2', 3'-dideoxythymidine (zidovudine, AZT). Later, other NRTI drugs were also used in therapy, including zalcitabine (ddC), didanosine (ddI), lamivudine (3TC), stavudine (d4T), abacavir (1592U89), and adefovir dipivoxil [bis(POM)-PMEA]. These analogs are taken up by cells, become converted to the respective triphosphate forms, and presumably inhibit DNA synthesis as chain terminators. A group of non-nucleoside inhibitors (NNRTIs) have also been developed, including nevirapine, efavirenz (DMP266), and delavirdine (BHAP U90152S). Unlike NRTIs, these drugs interact with RT at an allosteric, non-substrate binding site. In spite of their structural diversity, NNRTIs bind to the same allosteric site of RT, close to the active site of the enzyme.
HIV PR inhibitors currently used in therapy are saquinavir, indinavir, ritonavir, amprenavir, and nelfinavir. A common feature of these compounds is that they are peptidomimetics, they mimic the substrates of the PR. The enzyme-inhibitor interactions involved, similar to the enzyme-substrate interactions, are primarily hydrophobic. Typically, these inhibitors contain a phenyl residue at the P1 position. Another common feature of these inhibitors is that they contain a nonhydrolyzable transition-state mimic, like a hydroxyl-ethylamine group, at the site corresponding to the cleavable bond in the substrates. Saquinavir was the first approved HIV-1 PR drug. It is base on a type 1 cleavage site, in which the Pro was replaced by a saturated isoquinoline ring. Indinavir and nelfinavir also mimic the type 1 cleavage site; ritonavir was developed from a symmetric molecule, while amprenavir is a sulfonamide compound. Most of the problems associated with HIV therapy are the consequence of the necessarily long-term use of the drugs. The first report on HIV drug resistance to AZT (Larder et al., 1989) was followed with a large number of reports documenting genotypic correlates of reduced drug susceptibility *in vitro* and virological failure *in vivo*. Mutation patterns that are

associated with resistance to AZT and 3TC have been extensively analyzed. Both of these inhibitors represent important components of drug regimens used in triple therapy. The acquisition of high level AZT resistance requires several changes in the RT (Larder, 1994), including several at amino acid positions 41, 67, 70, 215, and 219. Low-level resistance that is associated with the initial appearance of K70R is followed by the T215 Y or F, K219Q, D67N and M41L mutations that concomitantly confer higher levels of resistance (Boucher et al., 1992; Gao et al., 1992; Larder et al., 1991; Larder et al., 1989; Larder and Kemp, 1989; Rooke et al., 1989). The structure of HIV-1 RT, bound to a DNA primer/template substrate and an incoming nucleotide, suggests that amino acid substitutions associated with AZT-resistance can affect the interaction with the nucleotide (Huang et al., 1998a). The emergence of resistance to ddI and ddC occurs more slowly than for AZT, and the mutations (at positions 65, 69, 74 and 184) lead to only modest loss of potency (Pillay et al., 2000). A single amino acid substitution, i.e. M184V or M184I, is sufficient to confer high-level resistance to 3TC (Boucher et al., 1993; Gao et al., 1993; Schinazi et al., 1993; Tisdale et al., 1993). Resistance against abacavir is associated with mutations at RT positions 65, 74, 115 and 184 (Pillay et al., 2000). There is a variable cross-resistance between the approved drugs. In addition, combinational therapies may select for novel mutations not observed in monotherapy studies.

Resistance against NNRTIs develops rapidly, resulting from mutations at the amino acid residues surrounding the NNRTI-binding site. Failure of NNRTIs is often caused by a single mutation, therefore it is likely that these variants pre-exist as natural sequence variations. Emergence of NNRTI-resistant HIV-1 strains can be prevented if NNRTIs are combined with NRTIs and used from the beginning at sufficiently high concentrations (De Clercq, 1998).

Resistance also develops against PR inhibitors. In most cases mutations occur in the PR gene. While most of the natural variations are outside the substrate binding sites, several of the mutations conferring resistance involve residues of the substrate binding subsites and therefore alter the specificity and catalytic power of the enzyme. In patients receiving PR inhibitors, not only is the PR mutated, but mutations were also observed at the nucleocapsid/p1 and p1/p6 Gag cleavage site. These mutations were first described in *in vitro* studies, but later they were also found in patients undergoing indinavir therapy (Croteau et al., 1997; Zhang et al., 1997). These mutations occurred together with mutations in the PR gene at positions 46, 54, 71, 82, 89 and 90 (Zhang et al., 1997).

1.6.2 New targets for inhibition of HIV-1 replication

Although over a dozen drugs are now on the market, they all target only two viral enzymes. But up to 20% of patients cannot tolerate antiviral cocktails in the short term (Lucas et al., 1999). There is increasing concern about long-term metabolic side-effects of protease inhibitors (notably, poorly understood problems with fat metabolism). And drug-resistant HIV-1 variants are emerging and spreading at an increasing rate (Yerly et al., 1999). Because antiviral therapy cannot eradicate HIV-1 from infected people (Furtado et al., 1999), we need to identify a new class of drugs suitable for long-term use that can supplement, or partially replace, existing drug regimes.

Several stages of the viral life cycle are potentially vulnerable to specific inhibitors. These can be divided into the entry steps, which involve viral-envelope glycoproteins and their receptors, and the post entry steps involving viral accessory-gene products and the cellular proteins with which they interact. Although both viral and cellular factors can be targeted, it is usually less toxic to attack a viral factor than to disturb the function of a host protein.

Antagonists of viral entry, directed at the gp120 (CD4-IgG2) or gp41 (T20, T1249) glycoproteins or the CCR5 and CXCR4 co-receptors, are already in (or rapidly approaching) human clinical trials. A soluble CD4 derivative known as CD4-IgG2 is a tetrameric immunoglobulin-G fusion protein. Designated PRO 542, CD4-IgG2 has shown some ability to reduce plasma levels of virus in phase I clinical trials (Jacobson et al., 2000). T20 and T1249 function as peptides that substitute for one or more components of the gp41 trimer, thereby inhibiting the conformational changes and preventing the intermolecular interactions necessary for fusion (Jones et al., 1998). In clinical trials, they reduce viral load significantly (Kilby et al., 1998). Injectable CCR5 inhibitors, such as the monoclonal antibody PRO 140, may also soon enter the clinic, and an injectable CXCR4 blocker, AMD3100 (Arakaki et al., 1999; Donzella et al., 1998), has been tested as has the orally available CCR5 inhibitor, Sch-C. The various entry inhibitors may be especially useful in combination - they could act synergistically. But the development of post-entry inhibitors is less certain. The identification of clinically useful integrase inhibitors has been slow. Most potent and specific inhibitors contain a diketo-acid moiety, which confers undesirable pharmacological properties on the inhibitors, this moiety is not necessary for inhibition. The diketo-acid moiety of the integrase inhibitors will have to be eliminated from the next generation of compounds. RNase H also warrants closer attention, although convenient *in vitro* RNase H assays need to be developed first. Inhibition of accessory proteins is hindered by our poor understanding of their functions. The Vif protein is probably the most attractive target, given its obligatory function in viral replication. Identification of cellular ligands for Vif as well as the other accessory proteins will aid the design of inhibitor screening assays. Meanwhile, Tat and Rev have not received much attention as drug targets, probably because it is hard to recreate their activities *in vitro*. Nevertheless, cellular ligands for Tat and Rev have been identified, and this should help in designing screening assays.

1.6.3 Gene therapy of HIV-1 infection

Efforts in the field of gene therapy for HIV-1 infection have focused on the development of anti-HIV genes, delivery vehicles for these anti-HIV genes, and *in vitro* and *in vivo* inhibition assays. Almost every point of the HIV-1 life cycle has been targeted by the use of different kinds of anti-HIV-1 genes. These genes can be classified into two kinds: RNA-based and protein based. Among the first class are ribozymes and antisense RNAs, two versatile kinds of anti-HIV genes that can be engineered to aim a particular RNA sequence to trigger the specific degradation of the targeted RNA molecule. Other kinds of RNA-based anti-HIV genes are TAR and RRE RNA decoys, which are short stretches of RNA that bind and sequester the HIV-1 regulatory proteins, Tat and Rev, respectively. Among the protein based anti-HIV genes we find the transdominant negative proteins, single-chain antibodies, toxic proteins for suicide approaches, and CD4 to block either virus entry or virus maturation. These anti-HIV-1 genes have been delivered to target T cells or hematopoietic stem cells mainly in the context of Moloney murine leukemia virus (MoMLV), adeno-associated virus (AAV), and simian virus 40 (SV40) based vectors. These vectors can efficiently transduce hematopoietic cells (Bunnell et al., 1997) and they have proved to be safe in numerous clinical trials. Perhaps the most important advantage of these vectors is that they are not sensitive to the inhibitory effects of anti-HIV genes and therefore can be produced in stable packaging cell lines at high titers. However, they can integrate only into dividing cells (Miller et al., 1990), and cannot access nondividing T cells or macrophages, which considered to be important reservoirs of the virus and contribute to the persistence of HIV infection (Finzi et al., 1999; Finzi et al., 1997; Finzi and Siliciano, 1998). The use of lentiviral vectors based on HIV-1, HIV-2, SIV and FIV to block HIV replication and to deliver anti-HIV-1 genes is a rapidly evolving field. Lentiviral vectors based on HIV-1 or HIV-2 can

efficiently transduce both dividing and nondividing cells that are the natural targets of HIV-1 infection, such as dendritic cells, $CD34^+$ hematopoietic, T cells, and macrophages. Additionally, because these vectors contain the *cis*-acting sequences necessary to perform a complete viral life cycle, they can be mobilized to secondary cells by virions encoded by wild-type HIV-1. This trafficking of vector sequences *in vivo* would spread protection to previously untransduced cells with the same tropism as the wild-type virus. Also HIV-1 replication can be inhibited by the presence of sequences derived from HIV-1 or HIV-2 vectors. Ribozymes are small catalytic RNAs that can specifically bind and cleave other RNA species. Ribozymes directed against nucleotide sequence of the U5 region of the viral mRNA, conserved among several strains of HIV-1, may be an effective way to block HIV-1 gene expression. They catalyze the degradation of all the pre-mRNAs and mRNA classes, therefore preventing the expression of the early regulatory proteins Tat and Rev (Dropulic et al., 1996; Gervaix et al., 1997; Leavitt et al., 1994). However, as single nucleotide variations abolish the activity of ribozymes, the appearance of ribozyme-resistant variants of HIV-1 will likely limit the effectiveness of this approach. In other studies, tRNA-based ribozymes were developed (Gervaix et al., 1997; Thompson et al., 1995; Yu et al., 1993). tRNAs have the advantage of being relatively small and possessing long half-lives. Furthermore, they are expressed under the control of RNA polymerase III in excess over mRNAs. tRNA-driven transcriptional units were designed to express various RNAs as part of the tRNA anticodon loop (Cotten and Birnstiel, 1989; Ohkawa et al., 1993; Perriman et al., 1995; Yuyama et al., 1994) or as part of the 3' region of the tRNA(Gervaix et al., 1997; Leavitt et al., 1994; Thompson et al., 1995; Yu et al., 1993). A hammerhead ribozyme targeted against the HIV-1 *env* coding region was expressed as part of the anticodon loop of human $tRNA^{Lys3}$ without sacrificing tRNA stability or ribozyme catalytic activity. Retroviral vectors expressing this tRNA-ribozymes were tested in a human

$CD4^+$ T cell line and were shown to inhibit HIV-1 replication(Medina and Joshi, 1999).

Another way to target sequences that are present exclusively in the wild-type virus RNA is by the expression of antisense RNAs. Replication of HIV-1 has been successfully inhibited by plasmids or MoMLV vectors expressing antisense RNAs targeting different sequences of the HIV-1 genome such a splice acceptor sites, *tat*, *rev*, *gag*, pol, *env*, TAR, RRE, PBS, and portions of the HIV-1 LTR (Biasolo et al., 1996; Chadwick and Lever, 2000; Chuah et al., 1994; Cohli et al., 1994; Ding et al., 1998; Kim et al., 1996; Liu et al., 1997; Vandendriessche et al., 1995; Veres et al., 1996; Veres et al., 1998). Antisense RNAs can tolerate a high degree of nucleotide sequence divergence with the target RNA and that makes the appearance of antisense-resistant variants less likely.

More recently, RNA interference (RNAi) technology was used to suppress multiple steps of the HIV-1 life cycle. RNAi is a ubiquitous mechanism of gene regulation in plants and animals in which target mRNAs are degraded in a sequence-specific manner (Zamore et al., 2000). RNAi is initiated by the dsRNA-specific endonuclease DICER, which processes long double stranded (ds) RNA into small, 21-23-mer short interfering RNA (siRNA) (Zamore et al., 2000; Bernstein et al., 2001; Elbashir et al., 2001b; Hammond et al., 2000). An endonuclease complex uses siRNAs as a guide to cleave the target mRNA of homologous sequences, resulting in a decrease in the steady-state levels of the target mRNA. The requirement for DICER in maturation of siRNAs can be bypassed by introducing synthetic 21-23 nucleotide siRNA duplexes, which inhibit expression of transfected and endogenous genes in a variety of mammalian cells (Elbashir et al., 2001a). HIV-1 using RNA intermediates in its replication make it possible to use RNAi for inhibition of HIV-1 infection. siRNAs targeting various regions of the HIV-1 genome, including the viral LTR, the accessory genes *vif* and *nef*, and viral structural Gag protein, effectively inhibit pre- and post-integration infection events in the HIV-1

life cycle by specifically degrading genomic RNA (Jacque et al., 2002; Novina et al., 2002). Using siRNA to target the CD4 molecule, the study showed that an eight-fold decrease in CD4 expression led to a four-fold reduction in viral entry (Novina et al., 2002). SiRNA-directed HIV-1 inhibition provides a rationale for gene-therapy approaches for HIV that complement existing post-transcriptional approaches for inhibiting HIV, including ribozymes and antisense RNA.

Another possible treatment of HIV-1 infection is the use of oligodeoxynucleotides (ODN) to inhibit virus replication (Field, 1999). These include inhibition of virus absorption to the host cell, inhibition of transcription via antisense, or as a result of triple helix formation, and inhibition of viral encoded enzymes such as RT and IN. The particular mechanism of HIV inhibition depends on the ODN sequence and the ODN chemical modification. Various elements and steps in the HIV-1 life cycle have been targeted, including the HIV-1 PPT by triplex-forming ODN (Faria et al., 2000), integration by G-quartet ODN (Jing et al., 2002), and TAR by polyamide nucleic acid (PNA) (Mayhood et al., 2000). The specificity of reverse transcription initiation complex makes it an ideal target of antisense ODN. ODNs targeted to the U5 leader region, PBS and A-rich loop have been shown to block HIV-1 replication (Bordier et al., 1995; Freund et al., 2001; Kaushik et al., 2001; Lee et al., 1998) . Despite the favorable and encouraging antisense activity of ODNs, their biodelivery into the cell remains a major obstacle. Recently, several studies have reported methods for effective biodelivery of ODNs and their antisense effects in cells. Phosphorothioate ODNs were encapsulated into poly (D, L-lactic acid) nanoparticles. ODN-loaded nanopartices have been shown to be efficiently taken up by the cells and exhibit antisense activity on the target gene (Berton et al., 2001).

1.6.4 M184V

The M184V mutation, occurring within the highly conserved (Y183, M184, D185, D186) amino acid region that includes the active (catalytic) site of the p66 polymerase domain of reverse transcriptase (RT) (Balzarini, 1999), is unique in its effects on HIV-1 susceptibility to a variety of agents. In 1992, Gu and colleagues first observed that a single amino acid substitution, M184V consistently lead to high-level resistance among patients treated with 3TC (Gu et al., 1992). Several other teams of researchers also reported this finding (Boucher et al., 1993; Gao et al., 1993; Schinazi et al., 1993; Tisdale et al., 1993). Genotypic evidence of substitution of methionine with isoleucine (rapidly superceded by valine) at the codon 184, yielding the M184V mutation was found in all 3TC resistant viruses (Boucher et al., 1993; Wainberg et al., 1995).

The rapid emergence of M184V mutation in RT led to high-level (i.e. ≈ 1000-fold) resistance against 3TC (Boucher et al., 1993; Gao et al., 1993; Schinazi et al., 1993; Tisdale et al., 1993), in contrast with the more gradual development of resistance to AZT, abacavir and protease inhibitors. In addition to 3TC, M184V is the first resistance mutation to arise with abacavir both *in vitro* and *in vivo* (Harrigan et al., 2000; Tisdale et al., 1997). M184V has also been selected by ddI *in vitro* and reported in small numbers of patients on long-term ddI monotherapy (Gu et al., 1992; Winters et al., 1997). *In vitro* , M184V leads to an approximately 3-10 fold resistance against each of ddI, ddC (Gao et al., 1993), and abacavir (de Jong et al., 1997). In addition, this mutation confers to HIV each of the following:

(1) A marginally reduced viral fitness and RT processivity *in vitro* relative to wild-type enzyme in both primary cells and cell-free virions (Back et al., 1996; Boyer and Hughes, 1995; Quan et al., 1998; Sharma and Crumpacker, 1999). Reduced fitness is evident in a variety of studies demonstrating rapid reversion of an M184V viral population to wild-type *in vivo* on discontinuation of 3TC (Devereux et al., 2001; Devereux et al.,

1999). In growth kinetic studies, M184V results in an attenuated replicative phenotype in all genetic backgrounds, including those demonstrating high-level AZT resistance (Miller et al., 1999), among which, T215 Y or F, K219Q have been associated with an increase in RT processivity above wild-type (Arion et al., 1998; Naeger et al., 2001). Therefore, M184V is known to be an antagonistic suppressor mutation for a variety of NRTI mutations including those associated with AZT resistance.

(2) A modest increase in the "fidelity" of both the DDDP and RDDP activity in reverse transcription, the viral RT, such that viral RT may make fewer errors or be less error-prone than the RTs of wild-type or drug-sensitive viruses. Increased fidelity could potentially diminish genetic diversification, resulting in the slower development of resistance to other drugs *in vitro*. Several studies have shown that M184V increases RT fidelity as assessed by dNTP misincorporation and misinsertion (Feng and Anderson, 1999; Hsu et al., 1997; Rezende et al., 1998; Wainberg et al., 1996).

(3) A diminished ability of viral RT to enact the reverse reaction of polymerization, i.e. pyrophosphorylysis. RT removal of 3'-terminal AZT monophsphate from a blocked proviral genomic template is considered to be the primary mechanism of HIV-1 AZT resistance (Arion et al., 1998; Meyer et al., 1999; Meyer et al., 1998). M184V strongly inhibits the unblocking of AZT-terminated primer, which helps to explain the resensitization to AZT on the part of viruses that contain AZT resistance-conferring mutations as well as the M184V mutations in the RT gene (Götte et al., 2000).

(4) Hypersensitization to other NRTIs. M184V temporarily hypersensitizes HIV-1 to AZT by five-fold compared with wild-type, both in the absence and the presence of AZT-associated mutations. M184V is also associated with a two- or four-fold increase in sensitivity to adefovir, which is active *in vivo* against M184V virus (Miller et al., 1999).

M184I has been observed in patients receiving 3TC prior to appearance of M184V; however, the latter becomes dominant within weeks presumably because it affords greater fitness to HIV and permits faster growth (Back et al., 1996). Considering that the methionine lies in the center of the highly conserved 183YMDD186 motif, it was surprising to find viable HIV variants containing these types of mutations. M184 is part of the dNTP binding site of HIV-1 RT. Structural analysis suggests that the mechanism of resistance of HIV-1 RTs carrying the M184V and M184I mutation involves steric hindrance (Huang et al., 1998a; Sarafianos et al., 1999). 3TC has the ribose ring replaced by an oxathiolane ring; the stereochemical form of 3TC which is used to treat HIV-1 infections is the opposite enantiomer relative to normal dNTPs. The combination of introducing a sulfur atom into the ribose ring and choosing the opposite enatiomer causes the portion of the oxathiolane ring that carries the sulfur atom to project further than the normal ribose ring, thus creating an opportunity for steric hindrance (Huang et al., 1998a; Sarafianos et al., 1999). Because the wild-type enzyme readily incorporates 3TC, there can be no significant steric hindrance with M184. However, models developed based either on a ternary complex of wild-type HIV-1 RT, dsDNA and a bound dNTP or on the structure of the M184I mutant bound to dsDNA suggest that a β-branched amino acid at position 184 would interfere with the ability of 3TC to bind in the appropriate configuration at the polymerase active site. Such steric conflict may allow an improved discrimination among dCTP and 3TC-TP and represents a structural basis to explain the diminished incorporation rate of 3TC-MP by the mutant M184V/I enzymes.

In conclusion, while other mutations in the viral RT may also impact on "fitness", none apparently can do so to as great an extent as the M184V substitution. The fact that the above four distinct, possibly independent, effects are associated with the 184V substitution suggests that this mutation may possibly confer benefit by rendering HIV-1 less capable of

undergoing rapid replication and, by interference, of mutating into new forms. The "M184V fidelity hypothesis" articulates that such viruses might have a reduced capacity to become resistant to drugs that impose a "high genetic barrier", such as certain protease inhibitors, or to escape immunological effectors mechanisms as quickly as do wild-type viruses.

Chapter 2

The impact of the A-rich loop deletion and the M184V substitution in RT on the initiation of reverse transcription

This chapter was adapted from two articles:

1. The M184V mutation in HIV-1 reverse transcriptase reduces the restoration of wild-type replication by attenuated viruses. *By* Xin Wei, Chen Liang, Matthias Götte, and Mark A. Wainberg, 2002, AIDS. 16: 2391-2398.
2. Negative effect of the M184V mutation in HIV-1 reverse transcriptase on initiation of viral DNA synthesis. *By* Xin Wei, Chen Liang, Matthias Götte, and Mark A. Wainberg, 2002. Virology. (In press).

All data presented in this chapter were from experiments performed by myself under the supervision of Dr. Wainberg and Dr. Götte.

2.1 Preface

Initiation of HIV-1 reverse transcription requires specific recognition of the viral genome, primer $tRNA^{Lys3}$, and reverse transcriptase. In addition to the binding of the tRNA acceptor arm to the 18nt primer binding site (PBS), other regions within the viral genome also interact with $tRNA^{Lys3}$ to form a reverse transcription initiation complex. Previous results from our lab have shown that deletion of the A-rich loop causes diminished synthesis of DNA and reduced viral infectivity but can revert to near wild-type replication capacity following additional mutagenesis (Liang et al., 1997a). The M184V mutation located within the highly conserved (Y183, M184, D185, D186) region, that lies in the active site of the p66 polymerase domain of RT, can confer high level resistance to 3TC (Argos, 1988; Gao et al., 1993; Tisdale et al., 1993) and low level resistance to each ddC, ddI and Abacavir (Gao et al., 1993; Gu et al., 1992; Tisdale et al., 1997; Walter et al., 2002). This mutation also results in decreased HIV replication fitness, diminished RT processivity, and increased RT fidelity (Back et al., 1996; Feng and Anderson, 1999; Hsu et al., 1997; Larder et al., 1995; Wainberg et al., 1996; Wakefield et al., 1992). However, little or no evidence exists to suggest that M184V-containing viruses are less mutable than wild-type. A better criterion as to whether or not the M184V substitution can help to prevent viral evolution may be to ask whether HIV that is severely impacted in regard to replication ability can regain viral replication competence if the M184V substitution in RT is also present. Toward this end, we generated an A-rich loop deletion in HIV-1 provirus together with the M184V substitution. Long-term culture was employed to study viral replication fitness, and biochemical experiments were conducted to study the mechanism(s) to explain our *in vivo* results.

2.2 Abstract

The human immunodeficiency virus type-1 (HIV-1) recruits $tRNA^{Lys3}$ as primer to initiate synthesis of the first DNA strand during viral reverse transcription. This tRNA binds at its 18 terminal nucleotide stretch to a complementary primer binding site (PBS) near the 5' end of viral RNA. Previous studies had suggested that extended interactions between the anticodon loop of $tRNA^{Lys3}$ and an "A-rich loop" located upstream of the PBS ((+)169AAAA(+)172) can play important roles in regard to the initiation of reverse transcription, and we have shown that deletion of this A-rich loop caused diminished viral replication fitness. Long-term culture of these deleted viruses resulted in the emergence of breakthrough variants that contained compensatory mutations in the vicinity of the A-rich loop. Now, we have studied the mechanisms involved in the altered replication capacities of the deletion-containing viruses in the context of both wild-type HIV-1 and viruses also containing the M184V substitution in reverse transcriptase (RT). The M184V mutation, known to confer high-level resistance to lamivudine, is also associated with diminished replication fitness, as well as an increase in accuracy of DNA synthesis, and may thus compromise the emergence of revertants. We found that long-term culture of deletion-containing viruses with wild-type RT, yielded revertants that contained G→A substitutions upstream of the deletion, or T→A or C→A substitutions downstream of this region. In contrast, viruses containing the A-rich loop deletion together with M184V did not significantly recover replication ability over protracted periods. Analyses of $tRNA^{Lys3}$-primed DNA synthesis in cell-free assays showed diminished rates of initiation using an RNA template that contained the A-rich loop deletion. Clearance from pausing at position +3 was identified as a sensitive step in this reaction that could not be efficiently bypassed with the M184V mutant enzyme. Increased efficiency of initiation was seen with the deleted RNA templates that also contained mutations identified in the revertant viruses, provided that these mutations facilitated formation

of a competent binary tRNA/RNA complex. Thus, the initiation of $tRNA^{Lys3}$-primed DNA synthesis is an important rate-limiting step in reverse transcription.

2.3 Introduction

Reverse transcription is an essential step in the life cycle of the human immunodeficiency virus type 1 (HIV-1), during which the virus-encoded reverse transcriptase (RT) enzyme converts single-stranded genomic RNA into double-stranded proviral DNA that is later integrated into the host chromosome. The RT enzyme is multifunctional and possesses each of RNA- and DNA-dependent DNA polymerase (RDDP and DDDP) activities as well as a ribonuclease H (RNase H) function that degrades the transcribed RNA (Gilboa et al., 1979; Telesnitsky and Goff, 1997). Cellular $tRNA^{Lys3}$ is used as a primer in order to initiate RT activity and viral DNA synthesis (Götte et al., 1999; Mak and Kleiman, 1997; Marquet et al., 1995). This tRNA primer is incorporated into the virion during its assembly and binds via a stretch of 18 nucleotides at its 3' terminus to a complementary primer binding site (PBS) near the 5' end of viral RNA (Jiang et al., 1993; Mak et al., 1994).

The multifunctional character of RT and its key role in the life cycle of HIV-1 have made this enzyme an important target in antiviral chemotherapy. Nucleoside analogue RT-inhibitors (NRTIs), such as 3'-azido-3'-dideoxythymidine (AZT) and 2', 3'-dideoxy-3' thiacytidine (3TC), that act as chain terminators of nascent DNA synthesis, constitute important components of HIV therapeutic regimens (Emini and Fan, 1997). However, RT is extremely error-prone and this results in mutations in the viral genome including those that are associated with drug resistance (Bebenek and Kunkel, 1993). A M184V mutation located within a highly conserved (Y183, M184, D185, D186) motif that constitutes part of the polymerase active site of RT confers high level resistance (i.e. $\approx$ 1000 fold) to 3TC (Argos, 1988; Tisdale et al., 1993) and

low level resistance (i.e. ≈ 3-5 fold) to each of 2', 3'-dideoxycytidine (ddC), 2', 3'-dideoxyinosine (ddI), and abacavir (ABC) (Gao et al., 1993; Gu et al., 1992; Tisdale et al., 1997; Walter et al., 2002).

The M184V mutation also results in diminished RT processivity, and increased RT fidelity as measured in biochemical assays (Back et al., 1996; Feng and Anderson, 1999; Hsu et al., 1997; Larder et al., 1995; Wainberg et al., 1996; Wakefield et al., 1992). Using an *in vitro* mispair primer extension assay, we and others have shown that the fidelity of both the RDDP and DDDP steps of M184V-mutated HIV RT is significantly higher than that of wild-type enzyme (Feng and Anderson, 1999; Hsu et al., 1997; Wainberg et al., 1996). Rates of incorporation of incorrect nucleotides generally occur more slowly than accurate polymerization steps, and it appears that the M184V substitution may amplify this effect. Mutations at the strategic position of residue 184, located in close proximity to the enzymatic active site, may affect the formation of competent polymerization complexes that involve binding of inferior substrates. Both, the nature of the incoming nucleotide triphosphate, as well as the structure of the primer/template substrate are important parameters in this regard (Götte et al., 2001; Li et al., 1997).

RT must be able to accept a variety of distinct primer/template combinations that are utilized with varying efficiencies. The initiation of RNA-primed synthesis of minus- and plus-strand DNA synthesis represent rate-limiting steps of reverse transcription. The efficiency of incorporation of both correct and incorrect nucleotides is reduced with RNA compared with DNA primers (Götte et al., 2001; Lanchy et al., 1998; Thrall et al., 1998), and the sequence and structure of the RNA template in the region of the PBS also plays a key role during initiation (Isel et al., 1995; Isel et al., 1998). We have previously demonstrated that HIV-1 that is deleted of an A-rich loop, located upstream of the PBS, is initially impaired but can revert to near wild-type replication capacity following additional mutagenesis (Liang et al., 1997b). Both our group and others

have documented that interactions between this A-rich loop ((+169) AAAA (+172)), located 10nt upstream of the PBS and the anticodon loop of $tRNA^{Lys3}$ may be important in initiation of reverse transcription (Isel et al., 1995; Isel et al., 1998; Liang et al., 1997b; Zhang et al., 1998c). Indeed, the compensatory mutations that restored viral replication occurred at bases located immediately upstream of the deleted region and led to partial restoration of this A-rich region. In addition, HIV-1 is capable of using alternate cellular tRNAs to initiate reverse transcription, provided that the A-rich loop has been mutated to a sequence that allows binding to the alternative anticodon loop (Kang et al., 1997; Wakefield et al., 1996). However, additional mutations are required to fully establish the recruitment of other tRNA primers, and the M184 mutation may compromise the emergence of second site revertants (Li et al., 1997). We now show that HIV-1 variants containing the 184V mutation in a background of the A-rich loop deletion were severely compromised in regard to ability to regain viral replication competence, despite the fact that these viruses retained the ability to utilize the natural $tRNA^{Lys3}$ primer. Analyses of the efficiencies of tRNA-primed initiation revealed slow rates when using deletion-containing RNA templates, and it appears that the 184V-containing enzyme amplified these effects. Long-term culture of deletion-containing virus that included a wild-type codon at position 184 (184M) resulted in revertants with mutations at various positions in close proximity to the deletion, and we now show that a T→A transversion located downstream of the deleted A-rich loop plays an important role in the restoration of viral replication. The same mutation increased the efficiency of initiation under physiologically relevant conditions in the presence of the viral nucleocapsid protein (NCp7) that facilitates the placement of $tRNA^{Lys3}$ onto the PBS. These data suggest that decreased rates of tRNA-primed initiation of reverse transcription may directly correlate with diminished viral replication fitness which is an important parameter defining the replicative adaption to its enviroment.

2.4 Materials and methods

Chemicals, enzymes and nucleic acids. All chemicals were purchased from Bioshop Canada Inc., Toronto, Canada. Restriction enzymes and oligodeoxynucleotides were purchased from Gibco Products Inc., Mississauga, Ontario, Canada. Natural $tRNA^{Lys3}$ was purchased from Bio S&T., Montreal, Quebec, Canada. Synthetic $tRNA^{Lys3}$ was synthesized *in vitro* using the plasmid pT7hLys3 as template. This construct contains the 76bp-fragment that represents mature human $tRNA^{Lys3}$ under control of the T7 RNA polymerase promoter. A *Fok* I restriction site was introduced downstream of the tRNA gene to generate a DNA template that ensures run-off transcription of the tRNA with the correct 3' CCA terminus (Liu and Horowitz, 1993). *In vitro* transcription with T7 RNA polymerase was performed overnight at 37°C using 5 µg of the digested plasmid which was incubated in a total volume of 100µl containing 40 mM Tris-HCL (PH 8.0), 25 mM $MgCl_2$, 50 mM NaCl, 1 mM spermidine, 50 mM DTT, 4mM NTPs (each), 200U of T7 polymerase (MBI, Fermentas) and 20U of the placental RNase inhibitor RNasin (Pharmacia). The tRNA reaction product was purified using 8% polyacrylamide gels that contained 7 M urea and 50 mM Tris-borate EDTA (TBE), and was visualized under UV light, prior to elution from isolated gel slices using a solution of 0.5 M sodium acetate and 0.01 % SDS. Heterodimeric HIV-1 wild-type RT and M184V mutated RT were prepared and purified as described (Arts et al., 1998).

Cells and virus infection. MT-2 and COS-7 cells were maintained in RPMI1640 medium and Dulbecco's modified Eagle's medium, respectively, each being supplemented with 10% fetal calf serum. Virus stocks were prepared by transfecting COS-7 cells with HIV-1 wild-type BH10 strain DNA (HIV-1/WT) or with HIV-1 DNA containing the A-rich loop deletion (HIV-1/ΔA) or alternatively, with HIV-1 DNA containing both the A-rich loop deletion and the M184V mutation in RT region (HIV-1/ΔA-M184V), through use of Lipofectamine (Gibco Products Inc.,

Mississauga, Ontario, Canada). Production of progeny virus was assessed by measuring levels of p24 (CA) antigen released into culture fluids at 48 h after transfection using an enzyme-linked immunosorption assay (Abbott Laboratories, Abbott Park, Ill.). Similar quantities of virus based on p24 Ag levels (i.e. 2 ng of p24 antigen/10^6 cells), were treated with RNase-free DNase I in the presence of 10 mM $MgCl_2$ and were then used to infect 10^6 MT-2 cells. Cells were washed twice after 2 h with serum-free RPMI 1640 medium and were then maintained in serum-supplemented medium. Culture fluids were collected at various times for determinations of RT activity.

PCR amplification and sequencing analysis of breakthrough viruses. In the case of long-term cultures, when about 30% of cells presented with cytopathology, culture fluids were collected in order to initiate another round of infection. At the same time, infected cells were collected and suspended in lysis buffer containing 0.5% sodium dodecyl sulfate and 1 mg of protease K per ml. After 6 h at 37°C, the lysed suspensions were extracted twice with phenol-chloroform and precipitated with 2.5 volumes of 95% ethanol. The recovered total cellular DNA was resuspended in 20 µl double-distilled water, and 2 µl were used for 30-cycle PCR amplification of the region containing the A-rich loop deletion and the M184V mutation under the following condition: 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The primers employed were PΔA1 [5'-GACCAGATCTGAGCCTGGGAGCT-3'] and PΔA2 [5'-CACCATCCTCTCTCCTTCTAGCC-3'] for the A-rich loop region alone and P184a [5'-CATTAGATATCAGTACAATGTG-3'] and P184b [5'-TCCCCACCTCTAACAGATGTTG-3'] for the region in RT containing the M184V mutation. The PCR products were cloned into plasmid vector PCR2.1-TOPO (Invitrogen Corporation, Carlsbad, Cal.). After selection of white or light blue colonies, positive clones in regard to both the A-

rich loop region and M184V mutation were sequenced using primers PΔA1 and P184a.

Construction of RNA expression plasmids and *in vitro* transcription of HIV-1 RNA. Construction of the *in vitro* wild-type HIV-1 RNA template expression plasmid pHIV-PBS was carried out as described previously (Arts et al., 1998). Mutated forms of this expression plasmid were derived from positive clones containing the A-rich loop in the PCR2.1-TOPO vector. After digestion with *Bgl* II and *Bss*HII, the resulting DNA fragments that contained either the A-rich loop deletion, or different reversion sequence were cloned into pHIV-PBS digested with the same enzymes, to yield the plasmids pPBS/ΔA, pPBS/R0, pPBS/R12, pPBS/R1 and pPBS/R2. To prepare RNA transcripts, plasmids were linearized with *Bss*HII and used as templates in a Megashortscript kit (Ambion, Austin, Tex.) according to the manufacturer's instructions.

***In vitro* primer extension assays.** A cell free assay was used to study the effect of the A-rich loop and M184V mutated RT on the synthesis of minus-strand strong stop DNA [(-) ssDNA]. Heat annealing can ensure the complete hybridization of RNA or DNA primer to an RNA template and was performed in a 30 µl reaction mixture containing 50 mM Tris-HCl (pH7.8), 50 mM NaCl, 20nM natural or *in vitro* synthetic $tRNA^{Lys3}$ or DNA primer, and 40 nM template RNA. The DNA Primer used is PBS25D [5'-CTTTCAGGTCCCTGTTCGGGCGCCA-3'], which has 25 nucleotides complementary to the PBS region. This mixture was incubated for 2 min at 95°C followed by 20 min at 70°C and 20 min at 37°C. After incubation for 5 min at 37°C in the presence of 6mM $MgCl_2$, 100 mM of wild-type or M184V mutated RT was added into the reaction mixture and incubated for another 5 min at 37°C. Synthesis of (-) ssDNA was initiated by addition of a mixture of dNTPs to obtain a final concentration of 10µM of each of dATP, dGTP, and dTTP and 1µM dCTP. Reactions were monitored by

including [α-^{32}P]dCTP in the mixture, allowing us to study both the synthesis of the full-length (-) ssDNA as well as different pausing sites during each of the processes of initiation and elongation of the reaction. To further study the effect of the A-rich loop and M184V mutated RT on the early phrase of initiation, 10 μM ddATP was employed as a termination nucleotide instead of dATP to give rise to a 6 nucleotides elongation product. Aliquots of 2 μl were removed at different time points, and reactions were terminated in 8 μl of a solution containing 80% formanide and 40 mM EDTA. Products were separated on 8% polyacrylamide-7 M urea gels, which were dried and exposed to Kodak film at -70°C. The results were analyzed using Molecular Analysis software.

Placement of the tRNA^{Lys3} primer onto the RNA template by NCp7. The HIV-1 nucleocapsid protein (NCp7) used in this study contains 72 amino acids and was generously provided by Dr. Bernard Roques, Paris, France. A ratio of 6 nucleotides of template to 1 molecule of NCp7 was used in order to obtain maximal annealing activity (Li et al., 1996). 40 nM of natural tRNA^{Lys3}, 200 nM of various RNA templates, and 9 μM of NCp7 were incubated together in a buffer containing 50 mM Tris-HC1 (pH7.8), 50 mM NaCl, 5 mM $MgCl_2$ and 10 mM dithiothreitol for 1 h at 37°C. Reverse transcription was initiated under the same conditions as described above. Aliquots of 10 μl were removed at different times and were treated with 200 μg of proteinase K per ml at 37°C for 30 min and then extracted with phenol-chloroform. After precipitation with a 4 × volume of 95% ethanol, the products were separated on gels and visualized as described above.

Construction of revertant HIV-1 DNA. DNA fragments derived from the *in vitro* RNA expression plasmids pPBS/ΔA, pPBS/R0, pPBS/R12, pPBS/R1 and pPBS/R2, after digestion with *Bgl* II and *Bss*H II, were

cloned into an intermediate subcloning vector pSVU3R which contains the 5' U3 and R regions of HIV-1 genomic DNA. After further digestion with *Hpa* I and *Apa* I, the resultant 2kb fragments were cloned into HIV-1 wild-type BH10 strain DNA (HIV-1/WT) and M184V mutated HIV-1 DNA (HIV-1/M184V), to yield various HIV-1 clones that were then used to transfect COS-7 cells under the same conditions as described above. The viruses thus generated were harvested and used to infect MT-2 cells. Viral replication was monitored by determinations of RT activity in culture fluids.

2.5 The M184V mutation in HIV-1 reverse transcriptase reduces the restoration of wild-type replication by attenuated viruses.

2.4.1 Results

Effects of both the M184V mutation and the A-rich loop deletion on viral replication kinetics.

We generated plasmids that contained the deletion of the A-rich loop upstream of the PBS in backgrounds of wild-type HIV-1 as well as in a virus that additionally contained the M184V mutation in the RT gene. Plasmids were named HIV-1/ΔA and HIV-1/ΔA-M184V, respectively. These constructs, as well as wild-type HIV-1 (HIV-1/WT), were transfected into COS-7 cells to generate viruses for long-term culture experiments that were intended to generate viral revertants. For this purpose, viruses were passaged in MT-2 cells over protracted periods and viral replication capacity was assessed by measuring RT activity. Representative results shown in Fig. 2-1A document that MT-2 cells infected by either HIV-1/ΔA or HIV-1/ΔA-M184V generated far less progeny virus up to seven passages (results are shown for the fourth passage) than did cells infected by wild-type HIV-1. However, the HIV-1/ΔA viruses began to show increased infectivity after eight passages (Fig. 2-1B), and, after nine passages, were able to replicate with efficiency similar to that of HIV-1/WT (Fig. 2-1B). This result was consistent over 20 passages in subsequent analyses. In contrast, replication of the HIV-1/ΔA-M184V viruses remained stably impaired throughout this period.

Characterization of revertants.

We next sequenced the proviral DNA in the region of the PBS to identify putative genetic alterations that may have emerged during long-term culture of the HIV-1/ΔA virus. The first detectable change appeared after 8 passages; this mutation involved the substitution of (+173) A in place of (+173) T that is located immediately downstream of the former A-rich loop. However, only one of eight clones that had been sequenced contained this reversion, while the others remained unchanged and all clones maintained the (+169) AAAA (+172) deletion. These data are consistent with sustained defects in regard to viral replication kinetics

Fig. 2-1 Infection of MT-2 cells with viruses harvested after four passages (A) and nine passages (B). 10^6 cells were infected with HIV-1 wild-type BH10 strain, HIV-1/ΔA, or HIV-1/ΔA-M184V, all harvested from transfected COS-7 cells. An equivalent of virus to 2 ng of p24 antigen was used in each case and virus production was monitored on the basis of RT levels in culture fluids.

Fig. 2-2 Sequencing analysis of HIV-1/WT, HIV-1/ΔA and revertant viruses emergent in long-term tissue culture after 9 passages in MT-2 cells. The A-rich loop sequence and mutated nucleotides are highlighted in bold. The underlined TGGCG sequence represents the 5' end of the primer binding site (PBS).

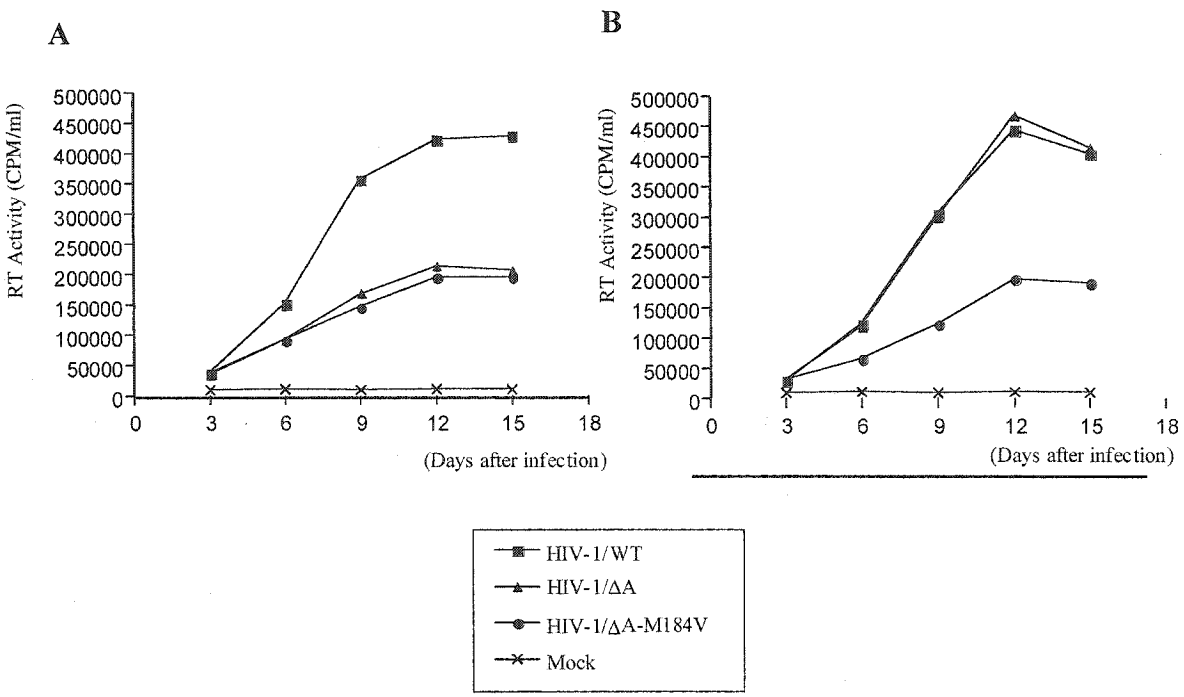


Fig. 2-1

Strain	Sequence	No. of different revertant/ 8 clones sequenced after 9th passage
HIV-1 wild type (BH10)	5' —— CA GTGTGG¹⁶⁹A¹⁷²AAA TCTCTAGCAGTGGCG —— 3'	
HIV-1/ΔA	5' —— CA GTGTGG -------- TCTCTAGCAGTGGCG —— 3'	1/8
HIV-1/R0	5' —— CA GTGTA¹⁶⁷G -------- TCTCTAGCAGTGGCG —— 3'	1/8
HIV-1/R1-2	5' —— CA GTGTGG --------¹⁷³A¹⁷⁶CTATAGCAGTGGCG —— 3'	1/8
HIV-1/R1	5' —— CA GTGTGG --------¹⁷³ACTCTAGCAGTGGCG —— 3'	3/8
HIV-1/R2	5' —— CA GTGTGG -------- TCT¹⁷⁶A TAGCAGTGGCG —— 3'	2/8

Fig. 2-2

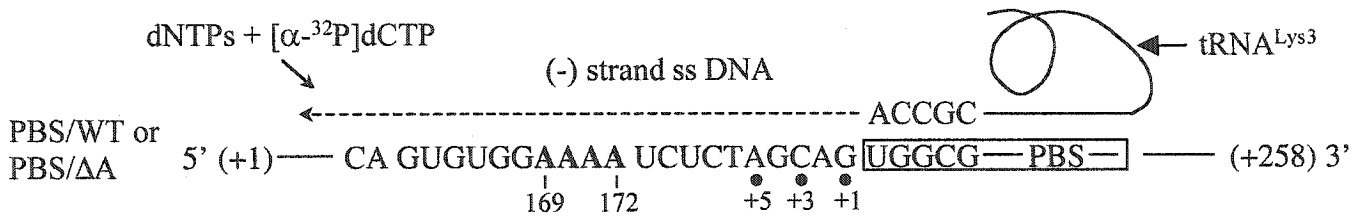
after eight passages (Fig. 2-1A). In contrast, after nine passages, at least seven of the eight clones sequenced possessed changes at positions adjacent to the deleted A-rich loop region (Fig. 2-2). A revertant termed HIV-1/R0 had a G→A substitution at position 167, and two other revertants, i.e. HIV-1/R1 and HIV-1/R2, contained single T→A and C→A substitutions at positions 173 and 176, respectively. In contrast, revertant HIV-1/R1-2 contained both of the latter mutations. After 11 passages, the revertant virus strain HIV-1/R1 became dominant in the outgrowth of viruses from the original HIV-1/ΔA stock. In agreement with the results of Fig. 2-1B, no revertant viruses were detected following long-term culture of HIV-1/ΔA-M184V. The M184V substitution in RT remained stable over 20 passages in these cases.

The impact of the A-rich loop deletion and the M184V substitution in RT on the initiation of minus-strand viral DNA synthesis.

To study biochemical mechanism(s) that might explain the negative impacts of both the A-rich loop and the M184V substitution on HIV-1 replication, we next studied the efficiency of initiation of minus-strand strong stop DNA [(-) ssDNA)] synthesis using a cell-free assay, as described in the legend to Fig. 2-3A. Natural $tRNA^{Lys3}$ was heat-annealed with *in vitro* synthesized RNA templates, that contained the PBS together with or without the A-rich loop, to form binary complexes that serve as substrates for HIV-1 RT. The preformed tRNA/RNA complexes were incubated with either purified wild-type HIV-1 RT or a mutant enzyme that contained the M184V mutation to study the efficiency of the tRNA-primed initiation reactions. A comparison of the time-dependence of formation of full-length (-) ssDNA revealed that reactions involving wild-type template (PBS/WT) yielded more product than those performed with PBS/ΔA (Fig. 2-3B). Furthermore, pausing at the early stages of DNA synthesis, at position +3 and +5, was significantly increased with use of the mutated template. Reactions conducted with the M184V mutant enzyme showed similar patterns; however, polymerization in general

Fig. 2-3 The effects of the A-rich loop deletion and M184V substitution in RT on synthesis of (-) ssDNA. (A) Graphic representation of the cell-free assay system for synthesis of full-length (-) ssDNA. The PBS/WT RNA template employed in this system consists of 258 nucleotides at the 5' end of the HIV-1 genome that contains the R, U5, and PBS regions. The PBS/ΔA template has the same sequences as found in PBS/WT, except for the deletion of the A-rich loop in the R region, which is highlighted in bold. (B) Synthesis of (-) ssDNA from either PBS/WT or PBS/ΔA template by each of HIV-1 WT RT and M184V mutated RT. A time-course experiment was performed by pre-forming a $tRNA^{Lys3}$/RNA template complex, and incubating this together with RT at 37°C for 5 min. Reactions were initiated by the addition of dNTPs and stopped after 1, 2, 4, 6, 8, 10, 15, 30 and 60 min (lanes 1-9). Reactions were monitored by incorporation of [α-^{32}P]dCTP into the growing DNA chain. (C) Synthesis of (-) ssDNA initiated by a DNA primer.

A


dNTPs + [α-32P]dCTP
(-) strand ss DNA
tRNALys3
ACCGC
PBS/WT or
PBS/ΔA
5' (+1)
CA GUGUGGAAAA UCUCTAGCAG UGGCG PBS
(+258) 3'
169
172
+5 +3 +1

B

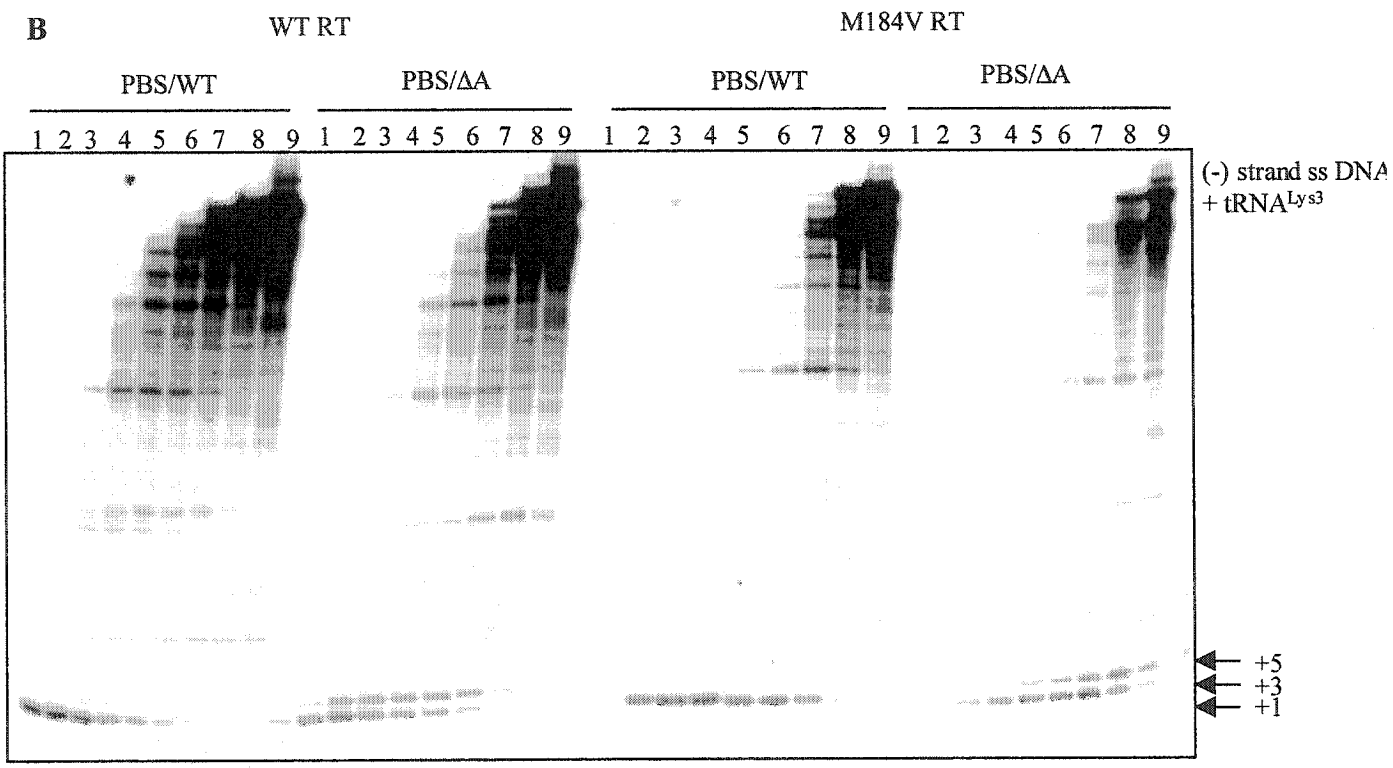

WT RT
M184V RT
PBS/WT
PBS/ΔA
PBS/WT
PBS/ΔA
1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9
(-) strand ss DNA
+ tRNALys3
+5
+3
+1

Fig. 2-3

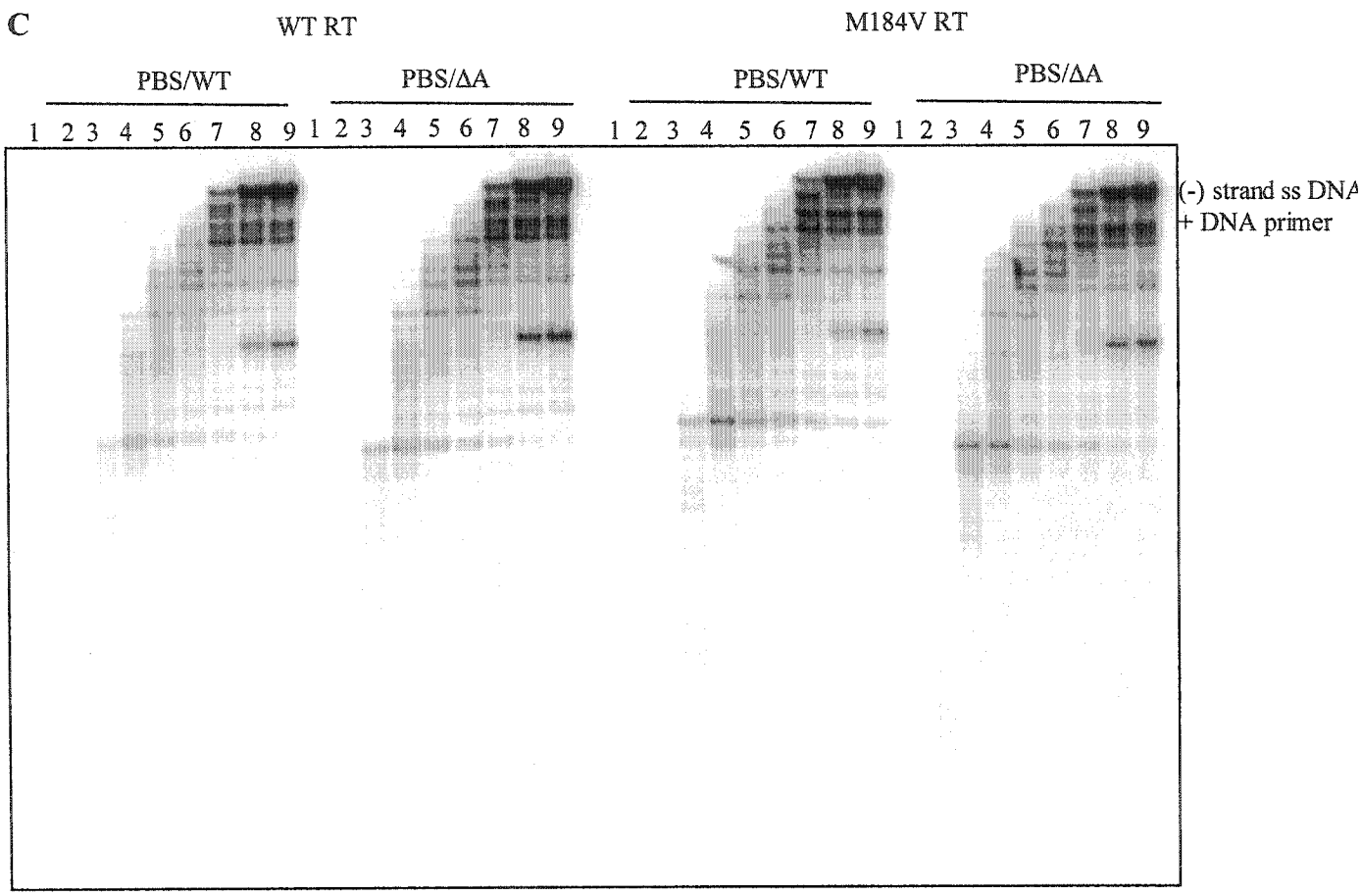

C
WT RT
M184V RT
PBS/WT
PBS/ΔA
PBS/WT
PBS/ΔA
1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9
(-) strand ss DNA
+ DNA primer

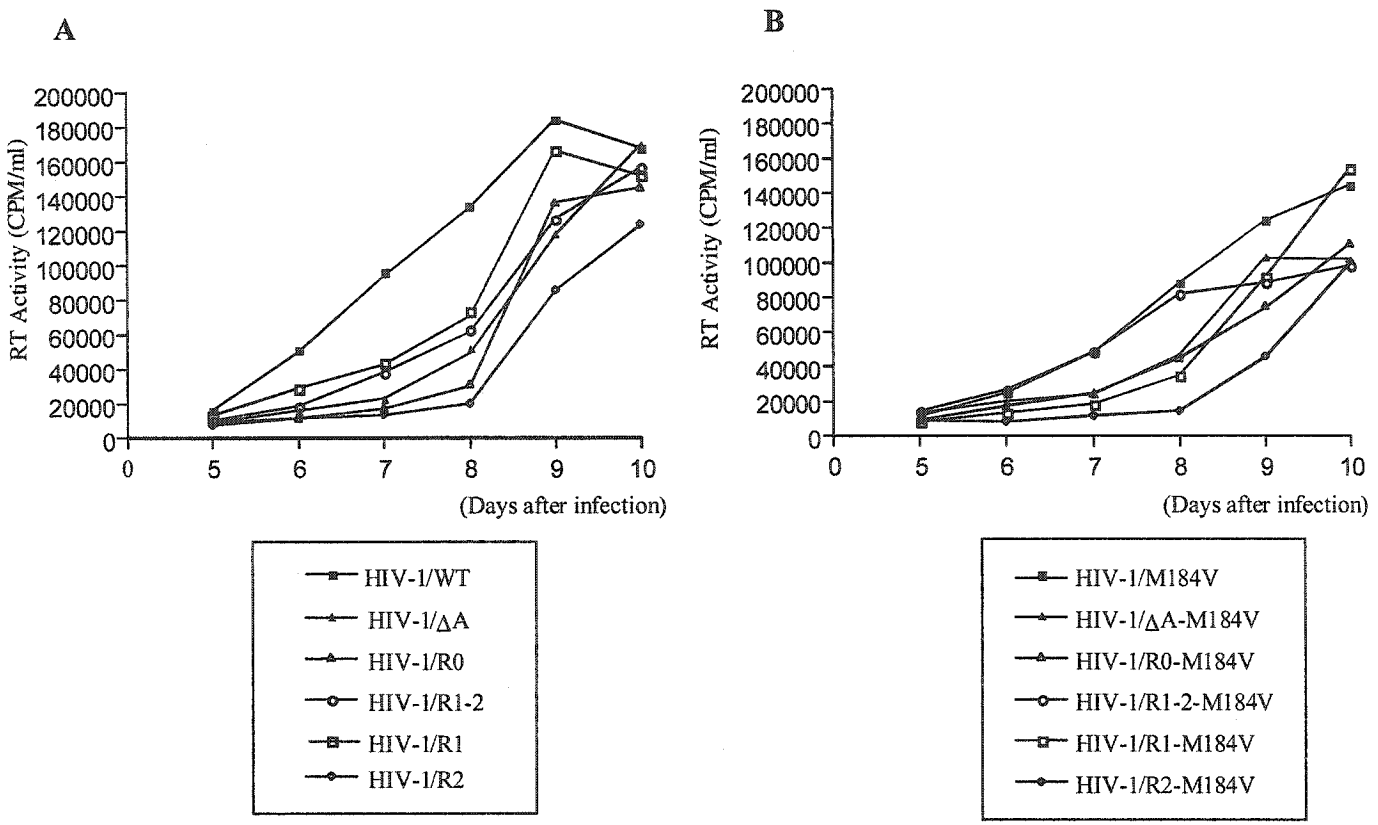
Fig. 2-3

proceeded with less efficiency and also involved increased pausing, as compared to reactions performed with wild-type RT. The strongest effects were seen in reactions conducted with both the mutant enzyme and the mutant template. Importantly, we did not detect any significant differences in these reactions when using a DNA primer, which contains its own PBS-binding sequence, instead of natural $tRNA^{Lys3}$ (Fig. 2-3C). These results suggest that both deletion of the A-rich loop and the M184V mutation can exert specific effects during early stages of tRNA-primed synthesis of (-) ssDNA.

***In vivo* infectivity of different revertant viruses.**

To confirm that the mutations identified in the revertant viruses, shown in Fig. 2-2, were indeed responsible for increased replication competence, we next constructed viruses containing these same sequences (HIV-1/R0, HIV-1/R1-2, HIV-1/R1 and HIV-1/R2). The M184V substitution was also included in the generation of a similar series of constructs, termed HIV-1/R0-M184V, HIV-1/R1-2-M184V, HIV-1/R1-M184V and HIV-1/R2-M184V, to determine whether the revertants identified in the context of wild-type RT would also be functional in the presence of M184V. Analyses of replication capacity revealed that HIV-1/WT replicated with the greatest efficiency of all the viruses studied, while HIV-1/R1 was the most efficient of all the mutant viruses, and HIV/R1-2 was the most impaired (Fig. 2-4A). In the presence of the M184V substitution, all of the mutated viruses showed a further diminution in replication capacity compared with their counterparts that contained a wild-type RT (Fig. 2-4B and 2-4C). These findings are consistent with those reported above that the additional presence of the M184V substitution in RT was not permissive for compensatory mutagenesis following deletion of the A-rich loop.

Fig. 2-4 (A) Infection of MT-2 cells by various recombinant viruses, based on 0.2 ng of p24 Ag per 5×10^5 cells in each case. Virus production was monitored by RT assay. (B) Infection of MT-2 cells with similar viruses as in (A) but also containing the M184V mutation in RT. (C) Percentage of virus replication in MT-2 cells after 9 days relative to HIV/WT.


A
B
RT Activity (CPM/ml)
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
0
5
6
7
8
9
10
(Days after infection)
HIV-1/WT
HIV-1/ΔA
HIV-1/R0
HIV-1/R1-2
HIV-1/R1
HIV-1/R2
HIV-1/M184V
HIV-1/ΔA-M184V
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HIV-1/R1-2-M184V
HIV-1/R1-M184V
HIV-1/R2-M184V

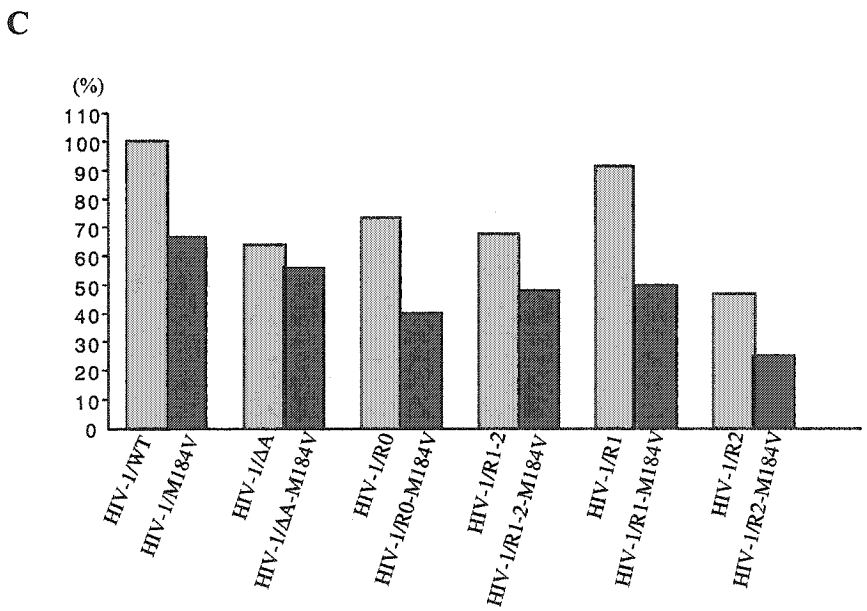

C
(%)
110
100
90
80
70
60
50
40
30
20
10
0
HIV-1/WT
HIV-1/M184V
HIV-1/ΔA
HIV-1/ΔA-M184V
HIV-1/R0
HIV-1/R0-M184V
HIV-1/R1-2
HIV-1/R1-2-M184V
HIV-1/R1
HIV-1/R1-M184V
HIV-1/R2
HIV-1/R2-M184V

Fig. 2-4

2.5.2 Discussion

Resistance of HIV-1 to antiretroviral therapy remains a significant obstacle in the long-term control of HIV-1 disease, and drug-specific mutations in the viral genome have been linked to the selection of viral strains with reduced susceptibility to antiviral drugs (Gunthard et al., 1998; Martinez-Picado et al., 2000). In the case of 3TC, a single amino acid substitution suffices to confer high-level resistance, but the mutation involved, i.e. M184V, is also associated with multiple changes in enzymatic properties that include diminished processive DNA synthesis (Back et al., 1996), moderate increases in accuracy of the polymerization process (Feng and Anderson, 1999; Hsu et al., 1997; Wainberg et al., 1996), and decreased excision of incorporated AZT- and 3TC-monophosphates from the primer terminus of newly synthesized viral DNA (Götte et al., 2000).

In this study, we have generated viruses that are deleted of an A-rich loop, located upstream of the PBS, and have asked how the presence of the M184V mutation might impact on the emergence of revertants. We hypothesized that the M184V mutation may diminish the formation of a competent RT initiation complex. This notion is also supported by earlier work that showed that M184V-containing viruses were less capable than wild-types of utilizing non-$tRNA^{Lys3}$ primers (Li et al., 1997). Here, we show that the presence of M184V can impact on the emergence of revertants, in the context of viruses deleted of the A-rich loop, which retain the ability to utilize the natural $tRNA^{Lys3}$ primer.

Our data show that deletion of the A-rich loop, when present in a background of wild-type HIV-1, caused reduced replication kinetics and was associated with the emergence of revertants in long-term culture. These reversions are attributable to a broad variety of genetic alterations in the vicinity of the deletion, that help to explain the structural and functional requirements for efficient $tRNA^{Lys3}$-primed initiation of reverse transcription. However, dually mutated viruses that contained both the A-

rich loop deletion as well as the M184V mutation in RT were unable to revert even after 20 passages. Thus, the M184V substitution impeded compensatory mutagenesis in our system.

How can we explain the severe impact of the M184V mutation on the efficiency of replication of the deletion-containing viruses? Analyses of early events of the tRNA-primed initiation reaction revealed that both of the genetic alterations described in this paper affected this reaction in specific but different ways. Earlier studies had shown that initiation is a slow process, accompanied by frequent pausing of the RT enzyme at positions +3 and +5 (Isel et al., 1996; Liang et al., 1998). Thereafter, a sharp transition occurs to a faster and more processive mode of polymerization after incorporation of the sixth nucleotide (Lanchy et al., 2000; Lanchy et al., 1998; Thrall et al., 1998). We found that the M184V mutation reduced the rate of tRNA-primed DNA synthesis and delayed release from pausing in cell-free reverse transcription assays performed with synthetic templates. These effects were specific for the tRNA-primed reaction, since the efficiency of DNA synthesis was, as expected, unaffected in reactions performed with a DNA primer. This supports our original hypothesis that the efficient usage of nucleic acid substrates that show reduced affinity to wild-type RT is further compromised in the presence of the M184V mutation. Together, these data show that the joint presence of the A-rich loop deletion and the M184V mutation affect initiation of reverse transcription in complementary and possibly synergistic fashion, resulting in greater impairment in synthesis of (-) ssDNA than seen with either alteration on its own.

In summary, this study suggests that the efficiency of tRNA-primed initiation of reverse transcription may correlate with viral replication competence. Both placement of $tRNA^{Lys3}$ onto the PBS and rates of initiation of DNA synthesis are important factors that determine the yield of (-) ssDNA. The A-rich loop probably plays a crucial role in these stages of reverse transcription, because it may be essential for the

formation of properly folded genomic RNA, and changes in viral RNA structure in close proximity to the PBS can affect tRNA placement and initiation of DNA synthesis. Synthesis of viral DNA with either mutated or a wild-type template is a far less efficient process with mutated M184V-containing RT than with wild-type enzyme. Detailed studies of the biochemical mechanisms whereby the reversion mutations identified here led to more efficient reverse transcription and viral growth are in progress.

2.6 Negative effect of the M184V mutation in HIV-1 reverse transcriptase on initiation of viral DNA synthesis

2.6.1 Results

A-rich loop and the M184V substitution in RT exert their effects on viral replication at the early stage of reverse transcription. We have previously demonstrated that deletion of this A-rich loop in viruses containing wild-type RT caused diminished viral replication fitness. The presence of M184V substitution in RT further amplified this effect. Biochemical experiments suggest that the enzyme containing both the M184V mutation and the deletion in the A-rich loop may have an impact on the early stage of HIV-1 reverse transcription. In this study, We devised an *in vitro* six-nucleotide extension assay to assess the events governing initiation of reverse transcription in more detail, in which a chain-terminating nucleotide ddATP was incorporated at position +6 during the synthesis of (-) ssDNA (Fig. 2-5A). The tRNA-primed reaction was analyzed in time-course experiments to visualize differences in pausing profiles. We found that reactions performed with mutated template (PBS/ΔA) yielded increased pausing at position +3 and +5 in comparing to reactions with wild-type template (PBS/WT). The M184V substitution in RT also contributed to decreased efficiency of DNA synthesis because of increased pausing at the +3 position, and product formation, in general, appeared to be diminished, suggesting that even the first nucleotide incorporation event was already compromised (Fig. 2-5B). These differences were further analyzed by comparing the relative formation of the +3 product, representing complexes that had reached the pausing site at position +3, and products at positions +5 and +6, representing complexes that had successfully passed the +3 pausing site. The results of Fig. 2-5C show that the M184V mutant enzyme caused a significant delay in clearance from pausing, and reactions performed with this enzyme only reached the +3 pause site at later time points. Escape from pausing was almost absent when using the deletion-containing template in these reactions. Similar conclusions were reached when analyzing the distribution of products at positions +1, +3, +5, and +6 after

Fig. 2-5 The effects of the A-rich loop deletion and M184V substitution in RT on initiation of reverse transcription. (A) Graphic representation of the cell-free six-base extension assay system for study of initiation of reverse transcription. The PBS/WT RNA template employed in this system consists of 258 nucleotides at the 5' end of the HIV-1 genome that contains the R, U5, and PBS regions. The PBS/ΔA template has the same sequences as found in PBS/WT, except for the deletion of the A-rich loop in the R region, which is highlighted in bold. (B) Initiation of synthesis of (-) ssDNA from each of the PBS/WT and PBS/ΔA templates using either wild-type RT or M184V mutated RT. Each panel shows a time course of DNA synthesis, monitored by incorporation of [α-^{32}P] dCTP into growing DNA chains. Reactions were terminated after extension of 6 nucleotides by the incorporation of ddATP. Samples were analyzed after 1, 2, 4, 6, 8, 10, 12, 15, 20, 30, 45 and 60 min (lanes 1-12). Relative RNA concentrations were determined using Molecular Analyst software. (C) Log scale plots of the percentage of the +3 pause product relative to total RNA product at each time point. (D) Relative percentage of the +1 and +3 pause products compared with total RNA product after 60 min.

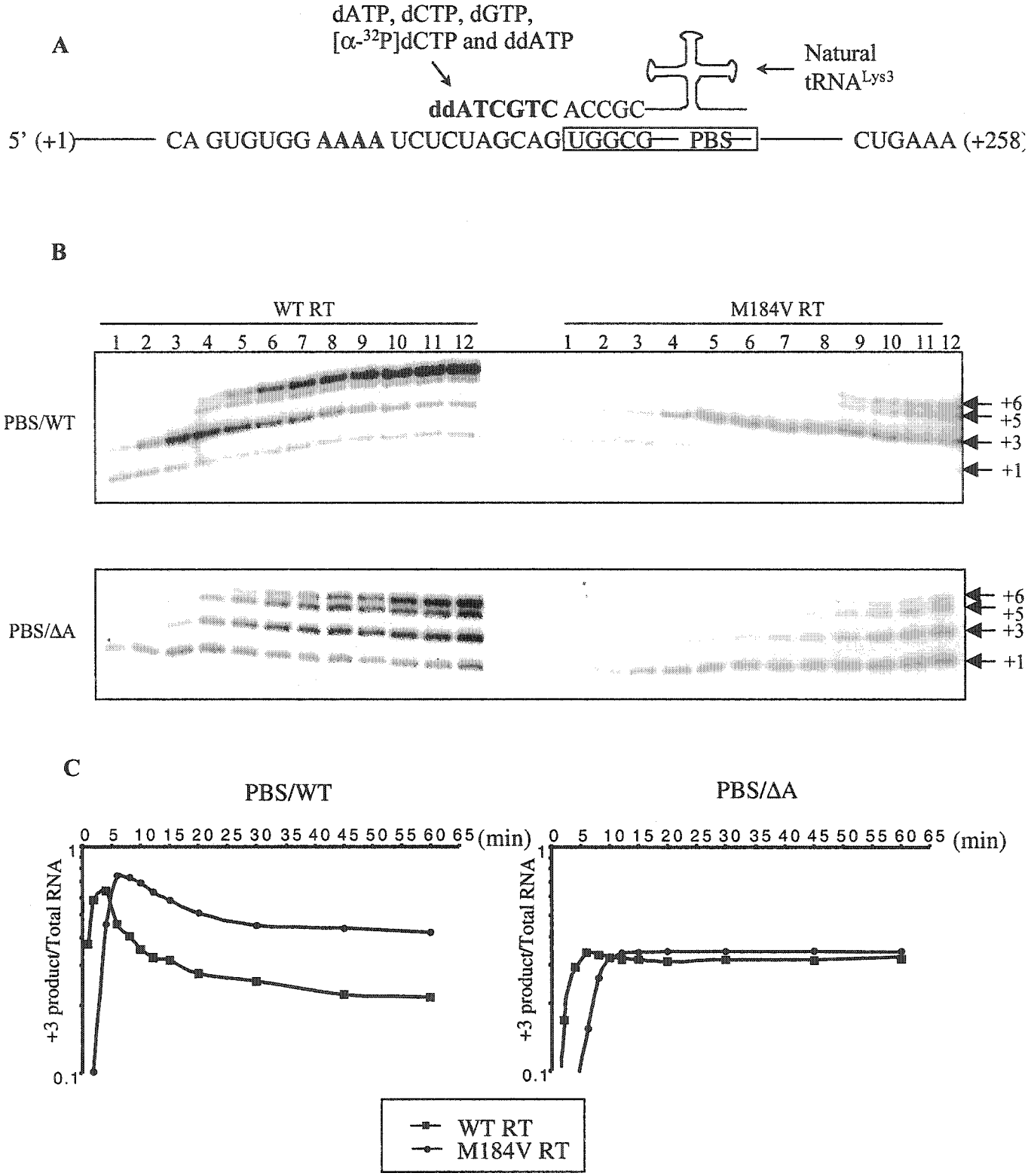

A
dATP, dCTP, dGTP,
[α-32P]dCTP and ddATP
Natural
tRNALys3
ddATCGTC ACCGC
5' (+1)
CA GUGUGG AAAA UCUCUAGCAG
UGGCG
PBS
CUGAAA (+258)
B
WT RT
M184V RT
1 2 3 4 5 6 7 8 9 10 11 12
PBS/WT
PBS/ΔA
+6
+5
+3
+1
C
PBS/WT
PBS/ΔA
0 5 10 15 20 25 30 35 40 45 50 55 60 65 (min)
+3 product/Total RNA
1
0.1
WT RT
M184V RT

Fig. 2-5

D

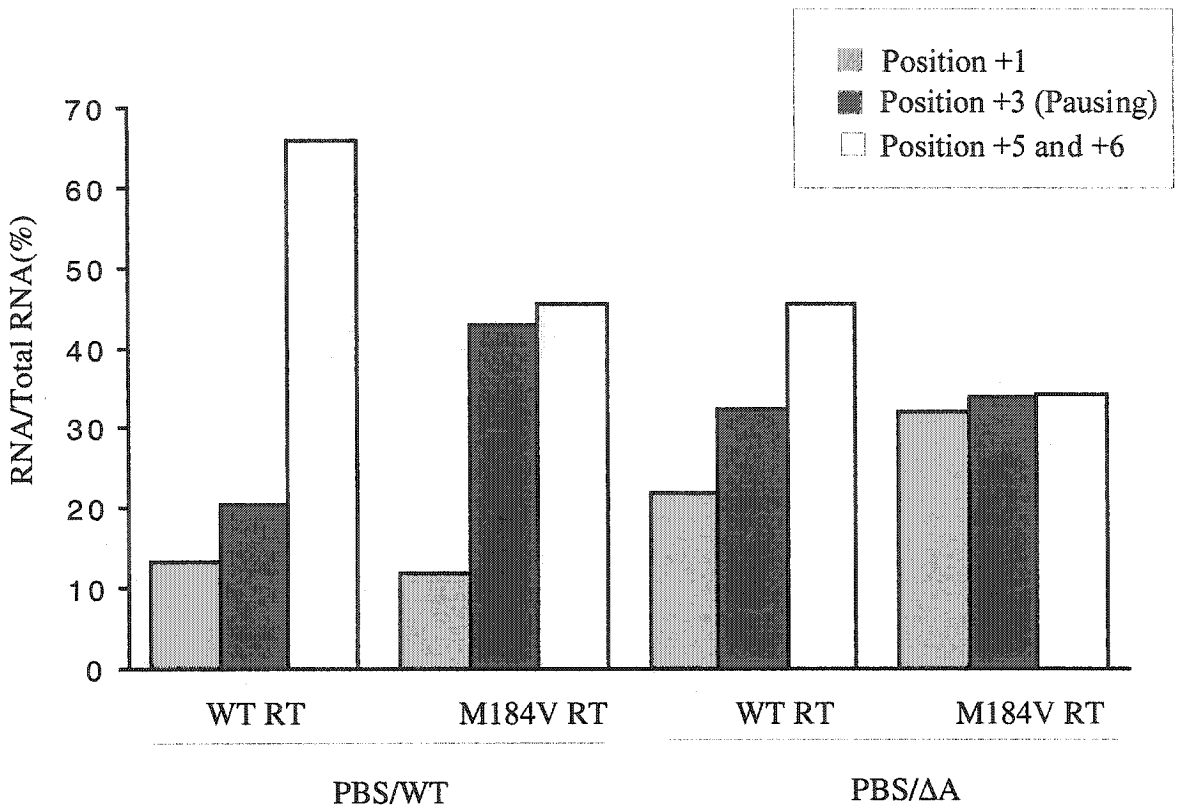

Position +1
Position +3 (Pausing)
Position +5 and +6
RNA/Total RNA(%)
70
60
50
40
30
20
10
0
WT RT
M184V RT
WT RT
M184V RT
PBS/WT
PBS/ΔA

Fig. 2-5

a 60 min reaction (Fig. 2-5D).

Efficiency of initiation of minus-strand DNA synthesis using mutated RNA templates. Long-term culture of viruses containing A-rich loop deletion resulted in the emergence of breakthrough variants that contained compensatory mutations in the vicinity of the deletion. A revertant termed HIV-1/R0 had a G→A substitution at position 167, and two other revertants, i.e. HIV-1/R1 and HIV-1/R2, contained single T→A and C→A substitutions at positions 173 and 176, respectively. In contrast, revertant HIV-1/R1-2 contained both of the latter mutations. Among these revertants, HIV-1/R1 was able to replicate with efficiency similar to that of wild-type HIV-1 and became dominant in the outgrowth of viruses from the original HIV-1/ΔA stock. In contrast, viruses containing the M184V mutation in RT together with the deletion of the A-rich loop neither reverted nor recovered replication ability over protracted period.
To study mechanisms whereby the various revertants established partial restoration of replication fitness, we next analyzed the efficiency of tRNA-primed (-) ssDNA synthesis using *in vitro* synthesized RNA templates containing the corresponding genetic alterations. Unexpectedly, the results revealed that the efficiency of initiation with PBS/R1, i.e. the mutant that showed the highest replication capacity, remains severely diminished (Fig. 2-6). The efficiency of pausing at position +3 appears to be increased, as compared to reaction conducted with the wild-type template PBS/WT. In contrast, PBS/R2, i.e. the mutant that showed the worst replication capacity, resulted in a significantly increased efficiency of (-) ssDNA synthesis, exceeding even that generated with PBS/WT. Similar results were obtained PBS/R0 mutant template, and both of these cases also resulted in a virtual elimination of pausing during the initiation stage. Thus, pausing appears to be an obstacle that results in diminished rates of tRNA-primed DNA synthesis, which may help to explain that the efficiency of initiation does not always correlate with viral fitness.

Fig. 2-6 Synthesis of (-) ssDNA from each of the PBS/WT and PBS/ΔA templates as well as RNA templates containing different reversions. Each panel shows a time-course of synthesis of (-) ssDNA performed by HIV-1 WT RT. Reactions were initiated by pre-forming the natural tRNA/RNA complex and were stopped after 1, 5, 10, 30 and 60 min (lanes 1-5).

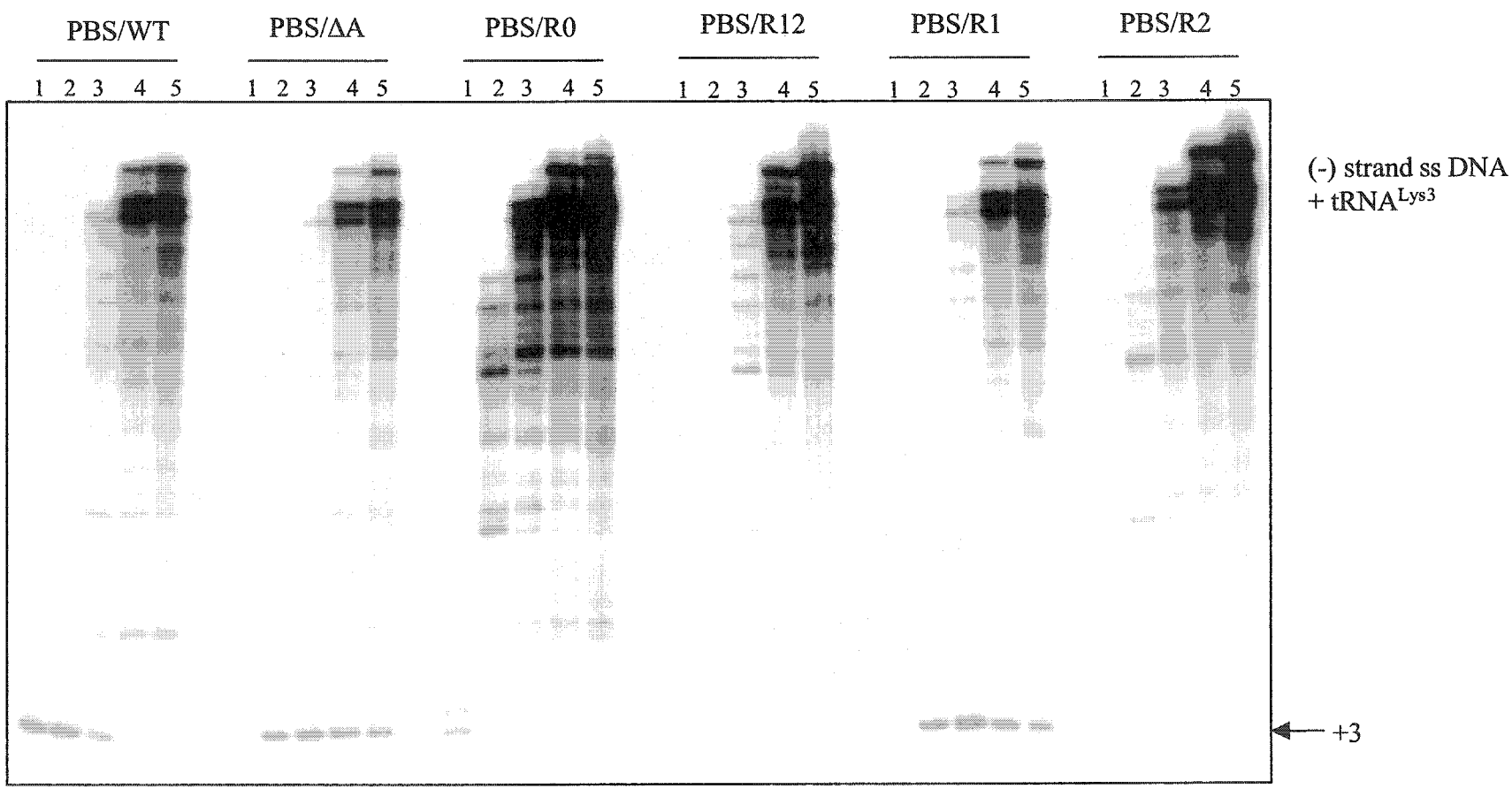

PBS/WT
PBS/ΔA
PBS/R0
PBS/R12
PBS/R1
PBS/R2
1 2 3 4 5
(-) strand ss DNA
+ tRNA^{Lys3}
+3

Fig. 2-6

However, an absence of pausing sites also points to significant structural rearrangements in the binary tRNA/RNA complex, and changes in sequence and structure of the RNA template in the vicinity of the PBS have been implicated in tRNA placement as well. Unwinding and annealing of $tRNA^{Lys3}$ is an important prerequisite for initiation, and are not revealed in assays that involve heat-annealed tRNA/RNA complexes. Previous studies have shown that specific placement of $tRNA^{Lys3}$ onto the PBS can be conducted at room temperature in the presence of mature viral nucleocapsid protein (NCp7) or its Gag precursor (Li et al., 1996; Rong et al., 1998). Therefore, we also analyzed the efficiency of initiation using our various mutant templates in the presence of NCp7 (Fig. 2-7). The data reveal that the PBS/R2 template yielded only low levels of full-length DNA product under these conditions, while initiation with PBS/R1 proceeded with far greater efficiency. Reactions performed with the M184V mutation showed the same patterns, although the efficiency of DNA synthesis was further diminished in each of these cases. These findings are in good agreement with our cell culture data and suggest that mutations in the RNA template do not only affect the efficiency of DNA synthesis but also the proper placement of $tRNA^{Lys3}$.

2.6.2 Discussion

HIV resistance to the antiviral drug 3TC is attributable to the M184V amino acid substitution in RT. However, this mutation is also associated with decreased viral replicative fitness as well as multiple changes in enzymatic properties that include diminished processivity of DNA synthesis (Back et al., 1996; Boyer and Hughes, 1995; Sharma and Crumpacker, 1999), moderate increases in the fidelity of polymerization (Feng and Anderson, 1999; Hsu et al., 1997; Wainberg et al., 1996), and decreased excision of incorporated AZT- and 3TC-monophosphates from the primer terminus of newly synthesized viral DNA (Götte et al., 2000).

Fig. 2-7 (A) Cell-free synthesis of (-) ssDNA after placement of primer $tRNA^{lys3}$ onto different RNA templates by NCp7. The tRNA/viral RNA complex was pre-formed at 37°C in the presence of NCp7. After incubating this complex together with wild-type RT or M184V RT at 37°C for 5 min, reactions were initiated by the addition of dNTPs. Samples were removed after 30 and 60 min (lanes 1-2). (B) The results depict the relative levels of radioactivity of final reaction products after 60 min. The amount of radioactivity of the final product of reactions performed with PBS/WT template and WT RT was standardized as 100%.

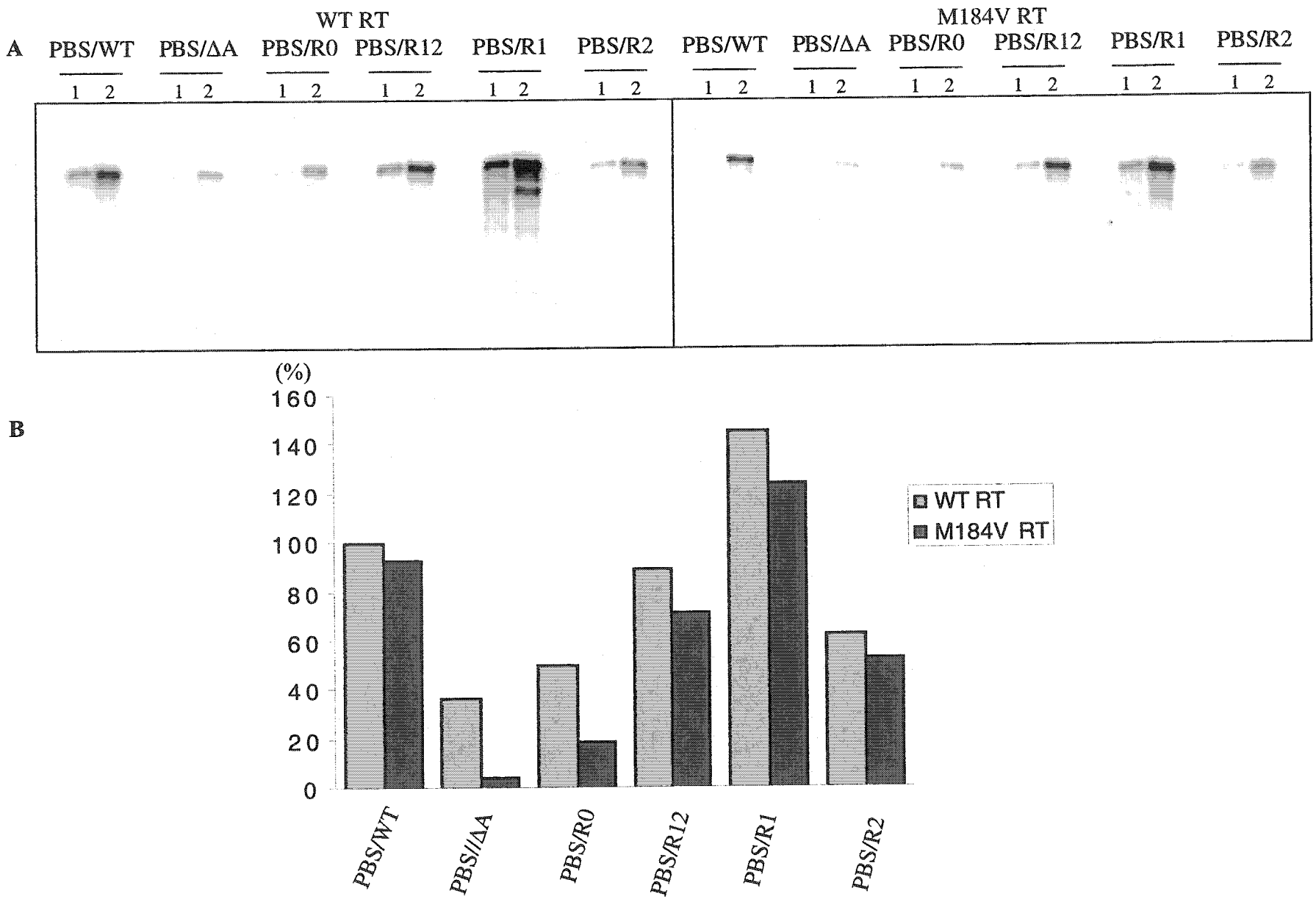

A
WT RT
M184V RT
PBS/WT
PBS/ΔA
PBS/R0
PBS/R12
PBS/R1
PBS/R2
1 2
B
(%)
160
140
120
100
80
60
40
20
0
WT RT
M184V RT
PBS/WT
PBS//ΔA
PBS/R0
PBS/R12
PBS/R1
PBS/R2

Fig. 2-7

Previous studies from our lab have shown that the M184V substitution impeded compensatory mutagenesis when present in viruses that were deleted of the A-rich loop, which is important for formation of a reverse transcription initiation complex. We hyposized that M184V might diminish the formation of this complex and have now studied the mechanisms involved using cell-free reverse transcription assays. Earlier studies had shown that the initiation reaction is a slow process, accompanied by frequent pausing of the RT enzyme at positions +3 and +5 (Isel et al., 1996; Liang et al., 1998). Thereafter, a sharp transition occurs to a faster and more processive mode of polymerization after incorporation of the sixth nucleotide (Lanchy et al., 2000; Lanchy et al., 1998; Thrall et al., 1998). In this paper, we have generated mutated RNA templates containing the A-rich loop deletion and different reversion sequences found in the long-term culture of mutated viruses. By using a cell-free six-base extension assay, we found that the M184V mutation reduced the rate of tRNA-primed DNA synthesis; notably, pausing at position +3 appeared at a later time compared with reactions performed with wild-type HIV-1 RT. In regard to the use of RNA templates deleted of the A-rich loop, we found that the rates of incorporation of the first 1 to 3 nucleotides were not significantly changed in comparison to studies performed with wild-type template; however, the probability of pausing at the +5 position was significantly increased when the A-rich loop was deleted. Delayed clearance from both pause sites was seen with both the mutant enzyme as well as the mutated RNA template. Clearly, these alterations result in reduced efficiency of initiation of synthesis of (-) ssDNA. These effects were specific for the tRNA-primed reaction, since the initiation of reverse transcription was unaffected in reactions performed with a DNA primer (data not shown). This result supports our hypothesis that the efficient use of nucleic acid substrates that show reduced affinity for wild-type RT is further compromised in the presence of the M184V mutation.

Increased pausing at the +5 position, in the context of the mutated template, is consistent with the notion that the interaction between the A-rich loop and the anticodon loop of $tRNA^{Lys3}$ may facilitate the transition from initiation to elongation after incorporation of the sixth residue (Lanchy et al., 2000). Deletion of the A-rich loop may have caused severe structural rearrangements in the binary complex between viral RNA template and $tRNA^{Lys3}$ that resulted in increased pausing at position +5, that, in turn, caused a delay in transition to the faster and more processive elongation phase. In contrast to the mutated RNA template, the M184V mutation may directly affect the interaction with the tRNA primer, which helps to explain the relatively late appearance of the pause product at position +3 in reactions performed with the mutated RT. Together, these data show that the deletion of the A-rich loop and the presence of M184V affect the initiation reaction in complementary and possibly synergistic fashion, resulting in greater impairment in synthesis of (-) ssDNA than seen with either alteration on its own.

Enzymatic and mutagenesis assays of the $tRNA^{Lys3}$/RNA complex have revealed the existence of a stem-loop structure, i.e. the U5-PBS hairpin, in which the A-rich loop is located in the center of the sequence (+163)GUGUGGAAAAUCUCU(+177) (Beerens et al., 2000b; Berkhout, 1996). Mutations that alter the stability of this hairpin structure and genetic alterations that cause severe structural changes in RNA template can inhibit virus replication (Beerens and Berkhout, 2000; Beerens et al., 2000a). We have previously shown that long-term culture of viruses containing a deletion of the A-rich loop in MT2-cells resulted in the restoration of one or two As either upstream or downstream of the deletion. Among the revertants studied, only HIV-1/R1which contains a single T→A replacement at position +173 was able to restore viral replication to nearly wild-type levels, while another single C→A substitution at position +176 in HIV/R2 resulted in further diminution of

viral replication. Thus, the positions of these various compensatory mutations can differentially impact on viral replication.

The restoration of the A at +173, located within the loop of the U5-PBS hairpin, may have helped to restore the stem-loop structure and hence to increase viral replication. The fact that position +176 is located within the stem may mean that restoration of an A at this site is not sufficient to re-form the loop structure, since, as for the deletion of the A-rich loop, the destabilization of the hairpin structure may have resulted in diminished viral replication. Interestingly, the doubly mutated HIV/R12 did not replicate as efficiently as either HIV/R1 or HIV/WT, indicating that the mutation at position +176 may play another structural and possibly functional role at other stages of viral replication. The efficiency of placement of the tRNA primer onto the PBS is a logical factor in this regard.

To address this issue, we also analyzed the efficiency of tRNA-primed DNA synthesis under physiologically relevant conditions, i.e. at 37°C, in the presence of the viral NCp7. Previous *in vitro* studies have shown that the mature viral nucleocapsid protein NCp7, as well as the $Pr55^{gag}$ precursor, can promote annealing between $tRNA^{Lys3}$ and PBS (Huang et al., 1998b; Huang et al., 1997a; Rong et al., 1998). We have now shown that levels of synthesis of (-) ssDNA, generated in the presence of NCp7, could be correlated with the efficiency of viral replication. In contrast to results obtained with heat-annealed complexes, template PBS/R2 generated less full-length (-) ssDNA than any of the other mutated templates, and the use of PBS/R1 facilitated the formation of (-) ssDNA. These data suggest that the mutation at position +176 impacted negatively on viral replication at the placement stage, and that the mutation at position +173 promoted the formation of a competent binary tRNA/RNA complex.

In summary, the results of this study suggest that the efficiency of tRNA-primed initiation of reverse transcription correlates with the efficiency of

viral replication competence. Both placement of $tRNA^{Lys3}$ onto the PBS and rates of initiation of DNA synthesis are important factors that determine the yield of (-) ssDNA. The A-rich loop plays a crucial role in these stages of reverse transcription, because it is essential for the formation of properly folded genomic RNA, and changes in viral RNA structure in close proximity to the PBS can affect tRNA placement and initiation of DNA synthesis. Synthesis of viral DNA with either mutated or wild-type template is a far less efficient process with mutated M184V-containing than wild-type enzyme. This result may be due to a diminished ability on the part of the mutated enzyme to recognize the initiation complex of tRNA and viral genomic RNA and further studies to address this topic are in progress. Furthermore, it is now of interest to study whether mutant enzymes with other amino acid substitutions, that are likewise associated with reduced viral replication fitness, may give rise to similar defects on the level of initiation of DNA synthesis. Such impaired initiation of synthesis of viral DNA was not observed with other mutated purified RTs, such as those containing substitutions at either positions E89G or K65R (results not shown).

Chapter 3

The initiation of HIV –1 reverse transcription as a target for the development of novel antiretroviral strategies

This chapter was adapted from two articles:

1. Human immunodeficiency virus type-1 reverse transcription can be inhibited by oligodeoxyribonucleotides that target the tRNA primer. *By* Xin Wei, Matthias Götte and Mark A. Wainberg, 2000, Nucleic Acids Research, 28: 3065-3074.
2. Inhibition of Human Immunodeficiency Virus type-1 reverse transcription using tRNA-like molecules that interfere with formation of the natural initiation complex. *By* Xin Wei, Elana Cherry, Balint Budai, Michael Stürzl, Mark A. Wainberg, Matthias Götte, 2002, (In preparation)

All data presented in this chapter were from experiments performed by myself under the supervision of Dr. Wainberg and Dr. Götte.

3.1 Preface

We have shown that the presence of the M184V mutation in HIV-1 reverse transcriptase compromises the ability of viruses containing a complex deletion to restore wild-type replication. We also showed that the rate of initiation of reverse transcription was severely diminished in the context of M184V mutated RT. This effect was significantly amplified when using an *in vitro* system that involved both the M184V substitution and a template that contained the deletion in the A-rich loop. Our data suggest that viral replication fitness may directly correlate with the efficiency of reverse transcription initiation, and specifically with the stability of the initiation complex.

Primer $tRNA^{Lys3}$ is selectively incorporated into the virion during its assembly where it binds to PBS with its acceptor arm. Chemical and enzymatic probing revealed that both the primer and viral RNA undergo extensive structural rearrangements upon binary complex formation (Isel et al., 1995; Isel et al., 1998; Isel et al., 1999). As a result, the tRNA/viral RNA complex fits into the nucleic acid binding cleft of RT and initiation of reverse transcription ensues (Isel et al., 1999). We have demonstrated that introducing mutations into the initiation complex may interfere with these interactions among tRNA, viral RNA and RT, which further caused diminished replication fitness. For the past decade, the emergence of HIV-1 drug-resistant strains has limited the success of current therapeutic strategies. There is an urgent need for novel antiviral approaches. Recently, gene therapy efforts including antisense nucleic acid, ribozyme, and RNA interference technology have been used to suppress multiple steps of the HIV-1 life cycle. However, such approaches requires highly conserved targets, the specificity of the HIV-1 initiation complex might make it a valuable target. Since one of the components of the initiation complex does not vary ($tRNA^{Lys3}$), the emergence of resistant strains may be limited with this approach.

3.2 Abstract

Reverse transcription of human immunodeficiency virus type-1 (HIV-1) is primed by cellular $tRNA^{Lys3}$, which is selectively packaged into viral particles where it is bound at its 3' terminus to a complementary sequence of viral RNA termed the primer binding site (PBS). Since the initiation of DNA synthesis was shown to be a highly specific process, it represents a potential target for the development of novel antiviral strategies. In this study, we have developed strategies for inhibition of the HIV-1 replication via interference with the specific binary $tRNA^{Lys3}$/RNA complex.

To target on the primer $tRNA^{Lys3}$, we employed oligodeoxyribonucleotides (ODNs) that are complementary to different parts of the tRNA primer and, therefore, may interfere with the initiation of RT-mediated DNA synthesis. The initiation of RT activity was almost completely blocked when using ODNs that interfered with intermolecular tRNA/RNA interactions that involved both the PBS and sequences outside the PBS. Both RNase H degradation of $tRNA^{Lys3}$ as well as the altered structure of the tRNA/RNA complex, due to the binding of the ODN, contributed to the inhibition of synthesis of viral DNA.

To target on the viral RNA, we devised a $tRNA^{Lys3}$-like molecule, termed $tRNA^{Lys*}$, which contained sequence alterations that will direct the initiation to a region distant from the natural PBS, designated PBS*. Since PBS* is involved in the formation of the natural tRNA/PBS complex, binding of $tRNA^{Lys*}$ is shown to interfere specifically with the initiation of reverse transcription. Cell lines that expressed $tRNA^{Lys*}$ were established in tissue culture and expression of mutant $tRNA^{Lys*}$ in a human $CD4^+$ T-cell line resulted in inhibition of HIV-1 replication. Moreover, cell free assays showed that $tRNA^{Lys*}$ can mis-direct the initiation of reverse transcription, which concomitantly initiated premature degradation of the genomic RNA by the RT-associated RNase H activity.

3.3 Introduction

A key step in the life cycle of all retroviruses is the conversion of single-stranded genomic RNA into double-stranded DNA. This process, termed reverse transcription, requires the virus-encoded reverse transcriptase (RT), a multifunctional enzyme that possesses DNA- and RNA-dependent polymerase activities as well as a ribonuclease H (RNase H) activity that specifically degrades the viral RNA template (Telesnitsky and Goff, 1997). As with other retroviruses, HIV-1 recruits a cellular tRNA primer in order to initiate DNA synthesis (Götte et al., 1999; Mak and Kleiman, 1997; Marquet et al., 1995).

Due to its central role in HIV-replication, HIV-1 RT is an important target for anti-retroviral drugs. Nucleoside analogue RT-inhibitors such as 3'-azido-3'-dideoxythymidine (AZT) and 2', 3'-dideoxy-3'-thiacytidine (3TC), as well as non-nucleoside RT-inhibitors, such as nevirapine, are used in the treatment of HIV disease (Emini and Fan, 1997). Although combinations of currently available drugs can often reduce viral burden to below the limit of detection (Fischl, 1999), these compounds cannot prevent the emergence of mutations in the RT or protease (PR) genes that confer drug resistance (Gunthard et al., 1998). Therefore, there exists an ongoing search for alternative strategies, that include gene therapy approaches based on antisense nucleic acids, decoy RNAs and ribozymes (Biasolo et al., 1996; El Dirani-Diab et al., 1997; Jendis et al., 1998; Jendis et al., 1996; Lee et al., 1998; Lee et al., 1995; Lisziewicz et al., 1993; Lisziewicz et al., 1994; Lu et al., 1997; Medina and Joshi, 1999). To avoid the problem of viral mutability and resistance, the targets of such approaches should include highly conserved sequences. The HIV-1 specific initiation complex fulfills this criterion, since it involves interactions among viral genomic RNA and a cellular component, i.e. primer tRNA.

Primer $tRNA^{Lys3}$ is incorporated into the virion during its assembly and is most likely through interaction with the Gag-Pol polyprotein (Mak et al.,

1994). Several studies indicate that RT sequences within Gag-Pol are involved in selective packaging of tRNA (Khorchid et al., 2000; Mak et al., 1994; Mak et al., 1997). RT deletion mutants and virus-like particles containing only Gag were unable to selective incorporate $tRNA^{Lys3}$ (Mak et al., 1994). The interaction between $tRNA^{Lys3}$ and HIV-1 reverse transcriptase is complex. *In vitro* studies using nuclease footprinting or cross-linking have demonstrated that RT contact the anticodon loop, D-loop and TΨC loop of $tRNA^{Lys3}$ (Mishima and Steitz, 1995). By introducing mutations into TΨC loop and D loop of $tRNA^{Lys3}$ (Oude Essink et al., 1995), Odue Essink *et al.* have further demonstrated that RT recognizes the central domain of the tRNA tertiary structure, which is formed by interaction of the D and TΨC loop (Oude Essink et al., 1995). Actually, there are three major $tRNA^{Lys}$ isoacceptors in mammalian cells. $tRNA^{Lys1}$ and $tRNA^{Lys2}$ variant which have 15 and 13 base changes compared with $tRNA^{Lys3}$, represent two $tRNA^{Lys}$ isoacceptors differing by 1bp in the anticodon stem due to their sequence and structural similarities (Mak et al., 1994). In HIV-1 produced from COS-7 cells transfected with HIV-1 proviral DNA, both primer $tRNA^{Lys3}$ and $tRNA^{Lys1,2}$ are selectively packaged into HIV-1 with equal efficiency (Huang et al., 1994b).

The initiation of reverse transcription requires specific interactions between HIV-1 RNA, primer $tRNA^{Lys3}$. The RT enzyme recognizes this binary tRNA/RNA complex and initiation of reverse transcription ensues. *In vitro* studies have shown that the mature viral nucleocapsid protein as well as the Gag precursor can promote annealing between $tRNA^{Lys3}$ and the PBS (Barat et al., 1989; De Rocquigny et al., 1992; Feng et al., 1999). However, PBS complementarity is not the only factor that determines specific $tRNA^{Lys3}$ primer usage. Additional interactions between genomic RNA and primer tRNA were found outside of the PBS (Isel et al., 1995; Isel et al., 1993). Chemical and enzymatic probing revealed complex interactions between the anticodon loop, the 3' portion of the anticodon

stem and part of the variable loop of $tRNA^{Lys3}$ with viral sequences located upstream from the PBS (Isel et al., 1995). For example, a 5' USUU 3' sequence in the anticodon loop of $tRNA^{Lys3}$ may be able to interact with an A-rich loop in the U5 region of viral RNA, located about 15nt upstream of the PBS. Deletion of the A-rich loop results in viruses with diminished levels of infectivity and reduced DNA synthesis, and this loop is restored at different sites upon long-term cell culture (Liang et al., 1997a). Further studies from other groups have also demonstrated that HIV-1 can stably use other cellular tRNA as primer, provided that PBS and A rich loop are simultaneously altered to provide complementarity to the 3' end as well as to the anticodon loops of these tRNA species (Kang and Morrow, 1999; Kang et al., 1997; Kang et al., 1999; Wakefield et al., 1996; Zhang et al., 1996; Zhang et al., 1998b; Zhang et al., 1998c). Both the primer and viral RNA undergo extensive structural rearrangements upon binary complex formation. In the viral RNA, structure changes occur upto 69 nucleotides upstream and 72 nucleotides downstream of the PBS. As a result, the tRNA/viral RNA complex fits perfectly into the nucleic acid binding cleft of RT (Isel et al., 1995; Isel et al., 1999). The specificity of the interaction of reverse transcription therefore directly linked to virus specific interactions between tRNA and genomic RNA and introduced mutations in this complex may interfere with these interactions. Therefore, it might represent a valuable target for antiviral strategies.

In this study, we first employed oligodeoxyribonucleotides (ODNs) that are complementary to $tRNA^{Lys3}$ to interfere with formation of the specific binary tRNA/RNA complex as a strategy to inhibit synthesis of (-) strand ssDNA. We have demonstrated in cell-free assays that efficient inhibition of initiation of reverse transcription requires ODNs that destabilize interactions between $tRNA^{Lys3}$ and the PBS as well as the extended contact between primer tRNA and viral RNA. Next, we devised a $tRNA^{Lys}$-like molecule, termed $tRNA^{Lys*}$, which shares structural features with human

$tRNA^{Lys3}$ and retains high-affinity binding to HIV-1 reverse transcriptase and viral encapsidation. Unlike natural $tRNA^{lys3}$, the mutant tRNA binds with its altered 3' terminus to a conserved sequence of the viral RNA located about 36 nucleotides upstream from natural PBS, designated PBS*, which is implicated in the formation of the natural binary $tRNA^{Lys3}$/viral RNA complex. The expression of $tRNA^{Lys*}$ in human T-cells results in inhibition of HIV-1 replication. Cell free assay showed $tRNA^{Lys*}$ inhibit viral replication specifically by interfering with the natural initiation process.

3.4 Materials and methods

Chemicals, nucleic acids and enzymes: All chemicals are purchased from Bioshop Canada Inc. Restriction enzymes are from Gibco Product Inc. (Mississauga, Ontario, Canada). Heterodimeric HIV-1 RT (p66/p51) was prepared and purified as previously described (Le Grice et al., 1995). Nucleocapsid protein (NCp7) was generously provided by Dr. Bernard Roques and was prepared by peptide synthesis as described previously (de Rocquigny et al., 1991). Natural $tRNA^{Lys3}$ was purchased from Bio S&T (Montreal, Quebec, Canada).

All ODNs used in this study were chemically synthesized and purchased from Gibco BRL (Canada). These ODNs are complementary to different segments of human $tRNA^{Lys3}$, including the 3' terminus, the TΨC stem-loop, and the variable loop. Sequences of antisense ODNs used are listed below; numbering refers to base positions of the tRNA to which the ODN shows complementarity and position 76 represents the 3' terminal residue of $tRNA^{Lys3}$ (Fig. 3-1):

ODN59-76: TGG CGC CCG AAC AGG GAC

ODN41-58: TTG AAC CCT GGA CCC TCA

ODN57-76: TGG CGC CCG AAC AGG GAC TT

ODN55-76: TGG CGC CCG AAC AGG GAC TTG A

ODN53-76: TGG CGC CCG AAC AGG GAC TTG AAC

ODN47-76: TGG CGC CCG AAC AGG GAC TTG AAC CCT GGA

ODN41-76: TGG CGC CCG AAC AGG GAC TTG AAC CCT GGA CCC TCA

Synthetic $tRNA^{Lys3}$ was synthesized *in vitro* using the plasmid pT7hLys3 as template. This construct contains the 76bp-fragment that represents mature human $tRNA^{Lys3}$ under control of the T7 RNA polymerase promoter. A *Fok* I restriction site was introduced downstream of the tRNA gene to generate a DNA template that ensures run-off transcription of the tRNA with the correct 3' CCA terminus (Liu and Horowitz, 1993). *In vitro* transcription with T7 RNA polymerase was performed overnight at 37°C using 5 µg of the digested plasmid which was incubated in a total volume of 100 µl containing 40 mM Tris-HCL (PH 8.0), 25 mM $MgCl_2$, 50 mM NaCl, 1 mM spermidine, 50 mM DTT, 4 mM NTPs (each), 200U of T7 polymerase (MBI, Fermentas) and 20U of the placental RNase inhibitor RNasin (Pharmacia). The tRNA reaction product was purified using 8% polyacrylamide gels that contained 7 M urea and 50 mM Tris-borate EDTA (TBE), and was visualized under UV light, prior to elution from isolated gel slices using a solution of 0.5 M sodium acetate and 0.01 % SDS. Radiolabeled tRNA was prepared using the same procedure, except that 4 mM UTP was replaced with a mixture of 50 µM UTP and 2.5µ Ci/µl [α-^{32}P] UTP. For mutant tRNAs, after digestion of the plasmid pT7hLys3 with *Pst* I and *Eco*R I, the human $tRNA^{Lys3}$ sequence was replaced by mutant tRNAs' DNA containing different mutations at the 5' terminus within the 76bp fragment (Fig. 3-9). Mutated tRNAs, $tRNA^{Lys*}$, $tRNA^{Lys*-15}$, $tRNA^{Lys*-12}$ and $tRNA^{Lys*-9}$, were prepared in a same fashion as synthetic $tRNA^{Lys3}$, after digestion of the plasmid with *Fok* I. An RNA template containing the 5' terminal sequence of genomic RNA, including the PBS, was prepared in similar fashion, after digestion of the plasmid pHIV-PBS with *Bss*H II (Arts et al., 1998).

Band shift assays: The ability of different antisense ODNs to bind to $tRNA^{Lys3}$ was analyzed under native conditions using 5% polyacrylamide

gels. A constant amount of $tRNA^{Lys3}$ (80 nM) was incubated with various concentrations of the ODN tested in a buffer containing 50 mM Tris-HCl (pH 7.8) and 50 mM NaCl. Reactions were allowed to proceed for 30 minutes at 37°C. Divalent cation has been shown to be able to stablize tRNA structure, to test the influence of divalent cations on the affinity between the tRNA and ODNs, this assay was also performed in the presence of 6 mM $MgCl_2$. Products were analyzed on 5% polyacrylamide gels containing 50 mM TBE or, alternatively, 50 mM TB and 500 μM $MgCl_2$ to assay ODN-tRNA interactions in the presence of the divalent cations. Results were analyzed by molecular image analysis. The ability of ODNs to bind to $tRNA^{Lys3}$ in the presence of the RNA template was analyzed following the same procedure.

Inhibition of synthesis of (-) ssDNA using ODNs: We used a cell-free assay to study the effects of different ODNs on the efficiency of synthesis of (-) ssDNA. Reverse transcription when initiated from the PBS of the RNA template normally yields a DNA product of 178nt that is joined to the 76nt $tRNA^{Lys3}$ primer. The yield of this (-) ssDNA product is significantly increased when the tRNA primer is heat-annealed to the RNA template prior to initiation of DNA synthesis. This procedure ensures complete hybridization and was performed in a 10 μl reaction mixture containing 50 mM Tris-HCl (pH7.8), 50 mM NaCl, 80 nM $tRNA^{Lys3}$, and 160 nM template RNA. This mixture was incubated for 2 min at 95°C followed by 20 min at 70°C. Synthesis of (-) ssDNA synthesis was initiated by addition of 240 nM RT in the presence or absence of 400 nM ODN, also in the presence of 6 mM $MgCl_2$ and 200 μM dNTPs (each). Aliquots of 2 μl were removed at different time points, and reactions were terminated in 8 μl of a solution containing 80% formamide and 40 mM EDTA. As indicated in the figure legends, reactions were monitored using radiolabeled $tRNA^{Lys3}$ or, alternatively, by including α^{32}P-dGTP in the reaction mixture. The latter method allowed us to study the inhibition of

DNA synthesis in the presence of the different ODNs, and, as well, the usage of ODNs as primers for the reverse transcription of the tRNA. In contrast, usage of internally labeled $tRNA^{Lys3}$ yielded information regarding the efficiency of RNase H mediated degradation of the tRNA primer. Products were separated on 8% polyacrylamide 7M-urea gels and analyzed as described above.

Synthesis of (-) DNA from mutant tRNAs and RNase H degradation assay: Cell free RT assay was used to study the effect of mutant tRNAs on the initiation of synthesis of (-) ssDNA. Heat annealing ensures complete hybridization of tRNA primer to RNA template and was performed in a 10 µl reaction mixture containing 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 80 nM *in vitro* synthesized $tRNA^{Lys3}$ or mutant tRNAs and 160 nM template RNA. This mixture was incubated for 2 min at 95°C followed by 20 min at 70°C and 20 min at 37°C. After incubated for 5 min at 37°C in the presence of 240 nM RT and 6 mM $MgCl_2$, synthesis of (-) DNA was initiated by addition of 10 µM dNTPs. Aliquots of 2 µl were removed at different time points, and reactions were terminated in 8 µl of stopping solution containing 80% formamide and 40 mM EDTA. Reaction was monitored by incorporating [α-^{32}P] dCTP into growing DNA chain and this allows us to study initiation sites of different tRNA primers. In contrast, for RNaseH degradation assay, the usage of 3' [α-^{32}P] pCp radiolabeld RNA template as monitor yielded information regarding the first RNaseH-mediated cutting site of different tRNAs. Products were separated on 8% polyacrylamide-7 M Urea gels and later analyzed using Molecular Analysis software.

To study the effects of $tRNA^{Lys*}$ on the efficiency of synthesis of (-) DNA primed by natural $tRNA^{Lys3}$, we performed another cell free RT assay in which a constant amount, 40 nM, of $tRNA^{Lys3}$ and increasing concentration of $tRNA^{Lys*}$ were simultaneously heat annealed onto RNA template. The rest of reaction was preceded in the same way as mentioned above.

Placement of $tRNA^{Lys*}$ onto $tRNA^{Lys3}$/RNA template complex by NCp7: The ability of mutant tRNA, $tRNA^{Lys*}$, to bind to $tRNA^{Lys3}$/viral RNA binary complex in the presence of nucleocapsid protein (NCp7) was demonstrated by band-shift assay. 80 nM $tRNA^{Lys3}$ and 160 nM RNA template were pre-hybridized by heat annealing as described previously. NCp7 was used to anneal $tRNA^{Lys*}$ to $tRNA^{Lys3}$/viral RNA binary complex. A ratio of 6 nucleotides to 1 molecule of NCp7 was used in order to obtain the maximal annealing activity (Li et al., 1996). The pre-hybridized binary complex was incubated with various concentrations of tRNALys* and NCp7 in a buffer containing 50 mM NaCl and 50 mM Tris-HCl (pH7.8) for 1 hr at 37°C. Products were analyzed on 5% polyacrylamide natural gels. Results were analyzed by molecular image analysis.
Cell free RT assay with NCp7 were also performed in which 40 nM $tRNA^{Lys3}$, 80 nM 497nt RNA template, 4 μM NCp7 and increasing concentration of $tRNA^{Lys*}$ were incubated together in a buffer containing 50 mM Tris-HCl (pH7.8), 50 mM NaCl, 5 mM $MgCl_2$ and 10 mM dithiothreitol at 37°C for 1 hr. After addition of 120 nM of RT, the reaction mixture was incubated at 37°C for another 5 min. The (-) DNA synthesis was initiated and monitored as mentioned above. After 30 min reaction, the product was treated with 200 μg of proteinase K pre ml at 37°C for 30 min and then extracted with phenol-chloroform. After precipitated with 4× volume of 95% ethanol, the products were separated on the 8% polyacrylamide natural gel as described above.

Construction of plasmid for *in vivo* tRNA expression: The plasmid for *in vivo* $tRNA^{Lys3}$ expression, pNeo-Lys3, which contains human $tRNA^{Lys3}$ expression DNA sequence under control of the eucaryotic RNA *pol* III promoter, is provided by Dr. Mattias Götte. The construct for mutant tRNA *in vivo* expression pNeo-Lys* were obtained by replacing a *Sal* I digested fragment within pNeo-Lys3 with a $tRNA^{Lys*}$ DNA sequence.

Construction of constitutively tRNA expression cell line: Jurkat and COS -7 cells were maintained in RPMI 1640 and Dulbecco's modified Eagle's medium (Gibco BRL, Montreal, Quebec, Canada), respectively, each medium being supplemented with 10% heat- inactivated fetal calf serum, 2 mM L-glutamine, 100U/ml penicillin and 100 µg/ml streptomycin.

The plasmid for *in vivo* tRNA expression, pNeo-Lys3, pNeo-Lys* and pNeo were transfected into the Jurkat cell lines by electroporation at settings of 0.2K Voltage and 960 MicroFarad. Transfected cells were selected in 0.8 mg/ml G418 and subcloned by limiting dilution in 96-well plates, to yield J/Lys3, J/Lys* and J/pNEO clones.

PCR-assay for tRNA expression: Total RNA was extracted from tRNA expression J/Lys3, J/Lys*, and J/pNEO using RNeasy mini Kit (Qiagen, Ontario, Canada) and were [α-^{32}P] pCp radiolabeled. Products were separated on 8% polyacrylamide-7M urea gels and visualized by autoradiography using Kodak film. Fragment corresponding to the endogenous total tRNAs was excised and eluted from gel slices in a buffer containing 0.5M ammonium acetate (pH6.2) and 0.1% SDS. Total tRNAs products were used as templates for direct PCR identification of tRNA expression. Each PCR reaction was conducted in a final volume of 50 µl containing 1.25 unit of *Taq* DNA polymerase and a total of 1.2 µM of $tRNA^{Lys3}$ specific primers (Plys3-sense, 5'-GCC CGG ATA GCT CAG TCG GT-3'; and Plys3-antisense, 5'-TGG CGC CCG AAC AGG GAC TT-3') or 1.2 µM of $tRNA^{Lys*}$ specific primers (Plys*-sense, 5'-GAC TGG ATA GCT CAG TCG GT-3'; and Plys*-antisense, 5'- TGG CAA CTA GAG ATC CCT TT-3'). For each case, [γ-^{32}P] ATP radiolabeled antisense primer was used to monitor PCR product. 20 µl of the amplified PCR product was subjected to electrophoresis in a 5% polyacrylamide natural Gel and analyzed as described above.

Transfection and infection assays: Virus stocks were prepared by transfecting COS-7 cells with HIV wild-type DNA strain HXB-2D (HIV/HXB-2D) through use of Lipofectamine. Progeny viruses were collected at 48 hrs after transfection and clarified on a Beckman GS-6R bench centrifuge at 3,000 rpm for 30 min at 4°C. The amount of virus was determined by measuring the p24 antigen (Ag) level using an enzyme-linked immunosorption assay (Abbott Laboratories, Abbott Park, Ill, and U.S.A.).

The effect of endogenous $tRNA^{Lys*}$ expression on virus replication was examined by the infection of J/Lys3, J/Lys* and J/pNeo cells. An amount of virus equivalent to 5 ng of capsid protein (CA) p24 Ag was used to infect 0.5×10^6 cells in 2 ml of RPMI medium. Cells were washed twice at 2 hrs after infection and cultured in 10 ml of complete RPMI 1640 medium containing 0.8 mg/ml G418. Culture fluid was collected at various times, and RT activity was measured to monitor viral replication. For long-term infection assay, viruses were collected when RT activity reached the maximal value and same amount viruses were used to infect fresh cells as described above.

Amplification and sequence analysis of breakthrough viruses

Infected cell DNA was extracted using Qiagen Tissue Kit (Qiagen, Ontario, Canada) and resuspended in 20 µl double-distilled water and 1 µl was use for PCR amplification under the following conditions: 94°C for 1 min, 60°C for 90 s, and 72°C for 1 min. The primers employed were p15 [(+15) 5'-GAC CAG ATC TGA GCC TGG GA-3' (+34)] and p338 [(+338) 5'-CAC CCA TCT CTC TCC TTC TA-3' (+320)]. A 326-bp product was generated and inserted into PCR2.1-TOPO TA Cloning Vector (Invitrogen, Carlsbad, CA, USA). After transformation, positive clones were picked using white or light blue selection. Plasmid was extracted sequenced using primer p15.

3.5 Human immunodeficiency virus type-1 reverse transcription can be inhibited by oligodeoxyribonucleotides that target the tRNA primer

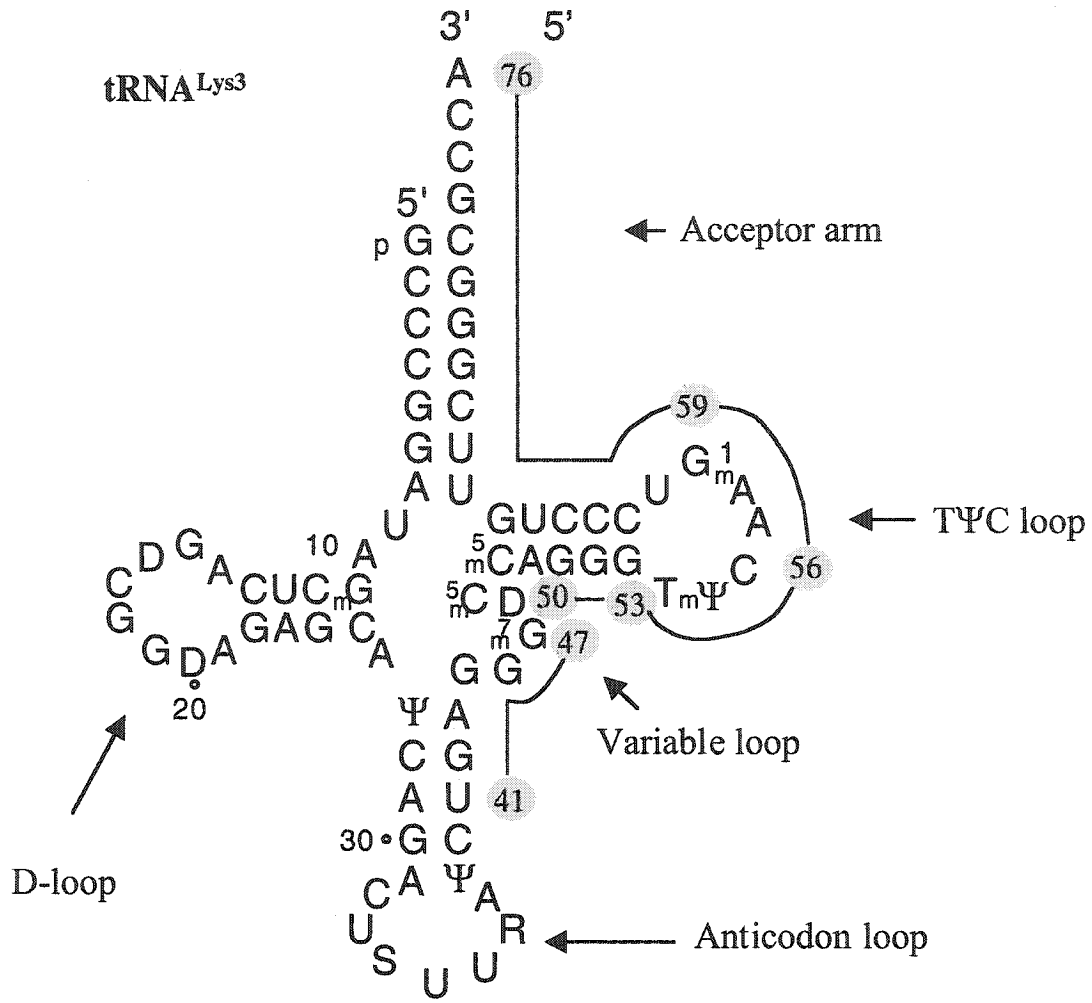
3.5.1 Results

The aim of this study was to identify ODNs that could bind efficiently to the $tRNA^{Lys3}$ primer in order to antagonize reverse transcription. Initially, two ODNs were employed, ODN56-76 that is complementary to the 3'end of $tRNA^{Lys3}$ and ODN41-58 that is complementary to the TΨC- and the variable loop (Fig. 3-1). While the first ODN may interfere with the interaction with the PBS, the latter may be required to destabilize intermolecular contacts outside the PBS. These ODNs were tested, both individually and in combination, for their ability to bind to $tRNA^{Lys3}$ and to inhibit synthesis of (-) ssDNA.

Efficient binding to $tRNA^{Lys3}$ requires ODN sequences that are complementary to $tRNA^{Lys3}$ nucleotide sequences involved in tertiary interactions

To evaluate the affinities of ODN59-76 and ODN41-58 for $tRNA^{Lys3}$, we first performed band-shift experiments in which a constant amount of free $tRNA^{Lys3}$ was incubated at 37°C with increasing concentrations of ODN. The isolated tRNA and complexes consisting of both $tRNA^{Lys3}$ and ODN were then separated on native gels. The results of Fig. 3-2 show that ODN41-58 possessed a higher affinity for $tRNA^{Lys3}$ than did ODN59-76 (Fig. 3-2A and B). A 2-fold molar excess of ODN41-58 over tRNA was required to obtain 50% complex formation, while the same level of band-shifting with ODN59-76 required an 8-fold excess. The simultaneous presence of both ODNs did not further improve the efficiency of complex formation between tRNA and ODN complexes (Fig. 3-2C and D).
These data suggest that destabilization of crucial tertiary structures within $tRNA^{Lys3}$ is necessary to obtain a stable ODN/tRNA complex under conditions that do not involve heat-annealing of the various reaction components. As a consequence, we reasoned that factors that stabilize the L-shaped structure of tRNA, such as divalent cations, might inhibit binding of the ODN. Fig. 3-3 shows that this was indeed the case and that

Fig. 3-1 Secondary structure of human $tRNA^{Lys3}$. Bases that involve tertiary interactions are highlighted in bold. The antisense ODNs 59-76 and 41-58 are complementary to the acceptor arm and TψC-loop of $tRNA^{Lys3}$, respectively, as indicated.



ODN59~76:	**3'-TGG CGC CCG AAC AGG GAC-5';**
ODN56~76:	**TGG CGC CCG AAC AGG GAC TT;**
ODN53~76:	**TGG CGC CCG AAC AGG GAC TTG A;**
ODN50~76:	**TGG CGC CCG AAC AGG GAC TTG AAC;**
ODN47~76:	**TGG CGC CCG AAC AGG GAC TTG AAC CCT GGA;**
ODN41~76:	**TGG CGC CCG AAC AGG GAC TTG AAC CCT GGA CCC TCA.**

Fig. 3-1

Fig. 3-2 Binding of ODNs to stRNA^{Lys3} in the absence of Mg^{2+}. Lanes 1-6 represent experiments performed in the absence of Mg^{2+}, with a constant concentration of tRNA^{Lys3} and increasing amounts of ODN, i.e. molar ODN/tRNA ratios 0, 1/2, 1/1, 2/1, 4/1, 8/1. (A) Binding of ODN59-76 to tRNA^{Lys3}. (B) Binding of ODN41-58 to tRNA^{Lys3}. (C) Binding of both ODN59-76 and ODN41-58 to tRNA^{Lys3}. (D) The graph summarizes the data shown in A, B and C.

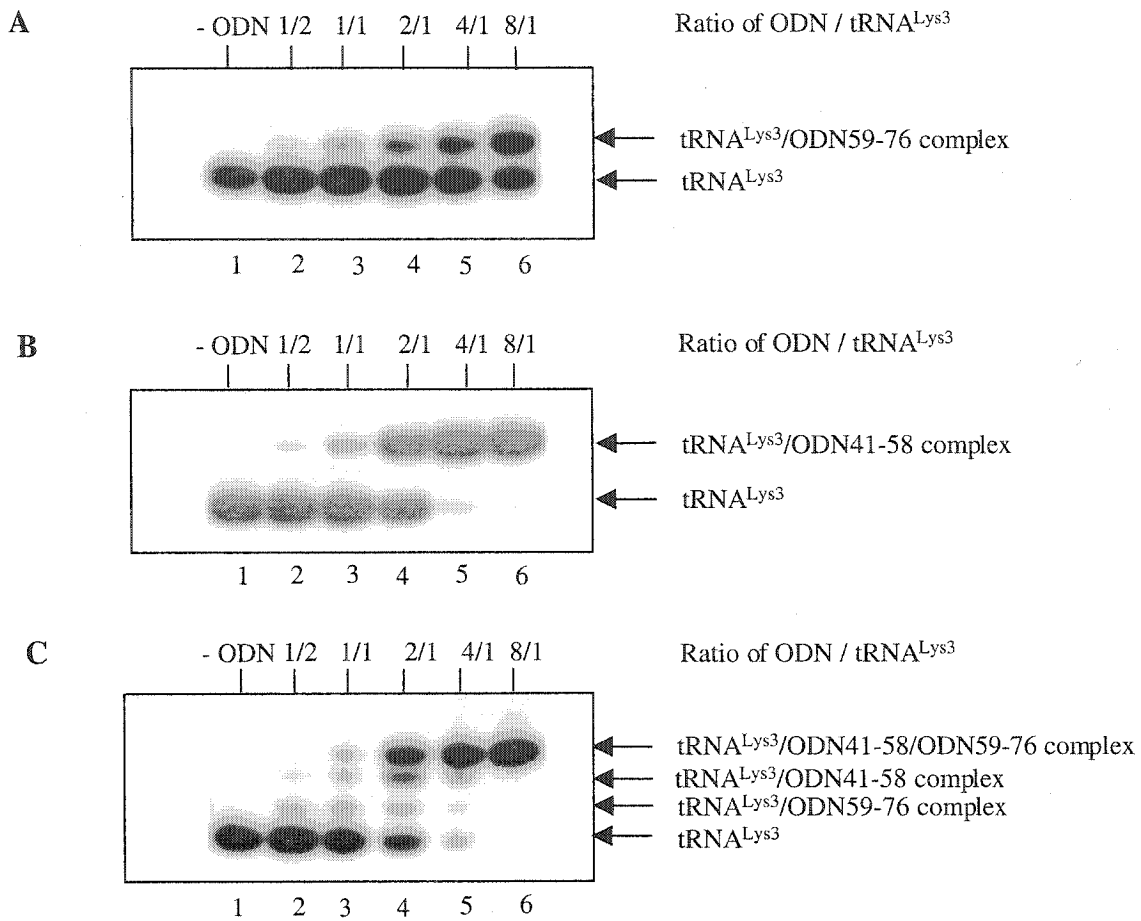
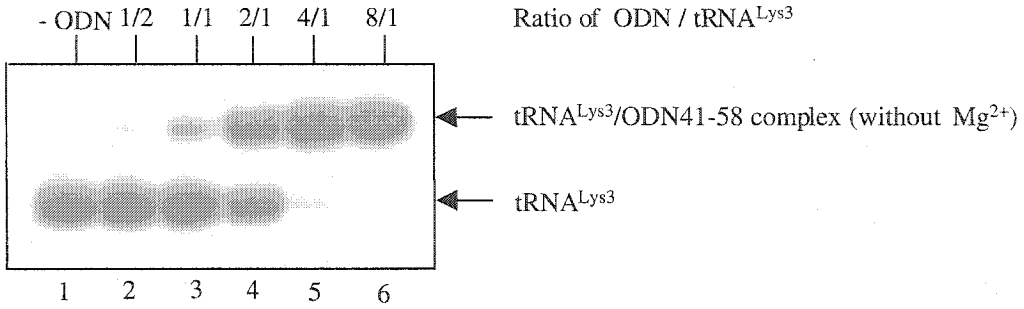

A
- ODN 1/2 1/1 2/1 4/1 8/1
Ratio of ODN / tRNALys3
tRNALys3/ODN59-76 complex
tRNALys3
1 2 3 4 5 6
B
- ODN 1/2 1/1 2/1 4/1 8/1
Ratio of ODN / tRNALys3
tRNALys3/ODN41-58 complex
tRNALys3
1 2 3 4 5 6
C
- ODN 1/2 1/1 2/1 4/1 8/1
Ratio of ODN / tRNALys3
tRNALys3/ODN41-58/ODN59-76 complex
tRNALys3/ODN41-58 complex
tRNALys3/ODN59-76 complex
tRNALys3
1 2 3 4 5 6
D
(%)
100
75
50
25
0
0.00
0.25
0.50
0.75
(μM)
tRNALys3 bound to ODNs/free tRNALys3 (%)
Concentration of ODNs
ODN59-76
ODN41-58
ODN59-76/ODN41-58

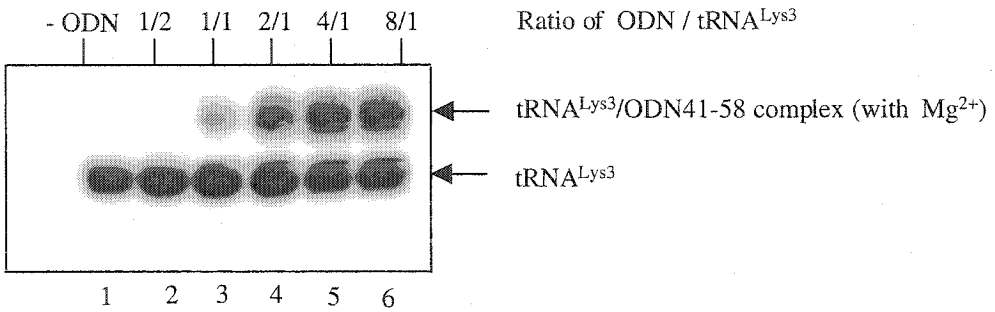
Fig. 3-2

Fig. 3-3 Effect of $MgCl_2$ on the affinity of ODNs for $stRNA^{Lys3}$. This experiment was performed as described in the legend to Fig. 3-2. Binding of ODN41-58 to $tRNA^{Lys3}$ in the absence (A) or presence (B) of Mg^{2+}. (C) The graph shows differences in the affinity of ODN41-58 for $tRNA^{Lys3}$ in the presence and absence of $MgCl_2$.

A


- ODN 1/2 1/1 2/1 4/1 8/1
Ratio of ODN / tRNA^{Lys3}
tRNA^{Lys3}/ODN41-58 complex (without Mg^{2+})
tRNA^{Lys3}
1 2 3 4 5 6

B


- ODN 1/2 1/1 2/1 4/1 8/1
Ratio of ODN / tRNA^{Lys3}
tRNA^{Lys3}/ODN41-58 complex (with Mg^{2+})
tRNA^{Lys3}
1 2 3 4 5 6

C

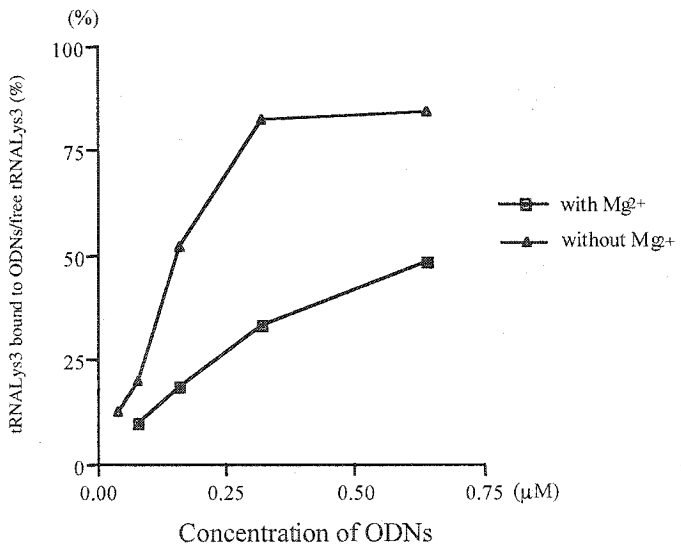

(%)
100
75
50
25
0
tRNALys3 bound to ODNs/free tRNALys3 (%)
0.00
0.25
0.50
0.75 (μM)
Concentration of ODNs
with Mg2+
without Mg+

Fig. 3-3

formation of a stable tRNA/ODN complex was compromised by approximately 3-fold in the presence of 6 mM Mg^{2+}.

$tRNA^{Lys3}$ bound to ODN is cleaved by RT-associated RNase H activity

To test whether ODN41-58 might also target $tRNA^{Lys3}$ as a component of the binary tRNA/PBS complex, band-shift assays were performed in which this ODN was added to a mixture of $tRNA^{Lys3}$ and an RNA template, containing the PBS. Fig. 3-4 demonstrates the existence of a ternary tRNA/PBS/ODN complex that was formed under native conditions at 37°C (compare lanes 3 and 4). Hence, under these circumstances, the initiation of DNA synthesis is expected to be severely compromised. Conceivably, binding between ODN41-58 and $tRNA^{Lys3}$ might provoke structural changes within the tRNA/RNA complex that may interfere with this initiation process. In addition, RT-associated RNase H domain may recognize the tRNA/ODN complex as an RNA/DNA substrate and initiate degradation of the tRNA. This activity can be used in a functional assay to further elucidate the efficiency of binding of ODNs to $tRNA^{Lys3}$.

RNase H degradation was analyzed in the presence of either ODN59-76 or ODNA41-58 in time-course experiments. The ODN was added under native conditions to the pre-formed binary complex in which the tRNA was quantitatively hybridized with the RNA template (see Materials and Methods). Heat-annealing of $tRNA^{Lys3}$ and the RNA template was performed in the absence, as well as in the presence of $MgCl_2$ to study the effects of divalent metal ions on the formation of the ternary tRNA/RNA/ODN complex. Fig. 3-5 shows that ODN59-76 was unable to initiate RNase H degradation of $tRNA^{Lys3}$, under conditions in which the tRNA/RNA complex was pre-formed in the presence of Mg^{2+} (Fig. 3-5A). Cleavages with considerable efficiency were seen only when $MgCl_2$ was omitted from the pre-incubation mixture and when these divalent metal ions were added to start the reaction (Fig. 3-5B). Thus, ODN58-76 can compete with the PBS of genomic RNA in the absence but not presence of

Fig. 3-4 Gel retardation with $stRNA^{Lys3}$, RNA template and ODN41-58. Lane 1: $tRNA^{Lys3}$ alone; Lane 2: formation of complex of $tRNA^{Lys3}$ /ODN41-58 with molar ratios of 1/5; Lane 3: formation of binary complex of $tRNA^{Lys3}$ and viral RNA template with molar ratios of 1/2; Lane 4: formation of ternary $tRNA^{Lys3}$/viral RNA/ODN41-58 complex with molar ratios of 1/2/5. This experiment was performed in the presence of Mg^{2+} (0.5mM) and the components of this reaction were not heat-annealed.

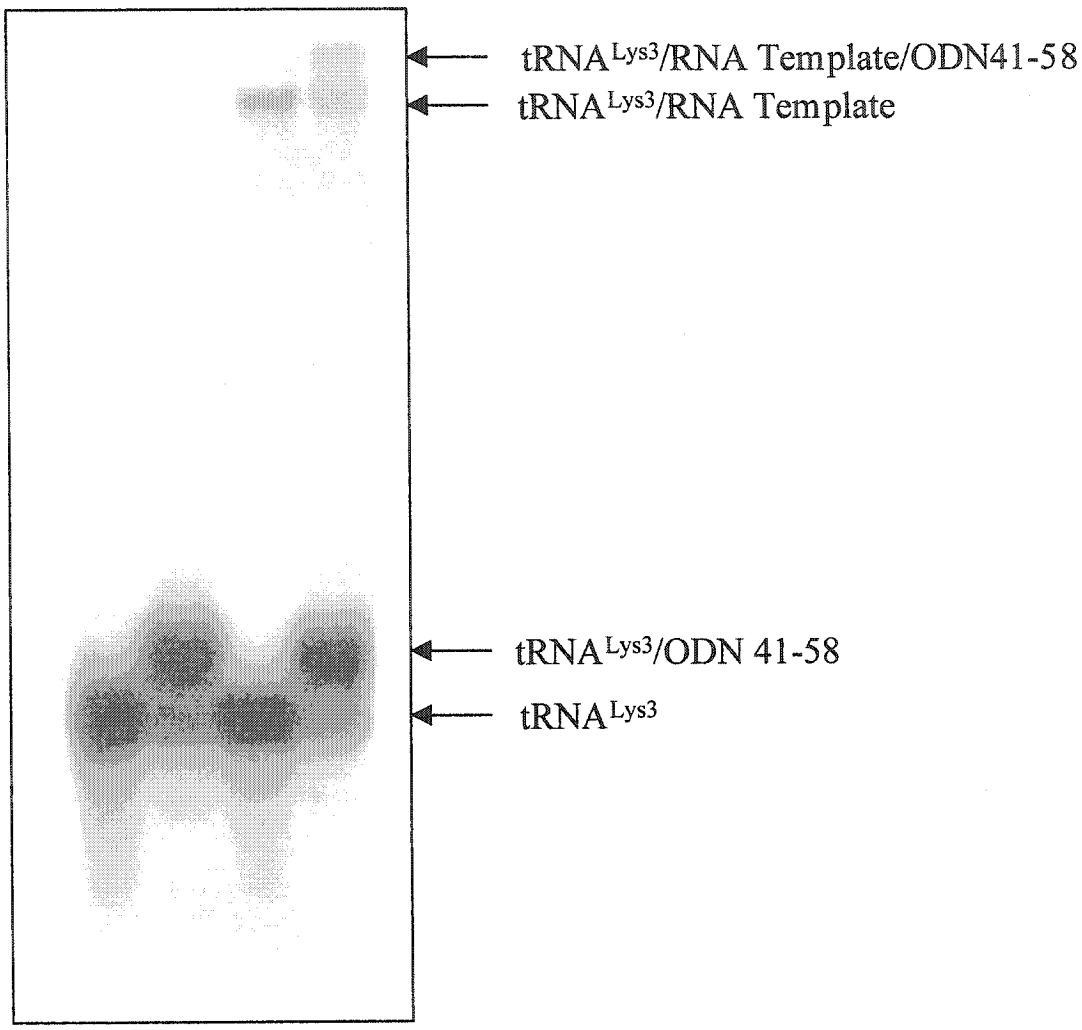

tRNA^{Lys3}/RNA Template/ODN41-58
tRNA^{Lys3}/RNA Template
tRNA^{Lys3}/ODN 41-58
tRNA^{Lys3}

Fig. 3-4

Fig. 3-5 Degradation of ODN-bound stRNA^{Lys3} by RT-associated RNase H activity. (A) A time-course experiment was performed by pre-forming the tRNA^{Lys3}/RNA complex (Materials and Methods), and incubating this substrate with ODNs at 37°C in the presence of Mg^{2+}. Reactions were initiated by the addition of RT. Samples were removed after 0, 1, 5, 10, 30, 60 minutes. (B) In this experiment, the tRNA/RNA complex was preformed in the absence of Mg^{2+} and incubated with ODNs and RT at 37°C. Mg^{2+} (6mM) was added to initiate the reaction. Each panel shows a time course of RNase H degradation, monitored with [α-32P]-UTP incorporated tRNA^{Lys3}.

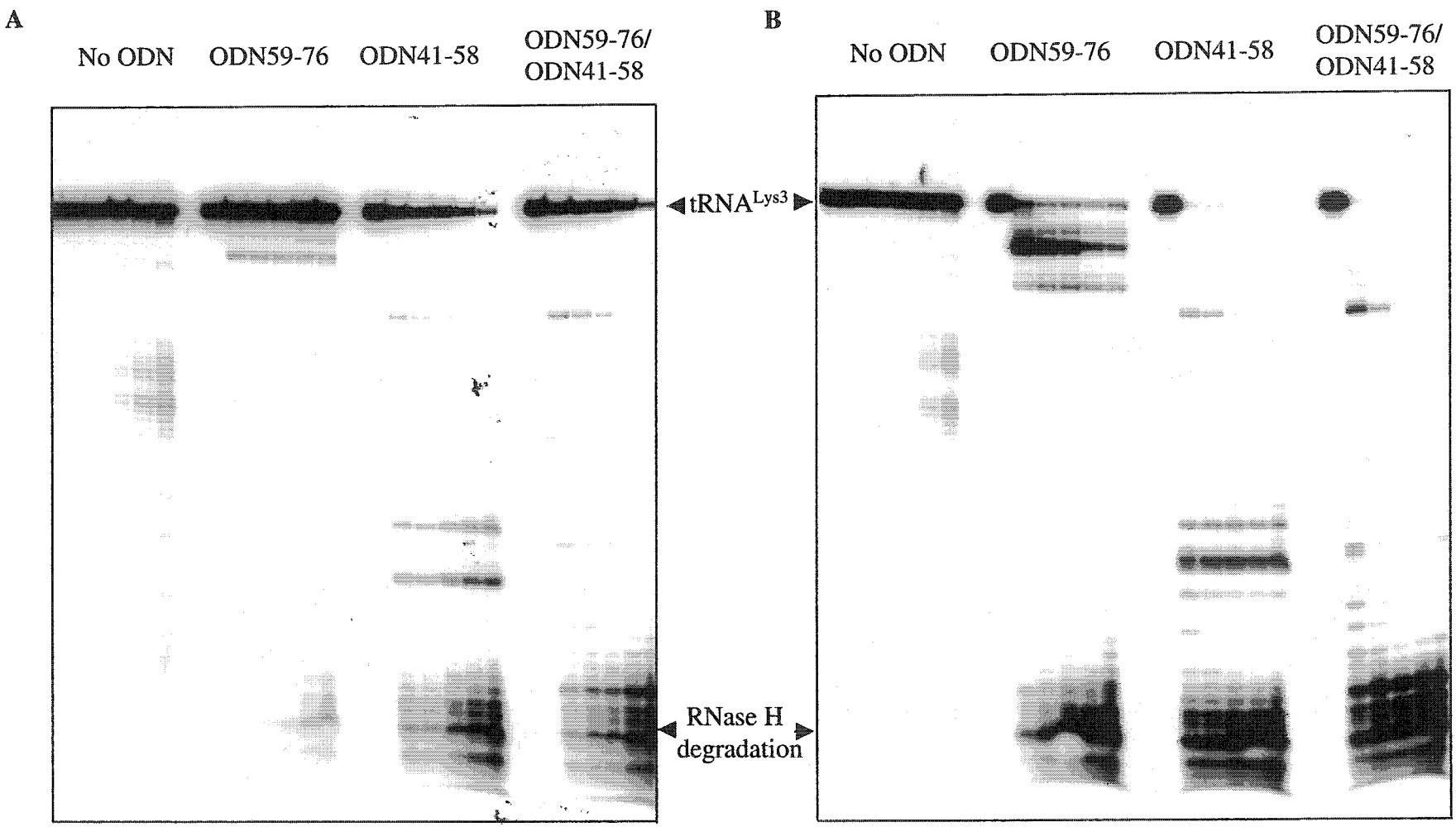

A
No ODN
ODN59-76
ODN41-58
ODN59-76/
ODN41-58
tRNA^{Lys3}
RNase H
degradation
B
No ODN
ODN59-76
ODN41-58
ODN59-76/
ODN41-58

Fig. 3-5

Mg^{2+}, suggesting that divalent cations stablize the binary tRNA/RNA complex, and, in turn, diminish binding of ODN59-76. In contrast, ODN41-58 was able to efficiently initiate RNase H degradation of tRNA, regardless whether $MgCl_2$ had been present during formation of the tRNA/RNA complex (Fig. 3-5A and B). Although the efficiency of initial RNase H cuts is lower as the tRNA/RNA complex is formed in the presence of Mg^{2+}, $tRNA^{Lys3}$ is almost completely degraded at longer time of reaction.

An ODN that is complementary to both the variable and TΨC-stem loops as well as the 3' terminus of $tRNA^{Lys3}$ can block the initiation of DNA synthesis

We next tested whether an ODN complementary to the PBS might be combined with the properties of ODN41-58 to inhibit the initiation of viral DNA synthesis. These studies employed a series of ODNs that contained the same 5' end as that found in ODN59-76 but with an increased number of residues at the 3' end. Considering the above results, this extension at the 3' end could be expected to facilitate the binding of ODNs to the tRNA primer. The longest ODN used, i.e. ODN41-76, is a combination of both ODN59-76 and ODN41-58. Time-course experiments show that ODNs of 18, 21, 24 and 27 residues were unable to block synthesis of (-) ssDNA in comparison with controls from which ODNs were omitted (Fig. 3-6). However, a set of bands of increased intensity, which corresponded to about 75nt, were seen as longer ODNs were added into the system. This shows that the HIV-1 RT recognized the 3' end of the ODN and copied the tRNA primer. Moreover, the longer the ODN employed, the better was the binding to the tRNA primer and the more efficient was its use as a template. These data are in good agreement with the efficient RNase H degradation of the tRNA observed in the presence of ODN41-58 (Fig. 3-5B).

Concomitant with the reverse transcription of $tRNA^{Lys3}$, the efficiency of synthesis of (-) ssDNA was drastically decreased, particularly when using ODNs that possessed complementarity to 30 or 36 residues at the 3' end of the tRNA primer. Moreover, longer ODNs further modulated this diminished initiation reaction and yielded shorter DNA products than that normally formed. This was a consequence of RNase H cuts in the tRNA region that now resulted in a full-length (-) ssDNA that is covalently attached to truncated version of $tRNA^{Lys3}$.

Finally, we have tested the effectiveness of the best inhibitor, i.e. ODN 41-76, using natural $tRNA^{Lys3}$ as a primer. Biochemical data have suggested that post-transcriptionally modified residues may contribute to the stability of the tRNA structure (Perret et al., 1990). Such increased stabilization could conceivably diminish complex formation between the natural tRNA and complementary ODNs, as compared to synthetic tRNA counterparts. Our results show that synthesis of (-) ssDNA, primed with natural human $tRNA^{Lys3}$, was strongly inhibited in the presence of ODN41-76 (Fig. 3-6B). As described for reactions performed with the *in vitro* synthesized primer (Fig. 3-6A), the presence of a truncated version of the strong stop product points to the likelihood that RNase H had cleaved the natural tRNA. However, in contrast to reactions primed with synthetic primer, we did not observe bands attributable to transcription of tRNA. Hence, it is possible that hypermodified residues in the anticodon loop of $tRNA^{Lys3}$ may have caused premature termination of DNA synthesis. To test this hypothesis directly, we labeled ODN41-76 at its 5' end and looked for extension products in the presence of both RT and the preformed binary tRNA/RNA complex (Fig. 3-6C). Complete transcription of $tRNA^{Lys3}$ using ODN41-76 was only seen with the synthetic molecule (Fig. 3-6C, right), consistent with the results of Fig. 3-6A. In contrast, the natural $tRNA^{Lys3}$ was specifically extended by only three nucleotides (lane 2-5, Fig. 3-6C, left), indicating that the hypermodified adenine, located at position 37 of the anticodon loop, caused premature termination of tRNA

Fig. 3-6 (A) Inhibition of synthesis of (-) ssDNA through use of a variety of ODNs of increasing length at the 3' terminus. Each panel shows a time course of DNA synthesis, monitored by incorporation of α^{32}P-dGTP into growing DNA chains. Reactions were initiated with stRNA^{Lys3} and stopped after 5, 15, 30 and 60 minutes (lanes 2 to 5); lane 1 is a control performed in the absence of dNTPs. (B) Inhibition of (-) ssDNA synthesis primed with natural tRNA^{Lys3}. The reaction was monitored in the absence (left) or presence of ODN53-76 (middle) and ODN41-76 (right); the latter reactions showed even stronger inhibitory effects than those initiated with synthetic tRNA^{Lys3}. (C) Time course of reverse transcription of the natural tRNA^{Lys3} primer and its synthetic counterpart, using 5' end-labeled ODN41-76 as primer.

A

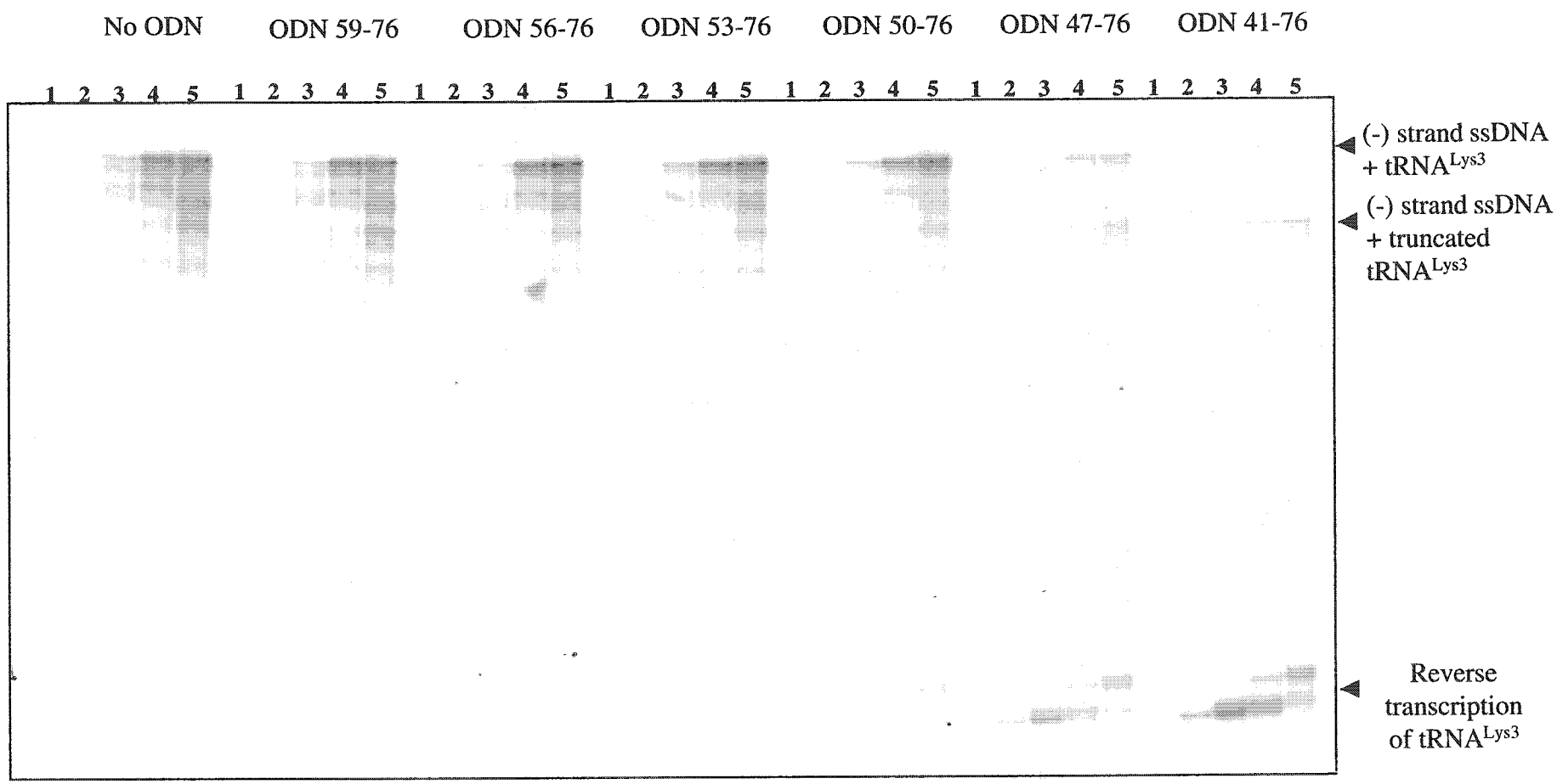

No ODN
ODN 59-76
ODN 56-76
ODN 53-76
ODN 50-76
ODN 47-76
ODN 41-76
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
(-) strand ssDNA + tRNA^{Lys3}
(-) strand ssDNA + truncated tRNA^{Lys3}
Reverse transcription of tRNA^{Lys3}

Fig. 3-6

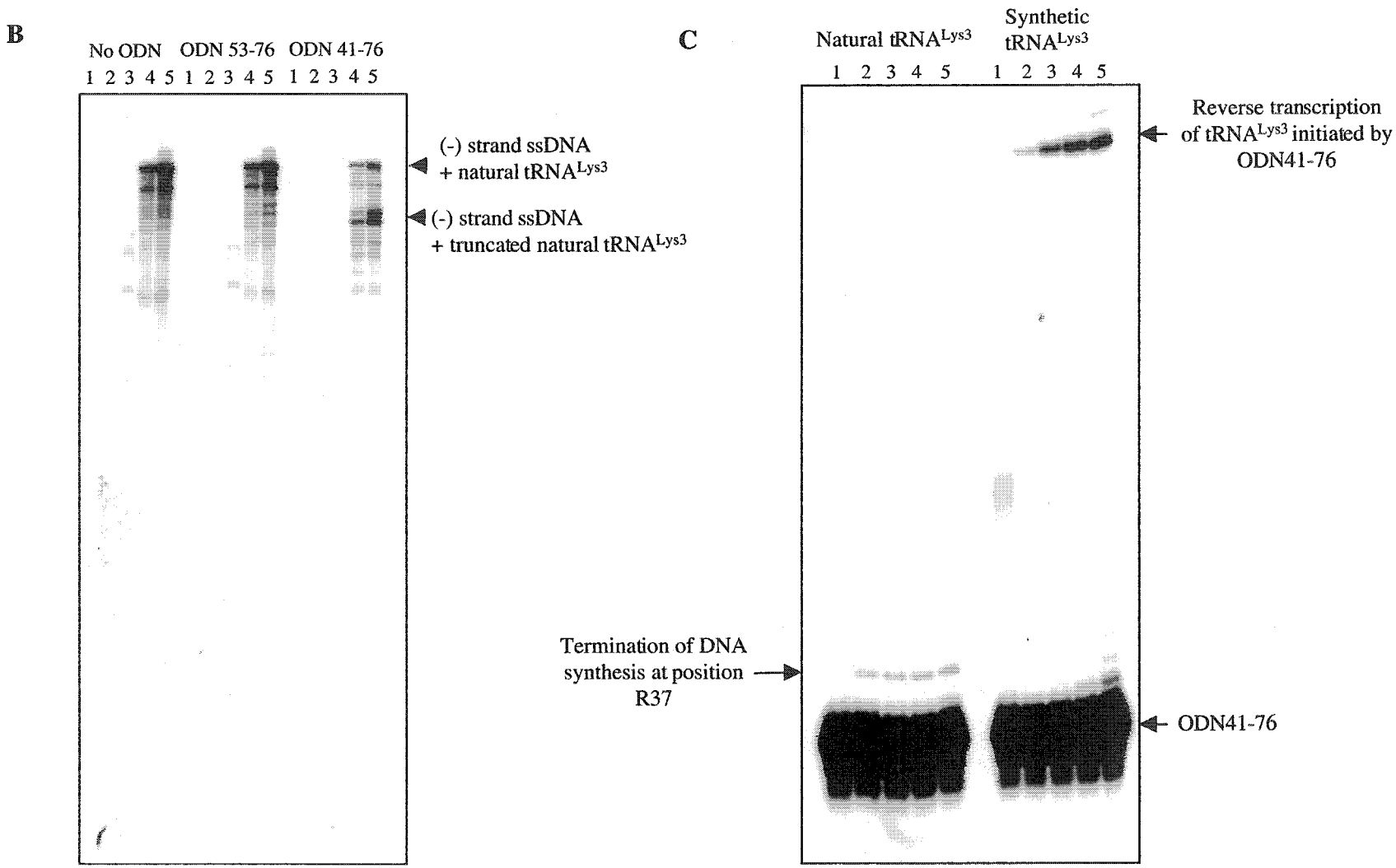

B
No ODN
ODN 53-76
ODN 41-76
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
(-) strand ssDNA
+ natural tRNA^{Lys3}
(-) strand ssDNA
+ truncated natural tRNA^{Lys3}
C
Natural tRNA^{Lys3}
Synthetic
tRNA^{Lys3}
1 2 3 4 5 1 2 3 4 5
Reverse transcription
of tRNA^{Lys3} initiated by
ODN41-76
Termination of DNA
synthesis at position
R37
ODN41-76

Fig. 3-6

transcription. Collectively, these assays demonstrate that ODN41-76 inhibits synthesis of (-) ssDNA by interfering with both the natural initiation complex as well as those involving synthetic primers.

3.5.2 Discussion

The emergence of HIV variants that display resistance to anti-retroviral drugs can limit the success of current therapeutic strategies. Thus, there is a need to identify novel targets that involve highly conserved structures and mechanisms, which may not easily undergo genetic alteration. The initiation of reverse transcription is a specific process that is sensitive to structural changes that involve participating molecules, i.e. RT, genomic RNA in the region of the PBS and primer tRNA (Arts et al., 1998; Kang and Morrow, 1999; Kang et al., 1997; Kang et al., 1999; Lanchy et al., 1996a; Liang et al., 1997a; Zhang et al., 1996; Zhang et al., 1998b; Zhang et al., 1998c). Both cell-free assays and tissue culture experiments have shown that the initiation of reverse transcription can be inhibited using antisense ODNs that bind to viral genomic RNA upstream of the PBS (Bordier et al., 1995; El Dirani-Diab et al., 1997).

In this study, we have evaluated the possibility of directly targeting the primer tRNA, since this cellular component is unlikely to undergo genetic alteration. We have identified antisense ODNs that bind specifically to $tRNA^{Lys3}$ and that are capable of inhibiting the initiation of (-) ssDNA synthesis in cell-free assays. Several factors must be considered in order to explain the observed diminution of viral DNA synthesis and to further evaluate the effectivities of this approach in biological systems. First, our data show that the antisense ODN can bind to the free $tRNA^{Lys3}$ primer as well as to tRNA when complexed with genomic RNA. One can not exclude that the first scenario may result in toxic side effects (see below). However, the latter binding event is expected to induce changes in the structure of the binary tRNA/RNA complex and may therefore specifically inhibit the initiation of (-) ssDNA. Second, the three components, i.e.

$tRNA^{Lys3}$, the RNA template and certain ODNs, can indeed form a stable ternary complex, provided that the ODN is complementary to the TΨC- and variable loops of the tRNA. Under these circumstances, the RT enzyme can recognize both the 3' end of the primer tRNA as well as the 3' end of the ODN. Competition between these recognition events additionally contribute to the inhibitory effect of these ODNs. Finally, interactions between the polymerase active site of RT and the 3' terminus of ODN position the RNase H active site over the tRNA primer; consequently, the latter serves as a template strand and can thus undergo RNase H degradation.

The ODN that showed the strongest inhibitory effect in regard to DNA synthesis, i.e. ODN41-76, possesses complementarity to the 3' end of tRNA as well as to its TΨC- and variable loops. Shorter ODNs that were truncated at their 3' ends did not effectively block the initiation of DNA synthesis. These data suggest that effective ODNs must interfere with interactions between $tRNA^{Lys3}$ and PBS, as well as those occurring outside the PBS. In particular, ODNs that were too short to bind to the variable loop, which is implicated in extended intermolecular interactions (Isel et al., 1995), did not inhibit (-) ssDNA synthesis (Fig. 3-7). Moreover, ODNs that bound to the variable loop effectively initiated RNase H degradation of the tRNA primer. Another consequence of this RNase H activity is that the low amount of (-) ssDNA that is generated was no longer attached to full-length tRNA. This shortened DNA product, that contains a truncated version of $tRNA^{Lys3}$, could conceivably have an inhibitory effect on the first strand transfer event that follows synthesis of (-) ssDNA. Indeed, previous studies have shown that $tRNA^{Lys3}$ facilitates this first strand transfer far more efficiently than a primer that contains only the first 18 nucleotides of $tRNA^{Lys3}$ (Arts et al., 1994; Brule et al., 2000). The effects seen with the various ODNs used in this study are summarized in the model shown in Fig. 3-8.

Fig. 3-7 Interactions of ODNs with residues of the tRNA primer that are implicated in binding to viral RNA. These residues are highlighted in bold. (A) ODNs ≤ ODN50-76 that are complementary to the 3' terminus and TΨC-stem loop fail to inhibit (-) ssDNA synthesis. (B) ODNs ≥ ODN47-76 may interfere with extended interactions between $tRNA^{Lys3}$ and viral genomic RNA. The latter ODNs severely compromised synthesis of (-) ssDNA.

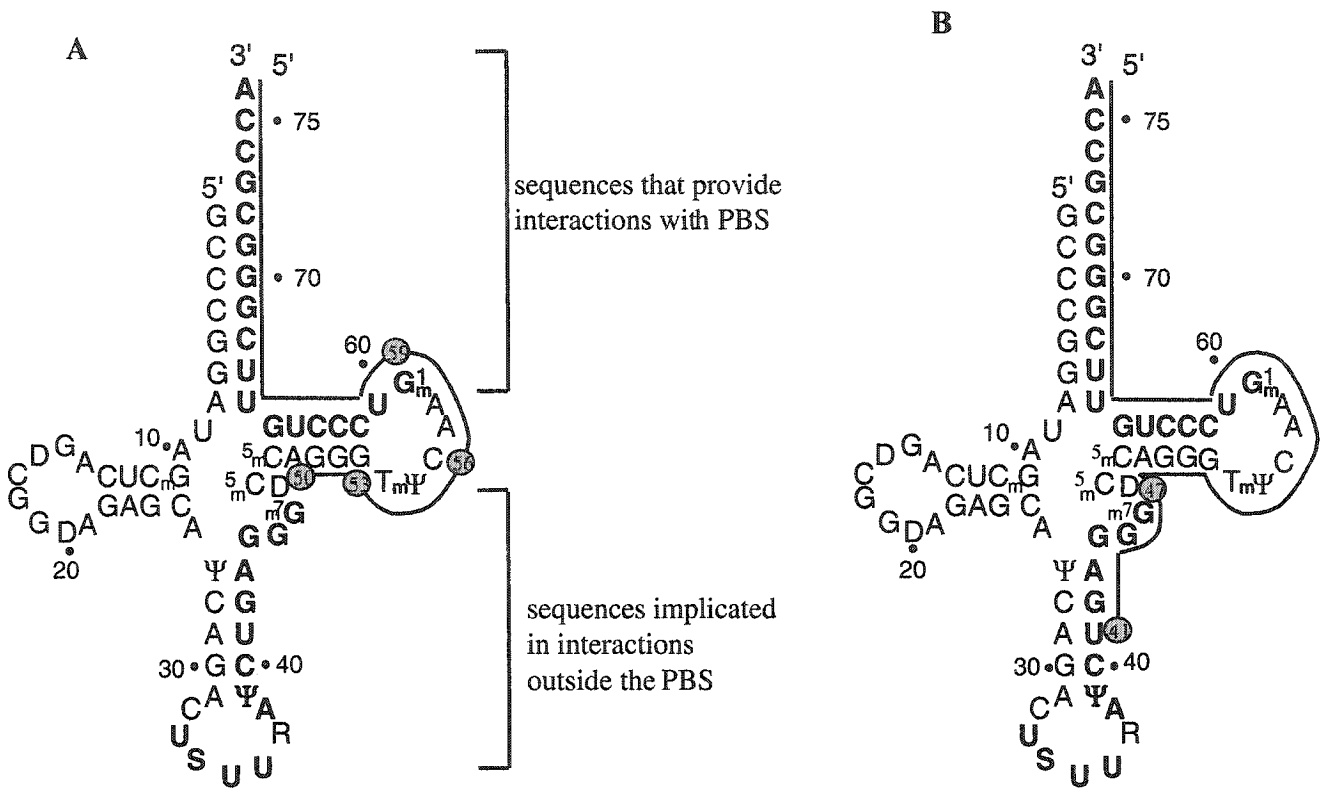

A
B
sequences that provide interactions with PBS
sequences implicated in interactions outside the PBS

Fig. 3-7

Fig. 3-8 Possible mechanisms involved in inhibition of (-) ssDNA synthesis by antisense ODNs ≥ ODN47-76. (A) Schematic representation of intermolecular interactions seen in the binary tRNA/viral RNA complex. (B) ODNs ≥ ODN47-76 can interfere with interactions outside the PBS (right), while shorter ODNs do not contact residues of the variable-loop or the anticodon stem-loop. The nature of the interaction between the 3' terminus of $tRNA^{Lys3}$ and ODNs is uncertain, since the antisense molecules compete with the PBS. This is shown by the dashed line. (C) The formation of a stable ODN/$tRNA^{Lys3}$ complex, with ODNs ≥ ODN47-76 (right), can initiate both reverse transcription as well as RNase H degradation of $tRNA^{Lys3}$. Thus, the tRNA primer serves as a template under these circumstances. Reverse transcription is indicated by the arrowheads. ODNs ≤ ODN50-76 are not sufficiently long to inhibit (-) ssDNA synthesis (left).

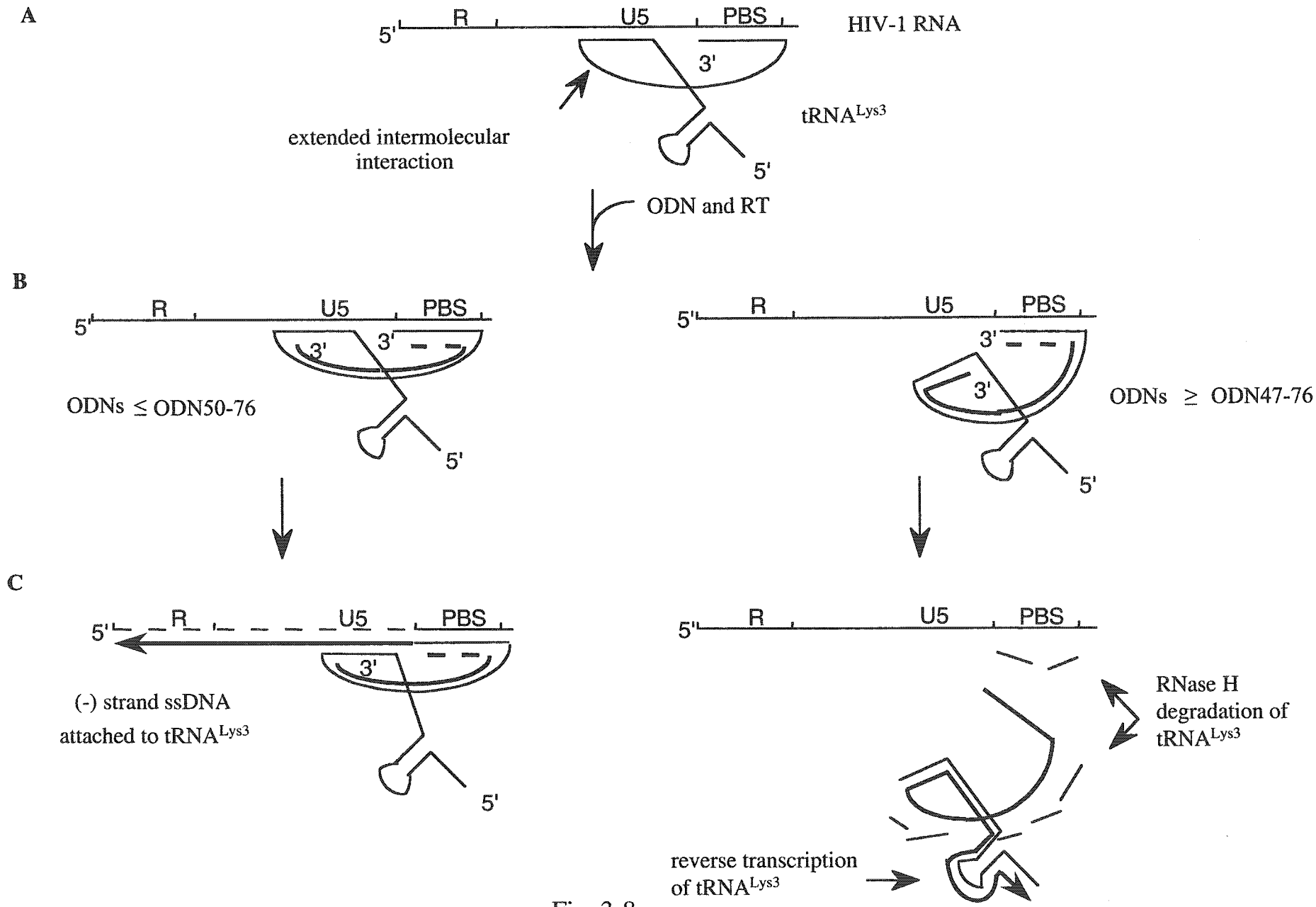

A
5'
R
U5
PBS
HIV-1 RNA
3'
tRNA^{Lys3}
5'
extended intermolecular interaction
ODN and RT
B
5'
R
U5
PBS
3'
3'
ODNs ≤ ODN50-76
5'
5'
R
U5
PBS
3'
3'
ODNs ≥ ODN47-76
5'
C
5'
R
U5
PBS
3'
(-) strand ssDNA attached to tRNA^{Lys3}
5'
5'
R
U5
PBS
RNase H degradation of tRNA^{Lys3}
reverse transcription of tRNA^{Lys3}

Fig. 3-8

In summary, we have identified an ODN that effectively blocks the initiation of synthesis of HIV-1 (-) ssDNA, by targeting the primer tRNA in cell-free assays. Of course, targeting of $tRNA^{Lys3}$ can interfere with cellular functions normally performed by this species and lead to cellular toxicity. However, it is possible that other $tRNA^{Lys}$ isoacceptors, e.g. $tRNA^{Lys1,2,4,5}$ (Das et al., 1997a; Raba et al., 1979), may be able to replace $tRNA^{Lys3}$, since the regions that are targeted by the ODNs used in this study differ among those tRNA species.

In addition, one might take advantage of the fact that the structure of $tRNA^{Lys3}$ in free form differs significantly from that which is bound to the RNA template (Isel et al., 1995; Skripkin et al., 1996). Despite earlier observations, that indicated a highly compact structure of the binary tRNA/RNA complex, our data make clear that antisense molecules can interfere with intermolecular interactions of the HIV-specific initiation complex. Inhibition of (-) DNA synthesis was seen regardless whether reactions were initiated with *in vitro* synthesized $tRNA^{Lys3}$ or with natural $tRNA^{Lys3}$. At the same time it should be stressed that these data do not imply identical structures of both complexes. This is important, as structural and functional studies have indicated that post-transcriptional modifications of human $tRNA^{Lys3}$ are required for extended intermolecular tRNA/RNA interactions, which, in turn, may facilitate initiation and subsequent elongation of reverse transcription (Isel et al., 1996; Isel et al., 1999). Considering the proposed compact structure of the binary complex, it may seem surprising that ODN41-76 can bind to $tRNA^{Lys3}$ and inhibit DNA synthesis of (-) DNA. However, since reverse transcription of this natural tRNA was specifically terminated at the first modified residue, that would need to be bypassed by RT, this shows that the binding of ODN41-76 was highly specific.

The mechanism of complex formation remains to be elucidated, and, it is conceivable that the presence of RT may help to facilitate binding. However, recent studies on the effects of nucleic acid structure on the

formation of complexes between natural $tRNA^{Phe}$ and a library of ODNs point to the importance of a few single-stranded, and accessible, residues that help to nucleate the hybridization process (Mir and Southern, 1999). The structural model of the tRNA/RNA complex predicts that such a single-stranded region is located directly upstream from the 3' terminus of tRNA and binds to the PBS (Isel et al., 1995).

Structural differences among free $tRNA^{Lys3}$ and bound tRNA might be of considerable advantage, since putative toxic effects of antisense-based strategies may be limited when specifically targeting the tRNA that is bound to the RNA template. Such an approach may be superior to those that only target $tRNA^{Lys3}$ in its free form (Shterman et al., 1995). Conceivably, an antisense molecule that is tethered to specific HIV packaging signals may permit colocalization with $tRNA^{Lys3}$, where it is bound to genomic RNA in the virion. Such an approach has been successful in the Moloney murine leukemia virus system, using a chimeric molecule that contained the Ψ-packaging signal as well as a ribozyme that specifically cleaved viral genomic RNA (Sullenger and Cech, 1993). Moreover, ribozymes fused to $tRNA^{Lys3}$ had been identified within HIV particles (Medina and Joshi, 1999).

3.6 Inhibition of Human Immunodeficiency Virus type-1 reverse transcription using tRNA-like molecules that interfere with formation of the natural initiation complex

3.6.1 Results

Design and construction of $tRNA^{Lys3}$-like molecules with altered 3'-terminus

We devised tRNA-like mutant tRNAs that provide complementarity to a sequence distant from the natural primer binding site, PBS. The 3' region of the acceptor arm and the TΨC arm of these tRNAs were altered to promote binding to a highly conserved region that is located 36 nucleotides upstream from the PBS, termed PBS* (Fig. 3-10). To determine sequence requirements that allow efficient initiation from PBS*, initially, we *in vitro* synthesized four tRNAs that provide PBS* complementarity via 18, 15, 12 and 9 nucleotides at 3' terminus. These tRNAs were designated $tRNA^{Lys*}$, $tRNA^{Lys*-15}$, $tRNA^{Lys*-12}$ and $tRNA^{Lys*-9}$ respectively (Fig. 3-10B). Additional substitutions were introduced in 5' sequences to maintain the tRNA secondary and tertiary structure. Possible secondary structures of $tRNA^{Lys3}$ and mutant variants are shown in Fig. 3-9.

Initiation of (-) DNA synthesis from PBS* can result in premature degradation of the genomic RNA by the RT-associated RNase H activity

Initially, to assess the function of *in vitro* synthesized mutated tRNAs in regard to their abilities to prime (-) DNA synthesis, we performed cell-free RT assays with HIV-1 RT (p66/p51), the different tRNA molecules and an *in vitro* synthesized RNA model template containing 258nt of the 5' end of HIV-1 RNA genome which include the PBS and PBS*. Monitored by [α-32P] dCTP incorporation, the data shows that the recombinant HIV-RT can recruit both $tRNA^{Lys3}$ and $tRNA^{Lys*}$ as initiation primer (Fig. 3-11). (-) DNA synthesis initiated from PBS generated a DNA product of 182nt that is joined to the 76nt $tRNA^{Lys3}$, DNA synthesis from PBS* yielded a 128nt product with $tRNA^{Lys*}$ at 5' end. However, the

Fig. 3-9 Possible secondary structure of human $tRNA^{Lys3}$ and $tRNA^{Lys3}$-like molecules $tRNA^{Lys*}$, $tRNA^{Lys*-15}$, $tRNA^{Lys*-12}$ and $tRNA^{Lys*-9}$. Regions that have been modified are highlighted by gray background. Nucleotides which are different from $tRNA^{Lys3}$ sequence are highlighted in bold.

Fig. 3-10 (A) Part of the sequence of the 5' end of HIV-1 RNA genome from position +113 to +221. The sequences of PBS and PBS* are boxed in and highlighted in bold. (B) Sequences of 3' end of $tRNA^{Lys3}$, $tRNA^{Lys*}$, $tRNA^{Lys*-15}$, $tRNA^{Lys*-12}$ and $tRNA^{Lys*-9}$, aligned to PBS and PBS*. Mismatches are highlighted in bold.

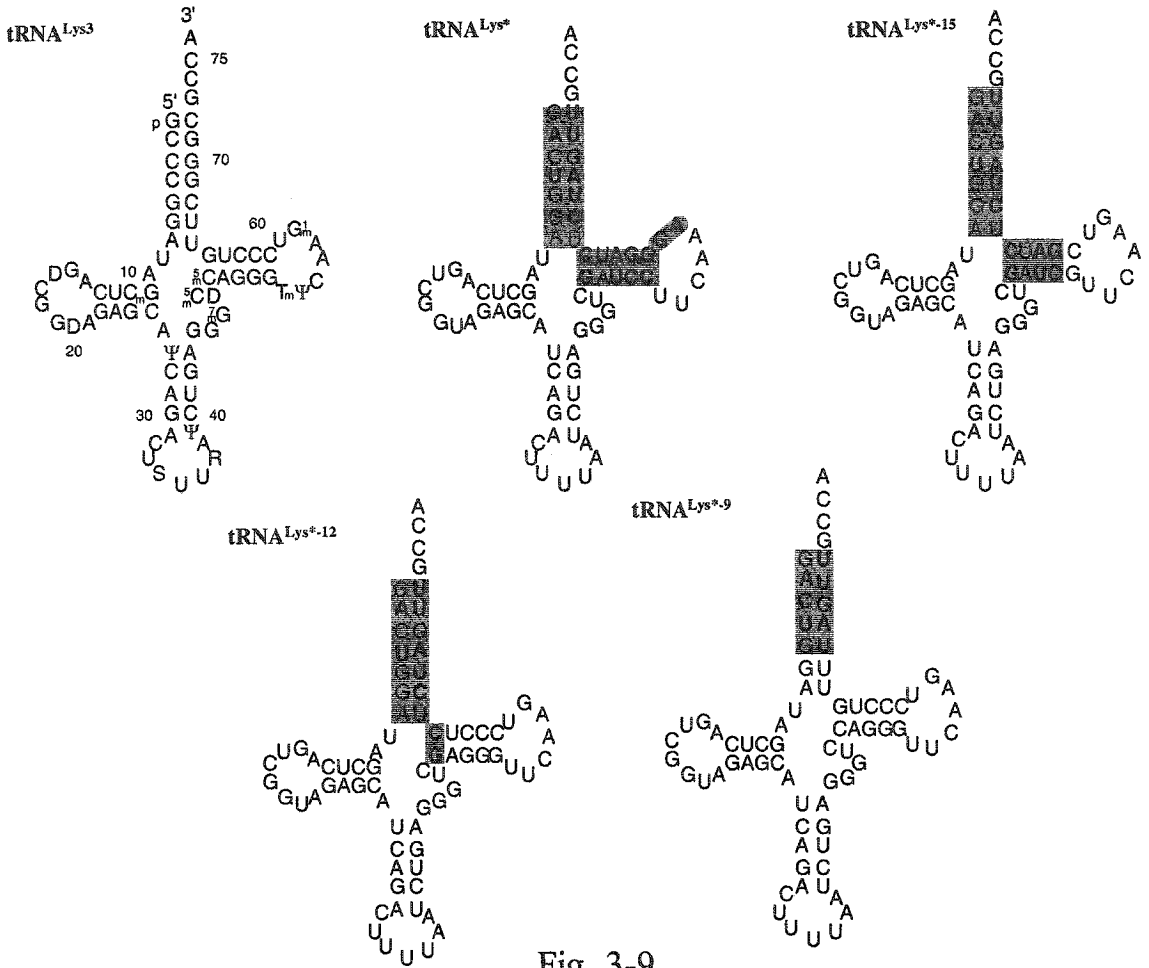


Fig. 3-9

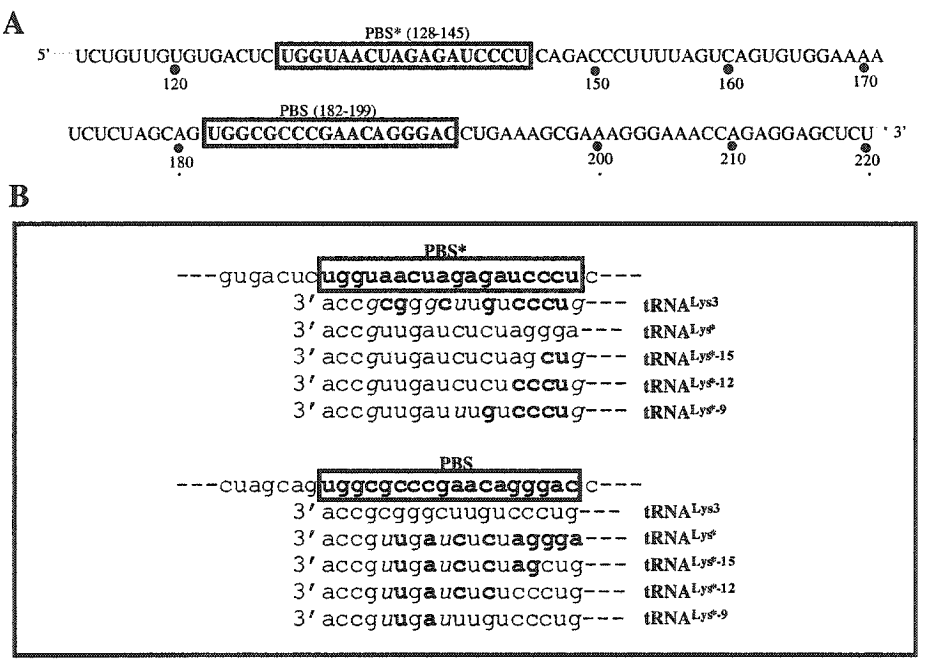


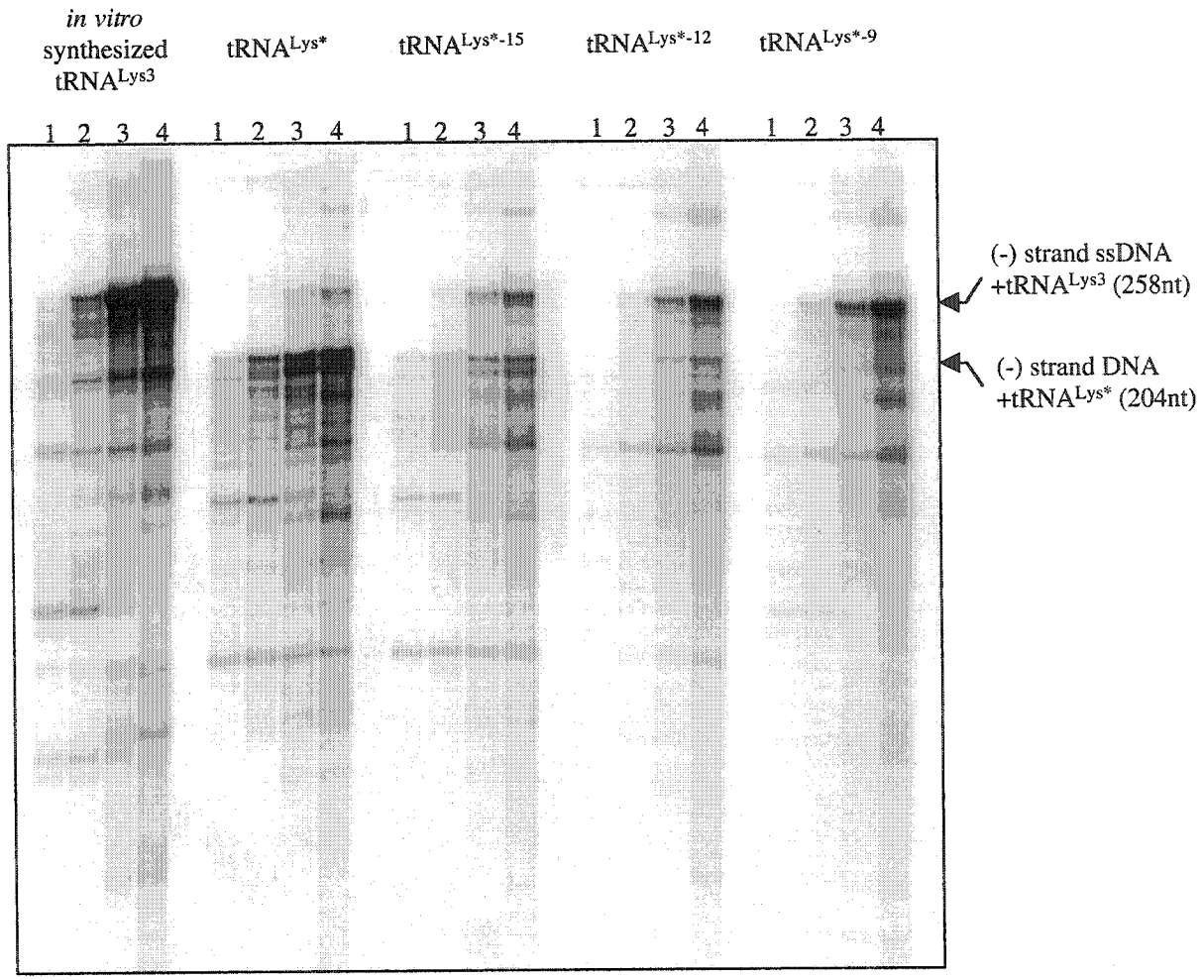
Fig. 3-10

efficiency with which DNA synthesis is primed from PBS* is slightly diminished, as compared to reactions primed with $tRNA^{Lys3}$ (Fig. 3-11A). Moreover, even though the acceptor arm of $tRNA^{Lys*}$ is fully complementary to PBS*, it still provides partially complementarity to the PBS. As a result, about 25% of the final (-) DNA product of the reaction primed by $tRNA^{Lys*}$ were initiated from PBS instead of PBS*. This percentage was even higher when other mutated tRNAs, which have more nucleotides complementary to PBS, were employed as primers. In the case of $tRNA^{Lys*-12}$ and $tRNA^{Lys*-9}$, about 60% of product was initiated from PBS (Fig. 3-11B).

Concomitant with (-) DNA synthesis, the RNA strand of the newly formed RNA/DNA hybrid is degraded by the RT-associated RNase H activity. Previous studies have shown that first cuts are seen directly upstream of the PBS(Götte et al., 1995). Using 3' [α-^{32}P] pCp labeled RNA template, we can monitor the cleavage of RNA template by RT. Cell free RT assay in Fig. 3-11C indicate that initiation at PBS by $tRNA^{Lys3}$ would result in RNase H-mediated cleavage of a longer fragment as compared to reactions that were initiated from PBS*. RNase H cleavage at 5' end of PBS generate a 77 nt fragment, and cleavage at 5' end of PBS* yield a 131 nt fragment instead. Our results show that $tRNA^{Lys3}$ and $tRNA^{Lys*}$ were the most efficient in inducing RNase H cleavage. As mutated tRNAs provide less complementarity to PBS*, more RNase H cleavage are seen at 5' of PBS and efficiency of RNase H cleavage were severely compromised. These results are in good agreement with (-) DNA synthesis assay. Taken together, our results suggest that $tRNA^{Lys*}$ prime (-) DNA synthesis resulted in premature degradation of the RNA template by RNase H activity of RT. The effects seen with $tRNA^{Lys3}$ and $tRNA^{Lys*}$ are summarized in the model shown in Fig. 3-11D. Based on these results, we conclude that tRNALys* is the most efficient mutant tRNA primer to initiate (-) DNA synthesis from PBS*, and it is therefore the best

Fig. 3-11 Efficiency of (-) DNA synthesis and RNaseH degradation. (A) (-) DNA synthesis through use of different *in vitro* synthesized tRNAs. A time course reaction was performed by pre-forming the tRNA/RNA template complex through heat annealing, and incubation with RT at 37°C for 5 min. Reactions were initiated by the addition of dNTPs and stopped after 5, 10, 30 and 60 min (lanes 1-4). Reaction was monitored by incorporation of [α-^{32}P] dCTP into growing DNA chain. (B) Relative percentage of (-) DNA products initiated from PBS and PBS* respectively in each reaction after 60 min, as quantified using Molecular Analyst. (C) RNase H degradation of the RNA template during (-) DNA synthesis. Each panel represents a time course reaction, monitored with 3' end radiolabeled RNA template. Reactions were initiated and stopped as described under A. (D) Schematic representation of possible mechanisms involved in the (-) DNA synthesis from PBS and PBS*, and RNase H degradation of the RNA template.

A


in vitro synthesized tRNA^{Lys3}
tRNA^{Lys*}
tRNA$^{Lys*-15}$
tRNA$^{Lys*-12}$
tRNA^{Lys*-9}
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
(-) strand ssDNA +tRNA^{Lys3} (258nt)
(-) strand DNA +tRNA^{Lys*} (204nt)

B

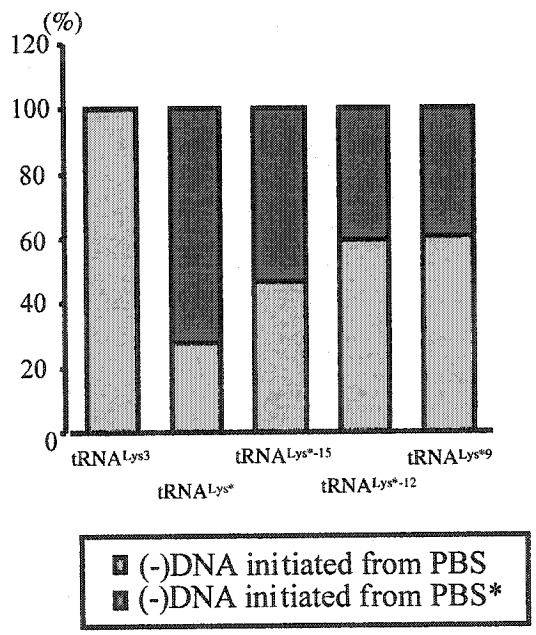

(%)
120
100
80
60
40
20
0
tRNA^{Lys3}
tRNA^{Lys*}
tRNA$^{Lys*-15}$
tRNA$^{Lys*-12}$
tRNA^{Lys*9}
(-)DNA initiated from PBS
(-)DNA initiated from PBS*

Fig. 3-11

C

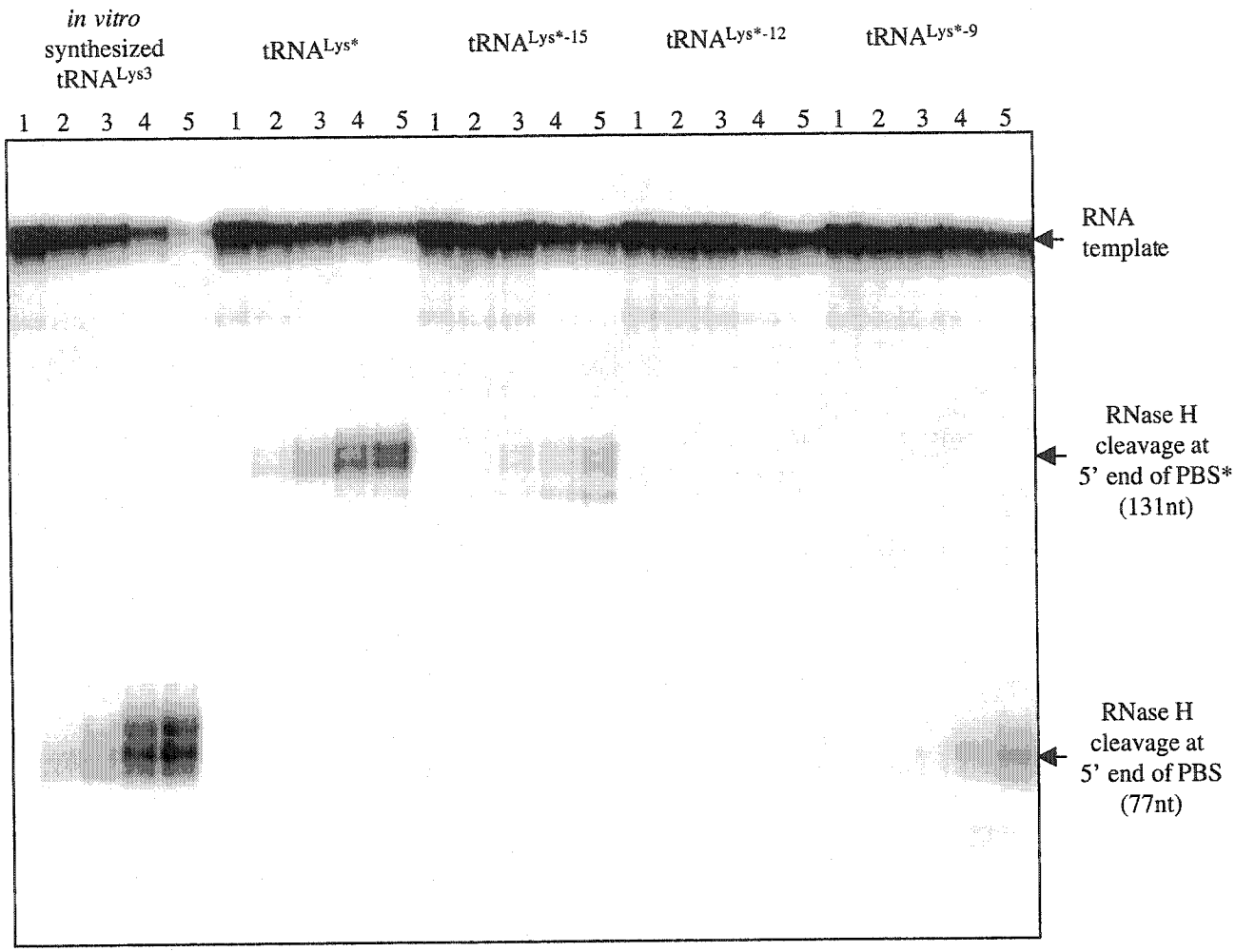

in vitro synthesized tRNA^{Lys3}
tRNA^{Lys*}
tRNA$^{Lys*-15}$
tRNA$^{Lys*-12}$
tRNA^{Lys*-9}
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
RNA template
RNase H cleavage at 5' end of PBS* (131nt)
RNase H cleavage at 5' end of PBS (77nt)

Fig. 3-11

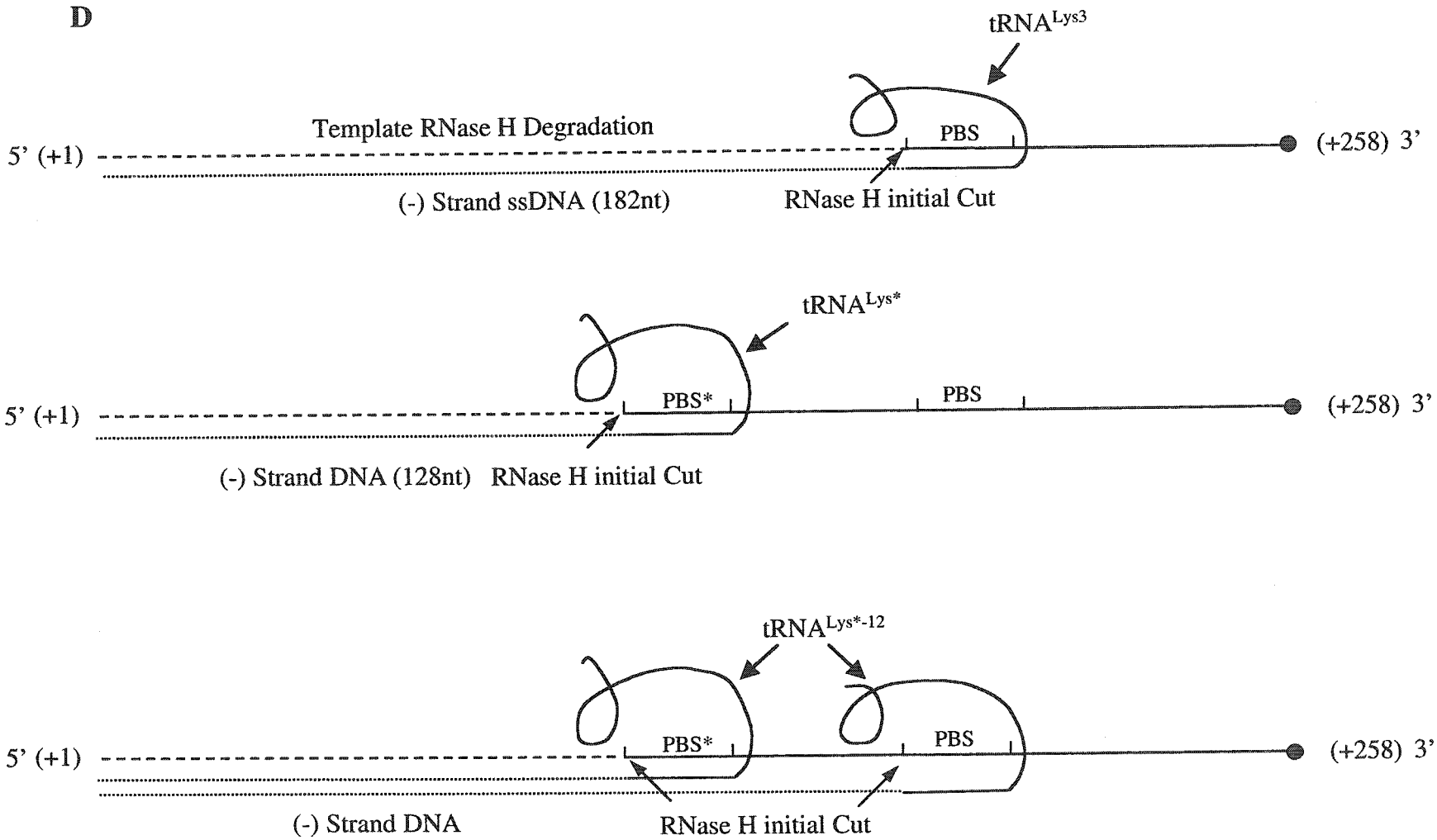

D
tRNA^Lys3
Template RNase H Degradation
PBS
5' (+1)
(+258) 3'
(-) Strand ssDNA (182nt)
RNase H initial Cut
tRNA^Lys*
PBS*
PBS
5' (+1)
(+258) 3'
(-) Strand DNA (128nt)
RNase H initial Cut
tRNA^Lys*-12
PBS*
PBS
5' (+1)
(+258) 3'
(-) Strand DNA
RNase H initial Cut

Fig. 3-11

candidate to study the effects of this type of inhibition in cell culture systems.

$tRNA^{Lys*}$ inhibits (-) DNA synthesis primed by $tRNA^{Lys3}$ in cell free RT assay

To initiate reverse transcription, $tRNA^{Lys3}$ and HIV-1 RNA must undergo extensive structural rearrangement to form a specific initiation complex. The modified nucleotide of natural tRNA play an important role during this process (Isel et al., 1993). Considering the above results, we anticipated that the $tRNA^{Lys*}$ would interfere with the natural $tRNA^{Lys3}$/RNA complex formation, which would result in diminished (-) DNA synthesis. A cell free RT assay was performed in which a constant amount of natural $tRNA^{Lys3}$ and increasing concentration of $tRNA^{Lys*}$ were simultaneously heat annealed to RNA template. The results in Fig. 3-12 show that the presence of $tRNA^{Lys*}$ inhibit $tRNA^{Lys3}$ primed (-) DNA synthesis. A 10-fold molar excess of $tRNA^{Lys*}$ over $tRNA^{Lys3}$ was required to obtain 50% inhibition.

NCp7 promote simultaneous binding of $tRNA^{Lys3}$ and $tRNA^{Lys*}$ to RNA template

In order to assess whether both $tRNA^{Lys3}$ and $tRNA^{Lys*}$ may bind simultaneously, under physiological condition, to the RNA template, we studied bindings in the presence of NCp7. Our ultimate goal in the NCp7 experiments was to determine if competition existed between $tRNA^{Lys3}$ and $tRNA^{Lys*}$ for binding the RNA template. A ratio of 6 nucleotides to 1 molecule of NCp7 was used to achieve highest annealing efficiency. The nucleotide value in this ratio corresponds to the total number of bases in the RNA template and the tRNA primer. *In vitro* synthesized $tRNA^{Lys3}$ has been heat-annealed with RNA template to form the binary $tRNA^{Lys3}$/RNA complex. This increased the stringency of $tRNA^{Lys*}$ binding due to the presence of a stable $tRNA^{Lys3}$/RNA template complex. Increasing

Fig. 3-12 Inhibitory effect of $tRNA^{Lys*}$ on the (-) ssDNA synthesis primed with natural $tRNA^{Lys3}$. Time course reactions were performed with constant amount of $tRNA^{Lys3}$, RNA template and increasing concentration of $tRNA^{Lys*}$. Reverse transcription were initiated by heat anneal $tRNA^{Lys*}$ and $tRNA^{Lys3}$ onto RNA template simultaneously and stopped after 1, 5, 10, 30 and 60 min (lanes 1-5).

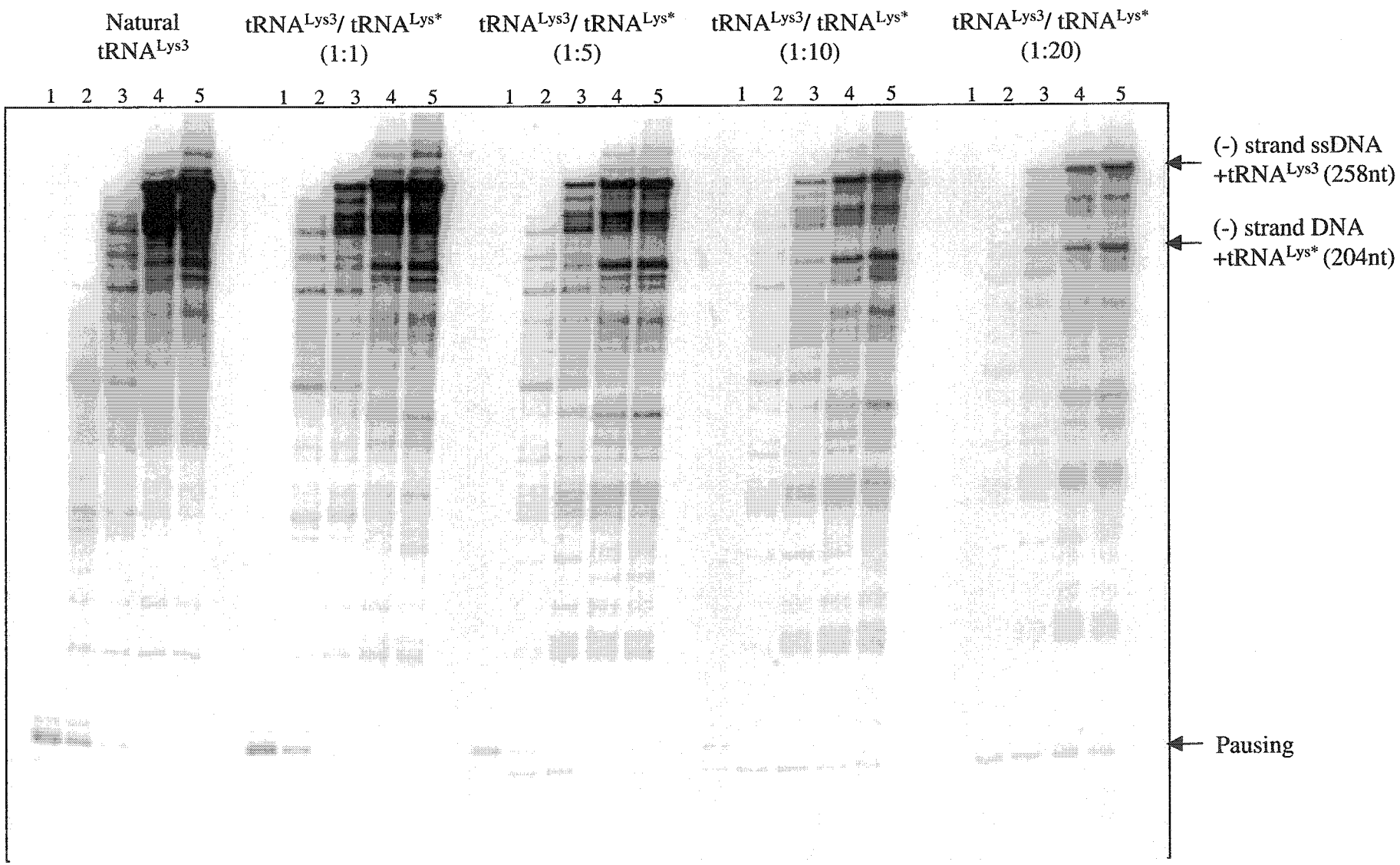

Natural
tRNA^{Lys3}
tRNA^{Lys3}/ tRNA^{Lys*}
(1:1)
tRNA^{Lys3}/ tRNA^{Lys*}
(1:5)
tRNA^{Lys3}/ tRNA^{Lys*}
(1:10)
tRNA^{Lys3}/ tRNA^{Lys*}
(1:20)
1 2 3 4 5
(-) strand ssDNA
+tRNA^{Lys3} (258nt)
(-) strand DNA
+tRNA^{Lys*} (204nt)
Pausing

Fig. 3-12

Fig. 3-13 (A) Placement of $tRNA^{Lys*}$ onto a pre-formed $tRNA^{Lys3}$/RNA template complex by NCp7. Lane 1, radiolabeled RNA template alone; lane 2, $tRNA^{Lys3}$/RNA template complex formed by heat annealing; lane 3, $tRNA^{Lys*}$/$tRNA^{Lys3}$/RNA ternary complex formed by heat annealing. Lanes 4-15, formation of $tRNA^{Lys*}$/$tRNA^{Lys3}$/RNA ternary complex by NCp7 with increasing concentration of $tRNA^{Lys*}$. This experiment was performed by incubating $tRNA^{Lys*}$ and preformed $tRNA^{Lys3}$/RNA complex together in the presence of NCp7 at 37°C for 1 hr. (B) Reverse transcription was initiated by placing tRNA primers onto RNA template using NCp7 and stopped after 60 min reaction. Lane 1: Reverse transcription of RNA template alone as control. Lane 2: (-) DNA synthesis primed with $tRNA^{Lys*}$. Lane 3: (-) ssDNA synthesis primed with natural $tRNA^{Lys3}$. Lanes 4-7: inhibition of $tRNA^{Lys3}$ primed (-) ssDNA synthesis using increasing amount of $tRNA^{Lys*}$ to obtain different molar ratio between $tRNA^{Lys*}$ and $tRNA^{Lys3}$, 1:1, 3:1, 6:1 and 10:1.

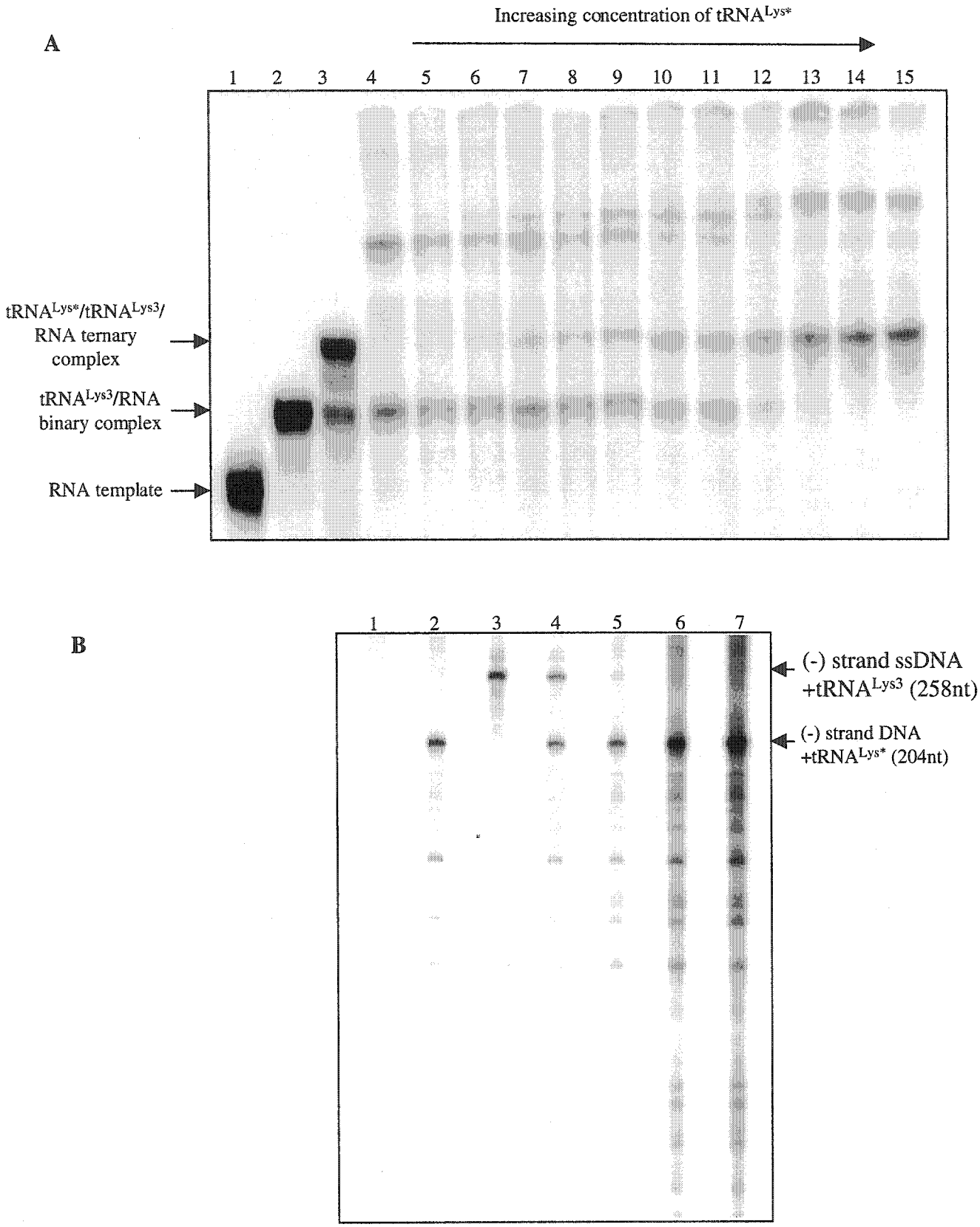

A
Increasing concentration of tRNA^{Lys*}
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
tRNA^{Lys*}/tRNA^{Lys3}/ RNA ternary complex
tRNA^{Lys3}/RNA binary complex
RNA template
B
1 2 3 4 5 6 7
(-) strand ssDNA +tRNA^{Lys3} (258nt)
(-) strand DNA +tRNA^{Lys*} (204nt)

Fig. 3-13

concentrations of $tRNA^{Lys*}$ resulted in the gradual formation of a supershifted band representing the $tRNA^{Lys*}$/ $tRNA^{Lys3}$/RNA ternary Complex (Fig. 3-13A). A-two-fold excess of $tRNA^{Lys*}$ over $tRNA^{Lys3}$ was sufficient to achieve complete supershifting. Also, phosphorimager analysis indicated that similar shift and supershift intensities exists at a 1:1 ratio between $tRNA^{Lys3}$ and $tRNA^{Lys*}$. These results suggest that NCp7 promotes simultaneous binding of $tRNA^{Lys3}$ and $tRNA^{Lys*}$ to RNA template.

Next we evaluate the effect of $tRNA^{Lys*}$ on (-) DNA synthesis under physiological condition in the presence of NCp7. NCp7 has been shown to bind preferentially to single-stranded nucleic acids and unwind primer tRNA *in vitro*, which stimulates synthesis of (-) DNA through enhancement of annealing between the tRNA primer and the viral RNA template. The result of a cell free assay is shown in Fig. 3-13B. The data indicate greater inhibitory effect on $tRNA^{Lys3}$ primed (-) ssDNA synthesis by $tRNA^{Lys*}$ as compared to reactions that were conducted in the absence of NCp7. At the molar ratio of 3/1 between $tRNA^{Lys*}$ and $tRNA^{Lys3}$, the (-) ssDNA synthesis were almost completely diminished.

$tRNA^{Lys*}$ is effectively expressed in Jurkat cells

By inserting $tRNA^{Lys3}$ and $tRNA^{Lys*}$ coding sequences into a eucaryotic expression vector termed pNeo, we obtained the constructs pNeo-Lys3 and pNeo-Lys*, that allow the expression of tRNAs *in vivo*. Together with pNeo, these construct were transfected into Jurkat cells by electorporation. Following G418 selection of transfected cells, we generated J/pNEO cell lines, as well as J/Lys3 and J/Lys* cell lines that stably expressed $tRNA^{Lys3}$ and $tRNA^{Lys*}$, respectively. Expression of $tRNA^{Lys3}$ and $tRNA^{Lys*}$ was detected using a PCR based technique. The total RNA was extracted from cells and radiolabled. The endogenous total tRNAs were separated and purified on a denaturing gel to eliminate the contamination of other RNAs and DNAs (Fig.3-14A) and used as

Fig. 3-14 Detection of $tRNA^{Lys*}$ expression in the J/Lys* cell line. Total RNAs were extracted from different cell lines and 3' end labeled. RNAs with different size were separated on 8% denature PAGE gel (A). The band corresponding to intracellular tRNAs, which might represent rRNAs, were excised and eluted from gel, after being precipitated, used as templates for PCR assay. One of the two primers was 5' end labeled and PCR products were separated on a 5% native PAGE gel (B). Lane1: *in vitro* synthesized $tRNA^{Lys3}$, lane 2: $tRNA^{Lys*}$ molecules. lane 3: total tRNAs from J/Lys3, lane 4: total tRNAs from J/Lys*, lane 5: total tRNAs from J/pNEO cells.

A

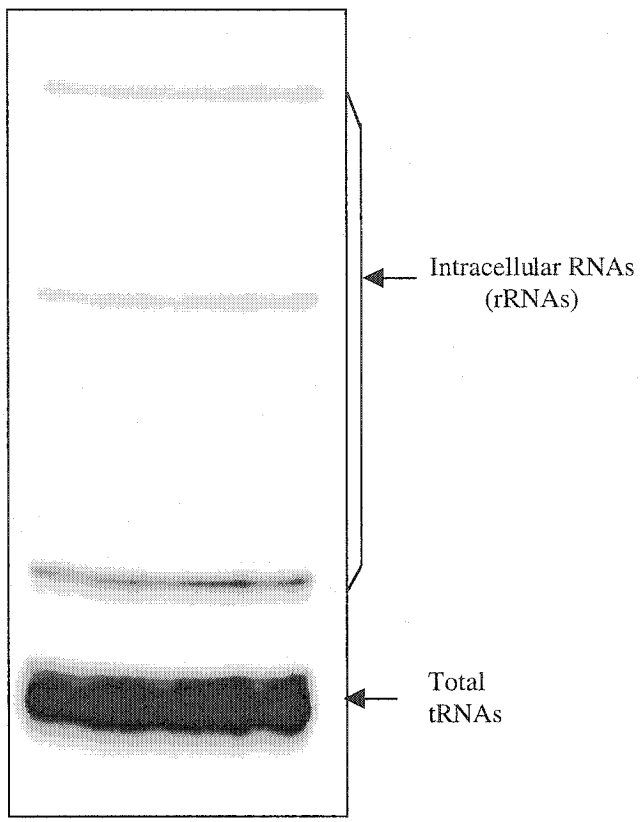


Fig. 3-14

B

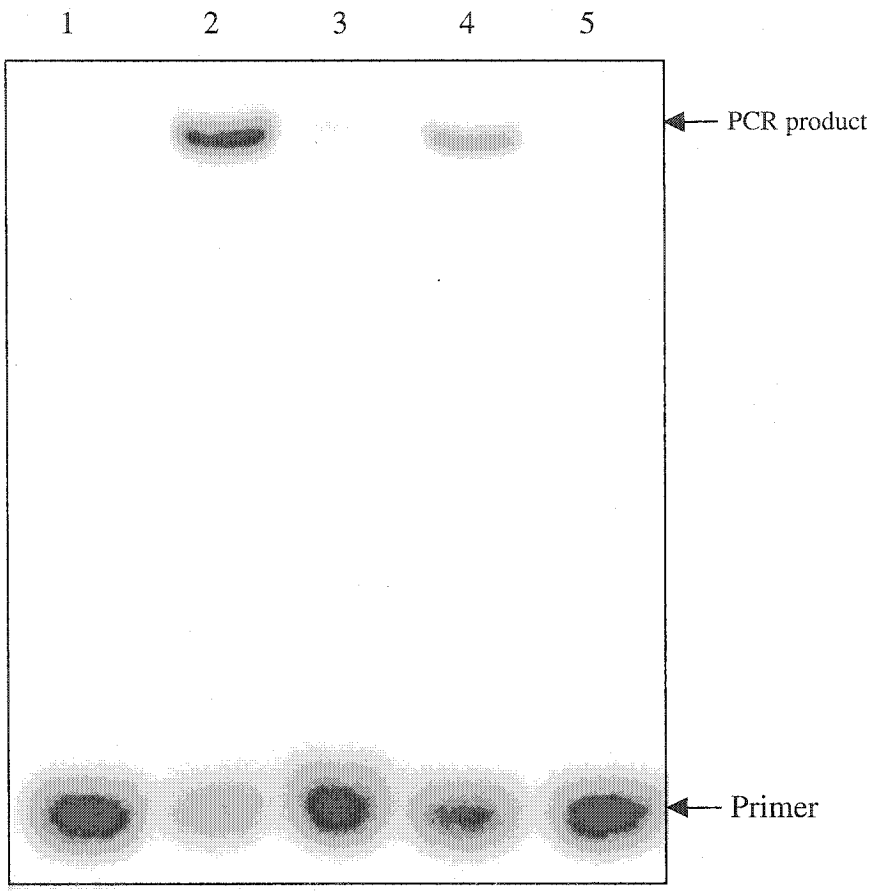


Fig. 3-14

templates for direct PCR identification of tRNA molecules expressed *in vivo*. The double-stranded PCR products were separated from single-strand radiolabled tRNAs template on a natural polyacrylamide gel. Using *in vitro* synthesized $tRNA^{Lys3}$ and $tRNA^{Lys*}$ molecules as control, the results shown in Fig. 3-14B indicate that the $tRNA^{Lys*}$ specific primers are sensitive enough to allow the specific detection of $tRNA^{Lys*}$ (lane 1 and 2). PCR amplification of endogenous total tRNAs clearly demonstrated the expression of $tRNA^{Lys*}$ in the J/Lys* cells (Lane 3-5).

Inhibition of HIV-1 replication.

To study the effect of $tRNA^{Lys*}$ expression on HIV-1 replication, we infected J/Lys3, J/Lys* J/pNEO cell lines with wild-type HIV-1 virus HXB-2D strain. Viruses equivalent to 5 ng of p24 antigen were used to infect 0.5×10^6 cells as described in Materials and Methods. The RT activity in culture supernatants was monitored to evaluate the replication capability in the different cell lines. The growth curve showed that the virus replication in J/Lys* cells was clearly diminished as compared to that in J/Lys3 and J/pNEO cells (Fig. 3-15). Moreover, in the long-term culture of HIV-1 HXB-2D viruses, after 30 days post-infection, we detected 3 different mutant viruses from 30 J/Lys* cellular DNA clones. One of these mutant viruses posses a 46bp deletion between PBS and PBS* that included the majority of PBS (Fig. 3-16). When we used mutated virus containing this 46bp deletion to infect J/Lys*, no virus replication was detected, which suggested that this virus is not a functional revertant.

Fig. 3-15 Comparison of HIV-1 HXB2D replication as assayed by RT activity in J/Lys3, J/Lys* and I/pNEO cell lines. For each infection, 0.5×10^6 cells were infected with HIV-1 HXB2D, equivalent to 5ng of p24.

Fig. 3-16 Sequences of mutated viruses after long-term culture of HIV-1 HXB-2D in the J/Lys* cell line. The identical sequence was shown by dashed line, while mutated nucleotides were shown in corresponding position. Deleted region was shown in bracketed.

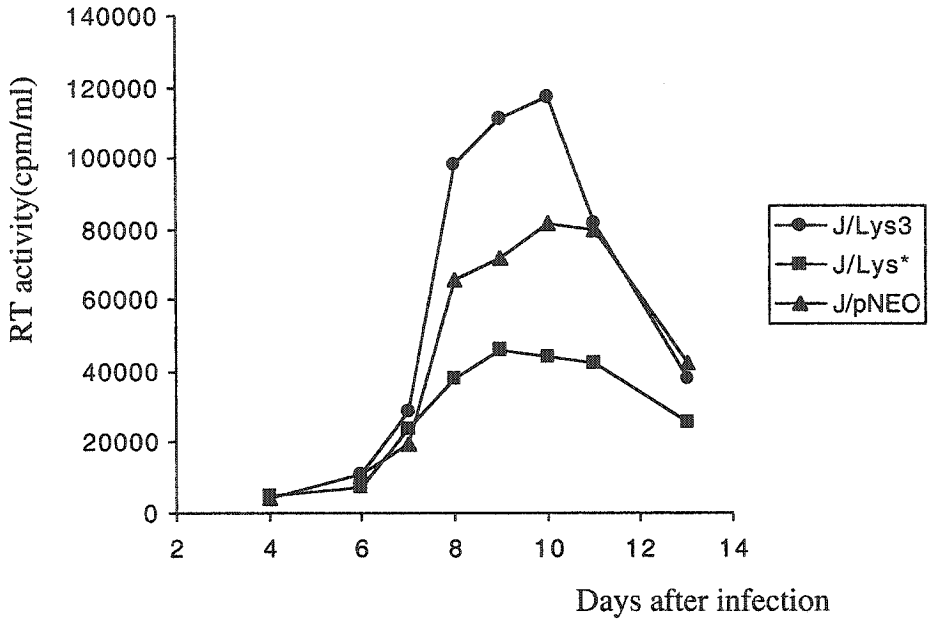


Fig. 3-15

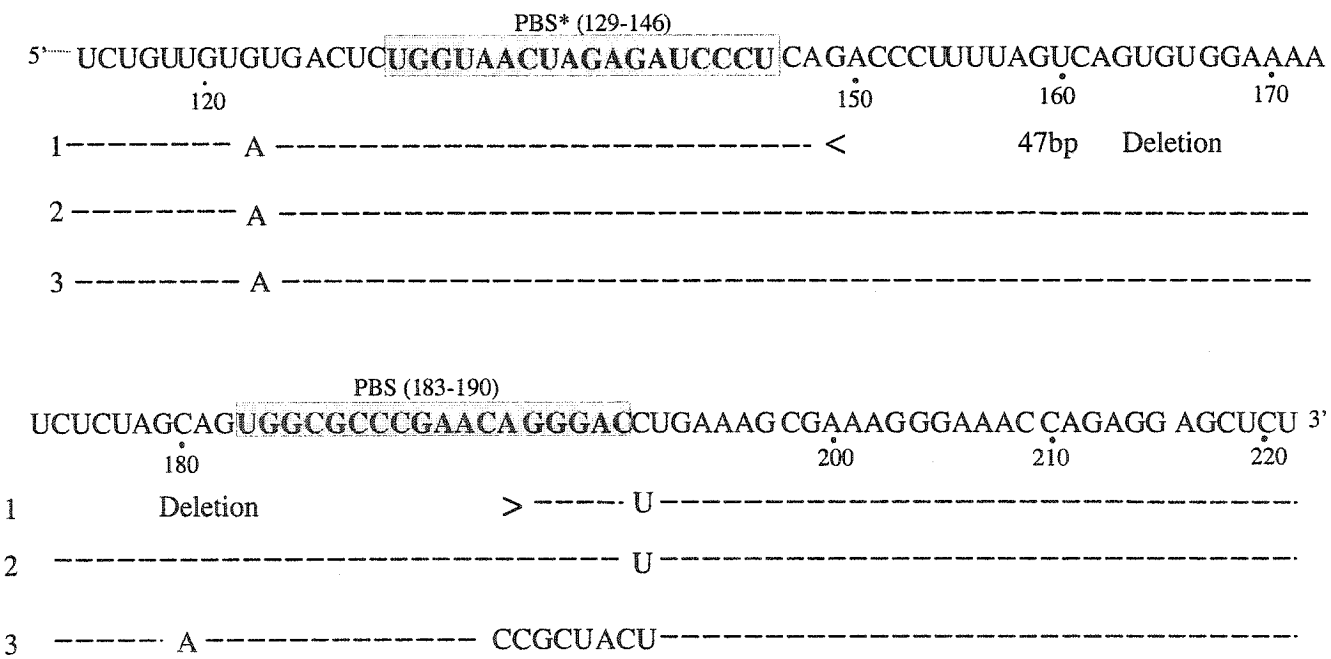


Fig. 3-16

3.6.2 Discussion

HIV-1 recruits a cellular component, $tRNA^{Lys3}$, as primer in order to initiate reverse transcription. tRNA primer is selectively packaged into HIV-1 virions, where it is found hybridized through 18 terminal nucleotides at its 3' end acceptor arm to a complementary PBS site near the 5' end of viral RNA. Together with interactions between the tRNA primer and viral RNA sequences outside the PBS, a specific tRNA/RNA complex is formed which is recognized by the RT enzyme to initiate DNA synthesis (Isel et al., 1999). Anti-retroviral strategies, which make use of the unique properties of the tRNA/RNA complex, have been proposed (Bordier et al., 1995; El Dirani-Diab et al., 1997; Freund et al., 2001; Kaushik et al., 2001; Lee et al., 1998; Lu et al., 1997; Renda et al., 2001). In this study, we designed a $tRNA^{lys3}$-like molecule, termed $tRNA^{Lys*}$, to interfere the formation of natural tRNA/RNA complex, which in turn resulted in inhibition of HIV-1 replication in cell culture.

The mutant tRNA contains modifications in the acceptor arm to redirect the binding to PBS*, a sequence 36bp upstream of natural primer binding site. Our data obtained with cell free RT assays showed that mutant $tRNA^{lys3}$ can prime (-) DNA synthesis from PBS*. However, 18 nt complementarity was needed to achieve efficient initiation of DNA synthesis from PBS*, in case of $tRNA^{Lys*}$, more than 70% of DNA products were from PBS*. Mutant tRNAs, $tRNA^{Lys*-15}$, $tRNA^{Lys*-12}$ and $tRNA^{Lys*-9}$ show increased (-) DNA synthesis initiated from PBS and decreased synthesis initiated from PBS*. Similar results has been observed by other group when using a $tRNA^{lys3}$-like molecule with 10 nt at 3' end complementary to the TAR stem-loop region upstream of the PBS (Lu et al., 1997). It has previously been shown that replication of HIV-1does not require the complete 18-nulceotide complementarity to initiate reverse transcription (Das and Berkhout, 1995; Liang et al., 1997b; Wakefield et al., 1994). This may help to explain why these three mutant tRNAs, which possess reduced nucleotide complementarity to the PBS,

can initiate DNA synthesis from PBS albeit with reduced efficiency. Chemical probing experiments revealed a U5-PBS hairpin, with PBS lying within the loop, while PBS* is located in a base-paired stem (Beerens et al., 2000a; Beerens et al., 2000b; Berkhout, 1996). This may serve as another reason to explain the diminished usage of PBS*.

Upon binding of $tRNA^{Lys3}$ to PBS of genomic RNA, both of these two highly structured components undergo extensive intermolecular interactions and major structural rearrangements, as shown by chemical and enzymatic probing. In addition to the well-known interaction between the anticodon loop of $tRNA^{Lys3}$ and the A-rich loop in the U5 region, it has recently been reported that a region, located about 50 nucleotides upstream of PBS, termed primer activation signal (PAS), may interact with the TΨC arm of the $tRNA^{Lys3}$ primer which provides a mechanism for positive and negative regulation of reverse transcription(Beerens and Berkhout, 2002b; Beerens et al., 2001). Our data show that $tRNA^{Lys*}$, can bind to the preformed $tRNA^{Lys3}$/RNA complex through use of NCp7. NCp7 is considered to be a nucleic acid chaperone that facilitate structural rearrangements in RNA or DNA to form thermodynamically stable conformations. This property is basically independent of specific sequences. NCp7 might be able to destabilize intermolecular interactions between $tRNA^{Lys3}$ and the viral RNA template, thereby promoting binding of $tRNA^{Lys*}$ to the PBS*.

The initiation of reverse transcription is a specific process that is sensitive to structural changes involve tRNA/RNA complex. We have shown that the (-) ssDNA synthesis can be inhibited using antisense oligodeoxynucleotides that bind to $tRNA^{Lys3}$ primer (Result 3.5). In this study, we have evaluated the effects of $tRNA^{Lys*}$ on the $tRNA^{Lys3}$-primed (-) DNA synthesis. Both heat annealing and NCp7 were used to promote binding of tRNAs to RNA template. We have demonstrated that under both conditions, the presence of $tRNA^{Lys*}$ in cell free assay inhibit (-) ssDNA synthesis primed from PBS. Several factors must be considered to explain

the observed inhibitory effect on viral DNA synthesis. First, PBS* is a highly conserved region which has been reported to participate in the formation of natural genomic RNA structure and $tRNA^{Lys3}$/RNA complex. This binding of mutated tRNA molecule to PBS* might interfere with the normal formation of tRNA/RNA complex, which is crucial for the normal initiation process. Second, PBS* contains part of PAS sequence, mutated tRNA binding and (-) DNA synthesis initiated from PBS* might further block PAS from interacting with $tRNA^{Lys3}$, which would play a positive role in the regulation of reverse transcription. Moreover, we have demonstrated above that (-) DNA synthesis initiated from PBS* resulted in premature degradation of RNA template, this might further aggravate the normal (-) DNA synthesis. Finally, when we used *in vitro* synthesized $tRNA^{Lys3}$ instead of natural $tRNA^{Lys3}$ to initiate (-) DNA synthesis in this inhibition assay, no inhibitory effect was detected (data not shown). The co-existence of two (-) DNA products initiated from PBS and PBS* respectively was observed even when 20 times more amount of $tRNA^{Lys*}$ over $stRNA^{Lys3}$ were used (data not shown). Since other groups have demonstrated the importance of modified nucleotides within natural $tRNA^{Lys3}$ for the formation of natural tRNA/RNA complex (Arts et al., 1996a; Isel et al., 1996; Isel et al., 1993), this might also suggest that $tRNA^{Lys*}$ inhibit $tRNA^{Lys3}$ primed (-) DNA synthesis by interfering with the formation of natural tRNA/RNA complex.

To ensure efficient expression of mutated tRNA inside eucaryotic cells, the conserved sequences in the so-called A (base pairs 8 to 19) and B boxes (base pairs 52 to 62) are needed for the binding of two transcription factors, TFIIIB and TFIIIC, respectively. This complex is the entity required for polymerase III-directed transcription. Since the 3' acceptor arm of mutated tRNA is altered to promote binding to PBS*, 6 nucleotides at position 52, 53, 59, 60, 61 and 62 within the B box were substituted, while the entire A box is kept intact. Our data showed that even though there are alterations in the B box, expression of mutated tRNA inside

Jurkat cells were detected by PCR-based assay. The expression tRNA in cultured Jurkat cells did not have an impact on cell growth rate and cellular morphology. The expression of $tRNA^{Lys*}$ in Jurkat cells results in decreased HIV-1 replication, as assayed by RT activity. However, the exact mechanism by which $tRNA^{Lys*}$ expression inhibit HIV-1 replication is not clear. Our *in vitro* cell free assays have demonstrated that $tRNA^{Lys*}$ interfere with the formation of natural $tRNA^{Lys3}$/ RNA complex to inhibit virus replication at early initiation stage. However, we can not exclude the possibility that $tRNA^{Lys*}$ execute its inhibitory effect during other stages of viral reverse transcription. Other groups have reported that HIV-1 can use tRNAs other than $tRNA^{Lys3}$ as primers to replicate as long as the PBS is altered to provide complementarity to this primer. However, in the long-term culture of viruses, mutated PBS is found to reverse back to wild-type (Kang et al., 1997; Wakefield et al., 1995). However even if HIV-1 recruits $tRNA^{lys8}$ to complete (-) DNA synthesis, it will generate a U5 deleted DNA which might render the DNA genome dysfunctional. Upon 30 days long-term culture of virus, there were no functional revertants detected.

The next question needed to be answered is whether $tRNA^{Lys*}$ expressed in cultured cells can be packaged into the virion. The design of mutated tRNA takes advantage of the natural mechanism for $tRNA^{Lys3}$-packaging. As altering the acceptor arm, we keep the entity of other essential features of natural tRNA molecule to maintain the secondary and higher level structure, i.e. L-shape structure. PBS sequence is not required for selective incorporation of $tRNA^{Lys3}$ into HIV-1 virus. It has been know that processor protein $Pr160^{gag\text{-}pol}$ play a key role in the packaging of tRNA primer through the association with both the anticodon and D-loop of tRNA. Most likely, it is the gross L-shaped tRNA structure that is recognized by Gag-Pol precursor. In this study, the most direct answer to the question whether $tRNA^{Lys*}$ was packaged into viral particle came from the results of long-term culture. The sequencing of revertant viruses after

30 days culture indicated the existence of a mutated viruses with most of PBS and sequence between PBS and PBS* deleted. This might imply that HIV-1 package mutated tRNA and used it as a dysfunctional primer to initiate reverse transcription.

Using mutated $tRNA^{Lys*}$-like molecules to inhibit HIV-1 replication has been achieved by other groups(Lu et al., 1997; Renda et al., 2001). In this study, by targeting HIV-1 natural tRNA/RNA reverse transcription initiation complex, we observed a major inhibition of HIV-1 replication, both *in vitro* and *in vivo*. Our results demonstrate that initiation complex represent a more sensitive target for antisense gene therapy approach.

Chapter 4

General Discussion

Reverse transcription is the central step in the HIV-1 life cycle and is initiated from a cellular tRNA, $tRNA^{Lys3}$, which binds with its 3' acceptor arm to a primer binding site (PBS) located at the 5' end of the HIV-1 RNA genome. Additional interactions between genomic RNA and primer tRNAs are found outside the PBS. Chemical and enzymatic probing have shown that both the RNA genome and primer $tRNA^{Lys3}$ undergo extensive structure change to form a binary initiation complex, which RT recognizes and uses to initiate reverse transcription (Isel et al., 1999).

The importance of formation of the initiation complex in viral reverse transcription

Previous results from our lab have shown that an interaction between an A-rich loop, located 10nt upstream of PBS, and the anitcodon loop of $tRNA^{Lys3}$ is important in term of HIV-1 replication fitness and reverse transcription. HIV-1 containing an A-rich loop deletion was initially impaired but can revert to near wild-type replication capacity following additional mutagenesis. Earlier studies had shown that the initiation reaction is a slow process, accompanied by frequent pausing of the RT enzyme at positions +1, +3 and +5 (Isel et al., 1996; Liang et al., 1998). Thereafter, a sharp transition occurs to a faster and more processive mode of polymerization after incorporation of the sixth nucleotide (Lanchy et al., 1998; Thrall et al., 1998). In this study, we demonstrated that the A-rich loop can impact on viral reverse transcription as early as at the initiation stage. We found that the rates of incorporation of the first 1 to 3 nucleotides were not significantly changed in comparison to studies performed with wild-type template; however, the probability of pausing at the +5 position was significantly increased when the A-rich loop was deleted (Chapter 2). Therefore, the impaired fitness caused by the A-rich loop deletion might be a result of reduced efficiency of initiation of synthesis of (-) ssDNA, which is the first DNA product during HIV-1 reverse transcription. Meanwhile, the initiation of reverse transcription

was unaffected in DNA primed reactions, which indicates that the formation of a tRNA/viral RNA binary complex is important for viral reverse transcription and viral replication fitness. Deletion of the A-rich loop may have caused severe structural rearrangements in the initiation complex that resulted in increased pausing at position +5, that, in turn, caused a delay in transition to the faster and more processive elongation phase.

The A-rich loop might also impact on the placement of $tRNA^{Lys3}$ onto the RNA genome. This conclusion comes from *in vitro* cell free assays using an RNA template containing different restoration of one or two As, either upstream or downstream of the deletion, yielded during long-term culture of deletion-containing viruses. Among the revertants studied, the only one that was able to restore viral replication to nearly wild-type levels contained a single T→A replacement at position +173. *In vitro* results showed that the mature viral nucleocapsid protein NCp7 could promote annealing between $tRNA^{Lys3}$ and PBS (Li et al., 1996). Under physiologically relevant conditions, i.e., at 37°C, in the presence of the viral NCp7, our results show that the efficiency of tRNA-primed DNA synthesis is in good agreement with the viral fitness assay. T→A replacement at position +173 restored the ability of the A-rich loop deleted RNA to form a competent binary tRNA/RNA complex.

Enzymatic and mutagenesis assays have predicted a model of the secondary RNA structure of the 5' untranslated leader region of the HIV-1 genome (Baudin et al., 1993; Clever et al., 1995; Das et al., 1997b; Harrison and Lever, 1992; Hoglund et al., 1997; McBride and Panganiban, 1996); (Berkhout, 1996), in which the A-rich loop is part of a so-called U5-PBS hairpin. Mutations that alter the stability of this hairpin structure and genetic alterations that cause severe structural changes in RNA template can inhibit virus replication (Beerens and Berkhout, 2000; Beerens et al., 2000a). The restoration of A at +173, located within the

loop of the U5-PBS hairpin, may have helped to restore the stem-loop structure and hence to increase viral replication.

Impact of the M184V substitution in RT on the initiation of reverse transcription

The M184V substitution in RT is associated with multiple changes in enzyme function and viral replication characteristics, probably as a consequence of the fact that this mutation is located within the RT catalytic site as part of the conserved YMDD motif. In this regard, the M184V substitution is unique among mutations that confer resistance to antiviral drugs. However, little or no evidence exists to suggest that M184V-containing viruses are less mutable than wild-type. In this study, we introduced the A-rich loop deletion into M184V containing virus, and analyzed the impact of the M184V mutation on the emergence of revertants (Chapter 2). Our results also show that M184V viruses might have a reduced capacity to become resistant to drugs that impose a "high genetic barrier" such as certain HIV-1 protease inhibitors as quickly as do wild-type viruses. Our results show that the presence of M184V compromised the ability of A-rich loop deleted viruses to restore wild-type replication. *In vitro* cell free assays provided an explanation, related to initiation of viral reverse transcription. We found that the M184V mutation reduced the rate of tRNA-primed DNA synthesis; notably, pausing at position +3 appeared at a later time compared with reactions performed with wild-type HIV-1 RT. These effects were found to be specific for the tRNA-primed reaction, since the efficiency of DNA synthesis was unaffected in reactions performed with a DNA primer. This result supports our original hypothesis that the efficient usage of nucleic acid substrates that show reduced affinity to wild-type RT is further compromised in the presence of the M184V mutation. In contrast to the mutated RNA template, the M184V mutation may directly affect the interaction with the tRNA primer, which helps to explain the relatively

late appearance of the pausing product at position +3 in reactions performed with the mutated RT. Since it is RT that recognized the tRNA/RNA binary initiation complex to start viral reverse transcription, this result may be due to a diminished ability on the part of the mutated enzyme to bind to it. This also provides further evidence for the importance of the initiation complex.

New antiviral strategies further reveal the importance of the HIV-1 initiation complex.

The results shown in Chapter 2 provided evidence that the initiation of reverse transcription is a specific process that is sensitive to structural changes among participating molecules, i.e., RT, genomic RNA. Another component of the initiation complex is a cellular molecule, $tRNA^{Lys3}$, which is non-variable. Previous results have shown that when HIV-1 PBS was altered to provide complementarity to a tRNA molecule other than $tRNA^{Lys3}$, it reverted back to $tRNA^{Lys3}$ PBS in cell culture (Kang and Morrow, 1999; Li et al., 1996; Wakefield et al., 1995). While the emergence of HIV variants that display resistance to anti-retroviral drugs remains a major obstacle of current therapeutic strategies, there is a need to find novel targets that involve highly conserved structures and mechanisms, which may not easily undergo genetic alteration. As mentioned above, the specificity of the viral initiation complex might render it a good target for novel antiviral strategies. Therefore, we targeted the primer tRNA with antisense ODNs and the HIV-1 RNA genome with a $tRNA^{Lys3}$-like tRNA molecule to inhibit the initiation of reverse transcription (Chapter 3).

In order to inhibit HIV-1 replication, the ODNs employed had to be able to destabilize interactions between $tRNA^{Lys3}$ and the PBS as well as the extended contact between primer tRNA and viral RNA. In this case, the ODN that showed the strongest inhibitory effect in regard to DNA synthesis, i.e. ODN41-76, possessed complementarity to the 3' end of

tRNA as well as to its TΨC- and variable loops. Furthermore, we have shown that antisense ODNs can bind to the $tRNA^{Lys3}$ as well as to tRNA when complexed with genomic RNA. Structural differences among free $tRNA^{Lys3}$ and bound tRNA might be of considerable advantage, since putative toxic effects of antisense-based strategies may be limited when specifically targeting the tRNA that is bound to the RNA template. Such an approach may be superior to those that only target $tRNA^{Lys3}$ in its free form (Shterman et al., 1995).

When using a $tRNA^{Lys3}$-like molecule, we targeted another component of initiation complex, viral genomic RNA. The target sequence was a highly conserved sequence, PBS*, which is located upstream of the PBS and participates in the extensive interactions between primer tRNA and viral RNA. Binding of the mutated tRNA molecule to PBS* might interfere with the normal formation of a tRNA/RNA complex. Moreover, part of the recently identified primer activation signal (PAS) is located within the PBS* sequence. It has been recently reported that PAS may interact with the TΨC arm of the $tRNA^{Lys3}$ primer which provides a mechanism for positive and negative regulation of reverse transcription(Beerens and Berkhout, 2002b; Beerens et al., 2001). Mutated tRNA binding and (-) DNA synthesis initiated from PBS* might further block the PAS from interacting with $tRNA^{Lys3}$, which could play a positive role in the regulation of reverse transcription. This inhibition effect was achieved only with natural $tRNA^{Lys3}$-primed DNA synthesis and clearly demonstrates that mutated tRNA primer inhibits virus replication by interfering with the formation of initiation complex.

In summary, we have demonstrated the crucial role of the HIV-1 initiation complex in viral reverse transcription and replication fitness. This complex is sensitive to structural changes and rearrangement. Our studies suggest that the efficiency of tRNA-primed initiation of reverse transcription correlates with the efficiency of viral replication

competence. Based on these results, we have developed novel antiviral strategies to target on the components of the initiation complex. By interfering with the formation complex, we achieved inhibition of viral reverse transcription and replication.

Contribution to original knowledge

The following is a summary of my original work and contribution to the scientific research community under the supervision of Dr. Mark A. Wainberg.

Chapter 2. HIV-1 reverse transcription primer $tRNA^{Lys3}$ is a cellular molecule. It forms an initiation complex with HIV-1 genomic RNA, which is recognized by HIV-1 RT to initiate reverse transcription. In this study, we found that the initiation complex is sensitive to structural changes. When this complex is destabilized by mutations, i.e., an A-rich loop deletion in genomic RNA and a M184V substitution in RT, viral replication was impaired. Using *in vitro* cell free assays, we demonstrated that viral replication fitness was correlated with the efficiency of reverse transcription initiation. Most important, the presence of M184V in RT compromised the ability of virus containing the deletion of A-rich loop to restore wild-type replication. M184V containing virus might have a reduced capacity to become resistant to drugs that impose a "higher genetic barrier", which, in turn, might confer future clinical benefit. Overall, these studies help to define the importance of the initiation complex in terms of HIV-1 reverse transcription and viral replication fitness.

Chapter 3. The specificity of the HIV-1 initiation complex makes it a good target for a gene therapy approach. This highly conserved structure may not easily undergo genetic alteration, which is the main obstacle in therapeutical strategies. In this study, we employed antisense ODNs and a $tRNA^{Lys3}$-like molecule to target primer tRNA and a highly conserved genomic RNA sequence, respectively. *In vitro* cell free and *in vivo* cell culture assays indicated that these antisense molecules function as

inhibitors of HIV-1 replication by interfering with the formation of the initiation complex.

Overall, our data clearly demonstrate that initiation of HIV-1 reverse transcription is a highly specific process which serves as a valuable potential target for future antiviral strategies.

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country	
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telephone	
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