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IN VITRO RECONSTITUTION AS A STRATEGY FOR
EVALUATING THE SUBUNITS OF
HUMAN IMMUNODEFICIENCY VIRUS TYPE 1
REVERSE TRANSCRIPTASE

by

PAMELA SUSAN JACQUES

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Thesis Advisor: Stuart F. J. Le Grice, Ph.D.

Department of Biochemistry
CASE WESTERN RESERVE UNIVERSITY
May, 1995
We hereby approve the thesis of

Pamela S. Jacques

candidate for the Ph.D.
degree.*

(signed)  
(chair)

[Signatures]

date 3/29/95

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IN VITRO RECONSTITUTION AS A STRATEGY FOR 
EVALUATING THE SUBUNITS OF 
HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 
REVERSE TRANSCRIPTASE

Abstract

by

PAMELA SUSAN JACQUES

Human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) is essential for viral replication and possesses DNA- and RNA-dependent DNA polymerase, ribonuclease H (RNase H), and RNase H* (hydrolysis of double stranded RNA) activities. The heterodimer (p66/p51) is the biologically relevant and the enzymatically active form of RT which initiates minus strand DNA synthesis from the HIV-1 replication primer, human tRNA\textsuperscript{Lys}.\textsuperscript{3} Any of the above enzymatic functions, including dimerization, could possibly be inhibited in an effort to inactivate the enzyme and therefore be used to develop therapeutic strategies for slowing the progression of AIDS.

In vitro reconstitution and mutagenesis of p66 and p51 HIV-1 RT were utilized in an attempt to localize specific activities within these subunits. Three truncated HIV-1 51 kDa subunits were constructed containing C-terminal deletions of 13, 19 and 25 amino acids. These p51 deletion mutants were then reconstituted with wild type HIV-1 p66 in an effort to determine a functional role of p51 in
the context of the biologically significant heterodimer. Deletion of the C-terminal 25 residues of p51 resulted in the inability to form a stable heterodimer. However, deletion of 13 or 19 amino acids did not disrupt dimerization, but significantly affected the various activities of heterodimer HIV-1 RT. Our study indicates the C-terminal region of p51 is necessary for retention of both tRNA\textsuperscript{Lys}, affinity and RNase H activity.

In a separate study, p66 HIV-1 mutants containing single amino acid substitutions between residues Glu\textsuperscript{224} and His\textsuperscript{235} were constructed. These altered 66kDa subunits were then reconstituted with wild-type p51 to generate p66\textsuperscript{E224A/p51}-p66\textsuperscript{E233A/p51} and p66\textsuperscript{H235A/p51} (reconstitution of p66\textsuperscript{L234A} and p51 did not result in a stable heterodimer). Each selectively-mutated heterodimer was assayed for retention of DNA- and RNA-dependent DNA polymerase activities, RNase H activity, and template-primer and tRNA\textsuperscript{Lys} primer binding. Our data indicates p66 residues Trp\textsuperscript{229}.Tyr\textsuperscript{232} are essential for both DNA polymerase activities and template-primer affinity.
DEDICATION

I dedicate this work to my loving and supportive parents who have always encouraged my education.
ACKNOWLEDGMENTS

Throughout my graduate education I have had support and guidance from a number of sources. I would first like to thank my advisor, Dr. Stuart Le Grice, for his thoughtful advice and encouragement. I also thank my thesis committee members, Drs. Nelson Phillips and Hsing-Jien Kung for their support and discussions, and particularly Dr. William Merrick who never failed to politely answer my departmental questions and concerns. I also thank Dr. David Setzer for participating in my defense.

I thank the following members of the Le Grice lab: Dr. Birgitta Wöhrl, an extraordinary post-doc whose patience and support was immeasurable and whose humor made the lab a brighter place; Nick Cirino, Nancy Richter-Cook, and Jason Rausch, my fellow graduate students and lunch dates who always knew when to laugh with me, when to laugh at me, and when it was time for a break; Kathy J. Howard, the backbone of the lab and the support network; Dr. Mita Ghosh for her contributions to this work; and newcomer Dr. Eric Arts for his sound advice and enthusiasm. For their collaborative efforts and experimental contributions, I also gratefully acknowledge Michele Ottmann, Dr. Jean-Luc Darlix, Janice Rose and Dr. C.-K. Shih.
I also thank my dear friends Paul and Marla Regelbrugge for all their long distance encouragement and contagious happiness, and my family for their seemingly endless support and understanding. Finally, I thank James Thomas, my dearest friend and confidant, who always manages to bring a smile to my face.
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<tr>
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<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
<td></td>
</tr>
<tr>
<td>CA</td>
<td>capsid protein</td>
<td></td>
</tr>
<tr>
<td>ddNTP</td>
<td>dideoxynucleoside triphosphate</td>
<td></td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
<td></td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>(ethylenedinitrilo)tetraacetic acid</td>
<td></td>
</tr>
<tr>
<td>E. Coli</td>
<td>Escherichia coli</td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>(N-[2-Hydroxyethyl]piperazine-N' [2-ethanesulfonic acid])</td>
<td></td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
<td></td>
</tr>
<tr>
<td>IN</td>
<td>integrase</td>
<td></td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
<td></td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>matrix protein</td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>nucleocapsid</td>
<td></td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
<td></td>
</tr>
<tr>
<td>NTA</td>
<td>nitrilotriacetic acid</td>
<td></td>
</tr>
<tr>
<td>pbs</td>
<td>primer binding site</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
<td></td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulphonyl fluoride</td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>protease</td>
<td></td>
</tr>
<tr>
<td>RNase H</td>
<td>ribonuclease H</td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
<td></td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

VIRAL LIFE CYCLE

Human immunodeficiency virus type 1 (HIV-1) is a genetically complex retrovirus in that its genetic information is encoded in RNA and must be converted into DNA before gene expression (Baltimore 1970; Temin and Mizutami 1970). Two copies of the viral RNA genome are located within the cone-shaped core of the HIV-1 virion (illustrated in Figure 1-1). The core is composed of the viral capsid protein (CA), and also contains the following viral proteins: reverse transcriptase (RT), nucleocapsid (NC), integrase (IN), and protease (PR), the functions of which are elucidated below. The glycoproteins, gp120 and gp41, are located on the cell surface, extending through the lipid bilayer of the virion, whereas the matrix (MA) protein forms the inner portion of the viral membrane, separating the lipid bilayer from the remainder of the virion.

Figure 1-2 illustrates the HIV-1 life cycle which consists of several specific steps (reviewed in Vaishnav and Wong-Staal 1991). HIV-1 infection begins with binding of virions to a target host cell, facilitated through an interaction between the HIV-1 envelope glycoprotein, gp120, and a CD4 molecule located on the surface of the host cell. Upon fusion of the viral and cellular membranes, the core of the virion is released into the cell cytoplasm. As outlined above, this
Fig. 1-1: HIV-1 Structure

A mature HIV virion is illustrated with the structural proteins and catalytic enzymes associated with the virion. Protein abbreviations are according to Leis et al. 1988. Figure adapted from Eric J. Arts.
Mature HIV-1 Virion

- gp120
- gp41
- Lipid bilayer
- Viral RNA
- MA
- CA
- IN
- NC
- PR
- RT
Fig. 1-2: The retroviral life cycle

In the first step of HIV infection, the virion binds to the outside of the host cell. After fusion of the viral and cell membranes, the core proteins and the double-strand RNA viral genome are released into the host cell. In the cytoplasm, reverse transcriptase (RT) transcribes the viral genome into double-strand DNA. The double-strand DNA then migrates to the cell nucleus where it is integrated into the host cell DNA. Following viral gene expression and protein translation, viral polyproteins and the full length double-strand RNA viral genome are assembled into a new virion that buds from the host cell. Polyprotein processing and maturation yields fully infectious virus. Figure courtesy of S.F.J. Le Grice.
core contains the two strands of genomic viral RNA as well as essential viral proteins. One of these proteins, reverse transcriptase (RT), converts the single-strand RNA genome into a double-strand DNA copy. The viral DNA, after migration to the cell nucleus, is inserted into the host chromosome via the action of another viral encoded protein, integrase. The production of new viral particles can begin after nucleotide sequences in the long terminal repeats (LTRs), located at each end of the viral genome, direct the host cell machinery to copy the viral DNA into RNA. This event produces both full length RNA providing the genetic information for the new generation of virus and messenger RNAs (mRNAs) encoding the structural proteins and enzymes of the new virus. Some of these transcripts are translated into virion precursor polypeptides, while some of the full length transcripts associate with these precursor polypeptides and assemble into immature virus particles. New infectious virions are produced following budding through the host cell membrane and processing of the precursor polypeptides by another viral encoded protein, protease.

**VIRAL PROCESSING**

The RNA genome of HIV-1 consists of three genes (*gag*, *pol*, and *env*) encoding structural proteins and enzymes, along with a number of regulatory genes (Ratner et al. 1985). Figure 1-3 outlines the genomic organization of HIV-1 as well as the processing of the viral proteins, while Table 1-1 describes the HIV-1 structural proteins and
Fig. 1-3: HIV-1 genome organization and viral protein processing

The HIV-1 genome consists of three genes encoding structural proteins and enzymes (gag, pol, and enu), along with several genes encoding regulatory and accessory proteins (vif, rev, tat, nef, vpr, and vpu). The genome is flanked on either end by long terminal repeat sequences (LTRs). Translation of the gag and pol genes produces a myristoylated Gag-Pol polyprotein precursor of 160 kDa from which three enzymes are released via viral protease processing, i.e. protease (PR), reverse transcriptase (RT), and integrase (IN). Likewise, a Gag precursor of 55 kDa is cleaved by the viral protease to release the four Gag structural proteins: matrix (MA), capsid (CA), p7, and nucleocapsid (NC). Translation of the env gene produces an Env precursor (gp160) which encodes two envelope glycoproteins (gp120 and gp41) and is processed by a cellular protease. The proteins encoded by the regulatory genes (Vif, Rev, Tat, Nef, Vpr and Vpu) are not processed. Figure adapted from Levy (1994).
Table 1-1: HIV-1 enzymes and structural proteins

The structural and catalytic proteins produced via proteolytic processing are listed and described. The approximate size of each protein is shown in kDa. Table adapted from Levy (1994).
## HIV-1 Enzymes and Structural Proteins

<table>
<thead>
<tr>
<th>Viral Protein</th>
<th>Size (kDa)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAG-POL Precursor</strong></td>
<td>Pr160</td>
<td></td>
</tr>
<tr>
<td><strong>Gag Precursor</strong></td>
<td>pr55</td>
<td></td>
</tr>
<tr>
<td>Capsid (CA)</td>
<td>p25 (p24)</td>
<td>Structural</td>
</tr>
<tr>
<td>Matrix (MA)</td>
<td>p17</td>
<td>Structural (myristoylated)</td>
</tr>
<tr>
<td>Nucleocapsid (NC)</td>
<td>p9</td>
<td>Potential RNA binding protein</td>
</tr>
<tr>
<td></td>
<td>p7</td>
<td>Potential RNA binding protein; aids in virus budding</td>
</tr>
<tr>
<td>Reverse Transcriptase (RT)</td>
<td>p66, p51</td>
<td>DNA polymerase, RNase H</td>
</tr>
<tr>
<td>Protease (PR)</td>
<td>p10</td>
<td>Post-translational processing of viral proteins</td>
</tr>
<tr>
<td>Integrase (IN)</td>
<td>p32</td>
<td>Viral DNA integration into host cell chromosome</td>
</tr>
<tr>
<td><strong>Env Precursor</strong></td>
<td>gp160</td>
<td></td>
</tr>
<tr>
<td>Envelope surface (SU)</td>
<td>gp120</td>
<td>Aids in viral binding to cell; Involved in viral budding</td>
</tr>
<tr>
<td>Envelope transmembrane (TM)</td>
<td>gp41 (gp36)</td>
<td>Aids in viral fusion to cell; Involved in viral budding</td>
</tr>
</tbody>
</table>
enzymes and their functions. The \textit{gag}-coding region is located at the 5' end of the genome and followed by the \textit{pol}-coding region. The \textit{gag} gene encodes the following structural proteins: matrix (MA), capsid (CA) and nucleocapsid (NC); whereas the \textit{pol} gene encodes the viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN) (Ratner \textit{et al.} 1985; Di Marzo Veronese \textit{et al.} 1986; Leis \textit{et al.} 1988; Lillehoj \textit{et al.} 1988). Interestingly, the \textit{pol} gene does not contain an initiation codon. Rather, translation of the \textit{pol} gene occurs only following a frameshift event during translation of the \textit{gag} gene to produce the Gag-Pol fusion protein (Jacks and Varmus 1985; Jacks \textit{et al.} 1987; Moore \textit{et al.} 1987; Jacks \textit{et al.} 1988). This event occurs only rarely (1-10\%) as usually protein synthesis is terminated at the \textit{gag} termination codon to produce the Gag polyprotein. Collectively, these events have the effect of producing much higher levels of structural proteins as compared to viral enzymes, thereby regulating the expression of the viral enzymes involved in replication. The \textit{env} gene, encoding the envelope glycoproteins gp120 and gp41, is located near the 3' end of the viral genome. Attachment to the cell, as well as viral entry, are mediated by these viral glycoproteins. The regulatory and accessory proteins of HIV-1, namely Tat, Rev, Nef, Vpr, Vpu, Vpx and Tev, are described in Table 1-2. These proteins are involved in regulation of viral replication, as well as viral infectivity.

HIV-1 PR is responsible for the various cleavage events necessary to produce both active structural proteins as well as the viral
Table 1-2: HIV-1 regulatory and accessory proteins

The HIV-1 proteins responsible for regulating viral gene expression and viral infectivity are listed and described. The approximate size of each protein is given in kDa. Table adapted from Levy (1994).
### HIV-1 Regulatory and Accessory Proteins

<table>
<thead>
<tr>
<th>Viral Protein</th>
<th>Size (kDa)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Regulatory</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tat</td>
<td>p14</td>
<td>Transactivates LTR-directed gene expression</td>
</tr>
<tr>
<td>Rev</td>
<td>p19</td>
<td>Regulates viral mRNA expression</td>
</tr>
<tr>
<td>Nef</td>
<td>p27</td>
<td>Down regulates CD4 surface expression in T-cells; effect on HIV-1 replication unclear</td>
</tr>
<tr>
<td><strong>Accessory</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vif</td>
<td>p23</td>
<td>Increases virus infectivity</td>
</tr>
<tr>
<td>Vpr</td>
<td>p15</td>
<td>Accelerates virus replication</td>
</tr>
<tr>
<td>Vpu</td>
<td>p16</td>
<td>Facilitates assembly and/or release of virus particles</td>
</tr>
<tr>
<td>Vpx</td>
<td>p15</td>
<td>Aids in infectivity</td>
</tr>
<tr>
<td>Tev</td>
<td>p26</td>
<td>Tat and Rev activities</td>
</tr>
</tbody>
</table>
enzymes, and the processing events of the HIV-1 PR are essential for production and assembly of new, infectious virus (Kohl et al. 1988; Peng et al. 1989). HIV-1 PR cleaves both the gag and the polyproenzyme to release MA, CA, NC, IN, RT and PR (Lillehoj et al. 1988). HIV-1 PR also generates the 51 kDa subunit of RT by proteolytic cleavage of the full-length RT gene product (p66) (Di Marzo Veronese et al. 1986; Lightfoote et al. 1986). Cleavage of p66 between Phe440 and Tyr441 releases a 15 kDa peptide containing most of the functional ribonuclease H (RNase H) domain (Le Grice et al. 1989; Mizrahi et al. 1989; Becerra et al. 1990; Graves et al. 1990).

OVERVIEW OF RT FUNCTION:

Reverse transcriptase is the only retroviral enzyme required to convert the single-stranded RNA retroviral genome into double-stranded DNA needed for integration into the host cell chromosome. The current model of reverse transcription is demonstrated in Figure 1-4 (Peliska and Benkovic 1992) and consists of the following steps:

- initiation of minus-strand synthesis
- first strand transfer event
- completion of minus strand synthesis
- RNase H degradation of the viral RNA genome
- plus-strand DNA synthesis
- RNase H removal of replication primers
- second strand transfer event
- completion of plus-strand synthesis
Fig. 1-4: Model of reverse transcription

Viral RNA is represented as thin lines and lower case letters; viral DNA as bold lines and upper case letters. HIV-1 reverse transcription begins upon binding of the viral replication primer, tRNA\textsuperscript{Lys},\textsuperscript{3}, to the primer binding site (pbs) of the viral RNA genome. Following initiation of minus strand DNA synthesis and RNase H degradation of the r and u5 sequences, the short nascent DNA strand is translocated to the 3' end of the same or a second viral RNA molecule (first DNA strand transfer). Minus strand DNA synthesis then continues through the pbs. The viral RNA template is concomitantly removed via RNase H hydrolysis, and plus-strand DNA synthesis initiates from an RNase H-resistant polypurine-rich RNA primer located near the U3 region. Plus-strand DNA synthesis is completed following a second strand transfer event. These events produce a double-strand DNA copy of the viral genome which is integrated into the host chromosome. Figure adapted from: Peliska and Benkovic (1992).
viral RNA

(-) strand DNA → 3' R U5 0 ← tRNA primer

RNase H

(-) strand DNA transfer

(+/-) strand DNA transfer

(+/-) strand DNA → 5' U3 R U5 PBS 3' PBS R U5

RNase H

(+/-) strand DNA transfer

(+/-) strand DNA → 5' U3 R U5 PBS 3' PBS R U5

RNase H

(+/-) strand DNA

(-) strand DNA
Minus strand synthesis initiates from a specific transfer RNA primer (tRNA\textsuperscript{Lys,3} for HIV-1) hybridized to the primer-binding site (PBS) near the 5' end of the viral genome and continues to the 5' end of the RNA (Ratner et al. 1985). Following unwinding of the tRNA acceptor stem, 18 nucleotides base-pair with the viral RNA. The RNA 5' to PBS is known as U5, whereas the RNA 3' to the PBS is referred to as the leader. Several viral RNA secondary structures have been determined to be important in initiation of HIV-2 and avian sarcoma/leukosis virus (ASLV) DNA synthesis (Cobrinik et al. 1988; Cobrinik et al. 1991; Aiyar et al. 1992; Berkhout and Schonveld 1993; Aiyar et al. 1994). These authors have suggested two different secondary structural elements located in the U5 region of the viral RNA, namely the U5-leader stem and the U5-inverted repeat stem, are necessary for efficient initiation of reverse transcription. Potentially, the T\textsuperscript{YC} loop of the tRNA primer interacts with 7 unpaired bases in the U5 region of the ASLV genome. However, HIV-1 replication may involve an additional interaction between the anticodon loop of tRNA\textsuperscript{Lys,3} and a loop in the viral RNA (Baudin et al. 1993; Isel et al. 1993). Furthermore, both RT and NC proteins are involved in assembling the initiation complex. RT has been shown to be required to anneal the tRNA primer to the PBS sequence (Aiyar et al. 1992), whereas, NC has been shown to have a functional effect on reverse transcription (Meric and Goff 1989), potentially stabilizing the
unwound tRNA acceptor stem annealed to the viral RNA (Khan and Giedroc 1992).

The short nascent DNA strand produced by initiation of minus strand synthesis from the PBS to the 5' end of the genome (termed minus strand strong stop DNA) translocates to the 3' end of the viral RNA genome through association with the repeat sequence located on both ends of the RNA. This translocation event occurs over multiple steps, requiring DNA polymerase, RNase H, and polynucleotide unwinding activities. Following completion of minus strand DNA synthesis and RNase H hydrolysis of the RNA template, initiation of plus strand DNA synthesis occurs at a polypurine-rich RNA primer (produced from the RT-associated RNase H degradation of the genomic RNA) located near the U3 region at the 5' end of the minus strand DNA (Champoux et al. 1984; Omer et al. 1984; Resnick et al. 1984; Huber and Richardson 1990; Wöhrl and Moelling 1990). This plus-strand strong-stop DNA terminates within the tRNA_Lys^3 primer (Swanstrom et al. 1982), and tRNA_Lys^3 and the plus-strand polypurine-rich primer are both removed by RNase H hydrolysis. An intramolecular plus strand DNA transfer then allows completion of reverse transcription, producing an intact double stranded proviral DNA.

**RT STRUCTURE**

HIV-1 RT has been crystallized with the non-nucleotide inhibitor Nevirapine (Kohlstaedt et al. 1992; Smerdon et al. 1994), a 19-base/18-base double-stranded DNA template-primer (Arnold et al.
1992; Jacobo-Molina et al. 1993), and as the unliganded p66/p51 heterodimer (Rodgers et al. 1995). The structure of RT complexed with dsDNA strongly resembles RT complexed with Nevirapine, and is presented in Figure 1-5. Due to the anatomical resemblance to a right hand, subdomains of the molecule have been designated (from the 5' terminus) fingers, palm, thumb and connection. The connection subdomain of the p66 subunit connects the RNase H domain to the rest of the molecule. The structure of the RNase H domain of heterodimer RT is in agreement with the isolated RNase H structure solved by Davies et al. (1991). Heterodimer RT is asymmetric, and the two subunits (p66, p51) are not related by a simple rotation axis. Rather, these have very different conformations.

The folding of the individual subdomains are highly conserved in p66 and p51; though the two subunits share the same sequence, the spatial relationship of these subdomains in relation to each other are quite different. p66 has more of an open conformation, where visualization of the nucleic acid binding cleft, consisting of the fingers, palm, and thumb subdomains of p66, is quite apparent. In contrast, the connection subdomain of p51 is folded up onto the palm subdomain, and the thumb subdomain is displaced from the palm, resulting in the loss of the template-primer binding cleft. The DNA polymerase active site (containing the catalytic triad of Asp185, Asp186 and Asp110) is essentially buried and inaccessible in the p51 subunit. However, Kohlstaedt et al. (1992) postulate that p51 may form a portion of the binding site for the tRNA primer, as well as part
**Fig. 1-5: Crystal structure model of HIV-1 RT**

Secondary structural elements of the p66 and p51 subunits of HIV-1 RT are illustrated in ribbon diagrams, and the amino acids corresponding to each element are presented below. Due to the anatomical resemblance to a right hand, the subdomains of each RT subunit are defined as fingers (cyan), palm (red), thumb (green), and connection (yellow) (Kohlstaedt et al. 1992). The RNase H domain is not pictured in the diagram, but corresponds to amino acids 436-560. Figure reprinted with permission from PNAS (Jacobo-Molina et al. 1993).
of the template-primer binding site. Furthermore, the thumb subdomain of p51 has extensive contacts with the RNase H domain of p66.

Nevirapine, a non-nucleotide and noncompetitive inhibitor of RT (Merluzzi et al. 1990), binds in a deep pocket adjacent to, but not overlapping, the proposed polymerase active site. The crystal structure of HIV-1 RT complexed with Nevirapine was partially refined to 2.9 Å allowing specific interactions between the protein and the drug to be defined (Smerdon et al. 1994). Nevirapine has been shown to bind in a hydrophobic pocket of RT contacting approximately 38 protein atoms from the p66 palm and thumb subdomains. Four of these residues, namely Phe\textsuperscript{227}, Trp\textsuperscript{229}, Leu\textsuperscript{234}, and Tyr\textsuperscript{319}, have not been selected as sites of drug-resistance mutations and may prove to be important, conserved residues necessary for an active, viable virus. It is believed Nevirapine may inhibit RT by affecting the movement of the thumb subdomain leading to a suppression of the translocation of substrate after nucleotide incorporation.

The structure of HIV-1 RT complexed with double-strand DNA (Jacobo-Molina et al. 1993) illustrates potential interactions with its template-primer, as shown in Figure 1-6. As mentioned above, the template-primer binding cleft is formed by the fingers, palm, and thumb subdomains of p66. The dsDNA template primer is both A-form and B-form with a 40-45° bend separating the two forms. The A-form DNA is near the polymerase active site, whereas B-form DNA is near the RNase H active site. The bend contains approximately
**Fig. 1-8:** Crystal structure model of HIV-1 RT complexed with double-strand DNA

HIV-1 RT heterodimer bound to a 19-base/18-base DNA template-primer is presented as a stereo ribbon diagram illustrating the interactions between HIV-1 RT and its substrate. The subdomains of p66 and p51 RT are as in Figure 1-4, with the RNase H domain shown in orange. Labels are as follows: T(thumb); F(fingers); P(palm); C(connection); R(RNase H). The primer strand is colored white and the template strand is colored blue. Bars connecting the two strands represent the approximate locations of the base pairs. Reprinted with permission from PNAS (Jacobo-Molina et al. 1993).
four nucleotides and is located near $\alpha$ helix H of the p66 thumb subdomain. Reverse transcriptase also uses duplex RNA in minus-strand DNA synthesis initiating from tRNA$^{Lys}$, and RNA/DNA in plus strand DNA synthesis initiating from the polypurine rich RNA primer. However, both substrates are suspected to have A-form geometry. It is unknown if these substrates would adopt similar bends or if this bending of the substrate is functionally significant.

Figure 1-7 presents an intricate view of template-primer at the polymerase active site. The model suggests the "floor" of the template-primer binding cleft consists of the connection subdomains of p66 and p51 and the thumb subdomain of p51, confirming an earlier prediction of Kohlstaedt et al. (1992) that p51 interacts with template-primer. Yet, the DNA and the floor of the cleft have only a few contacts, namely, p66 $\beta$ strand 18 interacts with both the template and the primer, while p51 $\alpha$ helix L contacts the primer strand, and p51 $\beta$ strand 20 contacts the template strand. The authors suggest that these interactions between p51 and the template-primer may be the only contacts p51 makes with the DNA. Certain regions of the RNase H domain also interact with the dsDNA, specifically $\alpha$ helix A, $\alpha$ helix B, the $\beta1-\beta2$ hairpin and the loop containing His-539. Importantly, dsDNA is not a substrate for RNase H and, in fact, is not in a position favorable for cleavage in this structure. The most extensive interactions between RT and the dsDNA, however, occur between the sugar-phosphate backbone of the DNA and the palm, thumb and fingers subdomains of p66. The palm and thumb subdomains of p66
Fig. 1-7: Model of the HIV-1 RT template-primer binding site

Structural elements of HIV-1 RT which are near the polymerase active site and could potentially form contacts with the double-strand DNA primer-template are illustrated. Reprinted with permission from PNAS (Jacobo-Molina et al. 1993).
have been described as a clamp, acting to position the template-primer correctly in the polymerase active site. Specifically, regions of the p66 palm (β strand 8 - α helix E connecting loop and β strand 5a) and the p66 fingers (β strand 4 and α helix B) form the template-grip, whereas the α helix H of the p66 thumb interacts with the sugar-phosphate backbone of the primer-strand, and the adjacent antiparallel α helix I makes contacts with the sugar-phosphate backbone of the template strand, possibly forming "tracks" for translocation of the template-primer. Similarly, the β12-β13 hairpin is designated the primer-grip as it is located near the phosphate joining the last two nucleotides of the primer 3' terminus.

Figure 1-8 illustrates the location of 3' primer nucleotides in relation to the catalytic triad of the polymerase active site. The primer-grip region of p66 may act to keep the terminal primer nucleotide in an optimal position such that the 3' hydroxyl of the primer terminus is available for nucleophilic attack on an incoming deoxynucleoside triphosphate. Furthermore, interaction between the primer strand and primer-grip is though to occur via the 5' phosphate of the terminal primer nucleotide as mentioned above. Significantly, the catalytic triad is not necessary for template-primer binding. The β12-β13 hairpin is also near the Nevirapine binding site (Kohlstaedt et al. 1992), suggesting this region may also have a role in binding the inhibitor. Interestingly, β11b, β12 and β13 are the only secondary structural elements not conserved among the two subunits. Rather
**Fig. 1-6: Illustration of the primer-grip of heterodimer HIV-1 RT**

The structure of the HIV-1 RT polymerase active site is illustrated in which the "catalytic triad" of Asp 110, Asp 185, and Asp 186 and the 3'-terminal nucleotides of the primer strand are prominently featured. The primer-grip of HIV-1 RT (β12-β13 hairpin of p66) is located at the top of the diagram in close proximity to the 3'-terminal nucleotide of the primer strand. Figure adapted from: (Jacobo-Molina et al. 1993).
Architecture of the primer-grip of p66/p51 HIV-1 RT
this amino acid stretch in p51 is either unraveled or, more likely, too flexible to be discerned by crystallography.

**RNASE H ACTIVITY**

RNase H function, the hydrolysis of RNA in an RNA/DNA hybrid, is essential in RT facilitated viral replication (Hansen et al. 1987). Following minus strand synthesis, the newly synthesized DNA strand remains hybridized to the viral RNA genome. As this DNA strand becomes the template for plus-strand synthesis, it must be removed from the RNA genome. The RNase H function of RT is responsible for this event as well as for unpairing the DNA/RNA hybrid at the 5' end of the genome during minus strand synthesis to allow the synthesized DNA strand to base pair with the complementary RNA sequence at the 3' end of the genome and complete minus strand synthesis. Retroviral RNase H also removes the RNA primers from the nascent minus and plus DNA strands (Omer et al. 1984; Panganiban and Flore 1988; Hu and Temin 1990). Interestingly, RNase H cleavage around a polypurine tract located near the 3' end of the viral genome, produces a specific primer for plus-strand initiation (upon hydrolysis of the RNA, RNase H activity produces 3' hydroxyls and 5' phosphates, with the resulting 3' termini being sufficient as DNA synthesis primers).

Retroviral RNase H was initially described as an endonuclease (Krug and Berger 1989). This activity of RNase H was later defined as a partially processive 3' to 5' endonuclease activity (DeStefano et al. 1991). However, others have reported that HIV-1 RNase H has both
endonuclease and 3′-5′ exonuclease activities (Schatz et al. 1990). The ambiguity in these results can probably be attributed to what has been described as polymerase dependent and independent RNase H activity (Peliska and Benkovic 1992). In other words, binding of the DNA polymerase to the substrate may act to lock the RNase H active site in a certain position so that cleavage can only occur specific distances toward the 3′ end of the RNA (polymerase dependent). However, not all cleavage sites are related to DNA polymerase binding to the primer (Furfine and Reardon 1991), leading to polymerase independent cleavages. The studies by DeStefano et al. (1991) and Finston and Champoux (1984) would suggest RNase H degradation of the template RNA and generation of the plus-strand polypurine rich primer are both polymerization independent. However, HIV-1 RNase H removal of tRNA from the viral genome involves specific cleavage of the RNA to leave one ribonucleotide A residue on the 5′ end of the DNA chain (Furfine and Reardon 1991; Pullen et al. 1992; Smith and Roth 1992).

HIV-1 RT is also known to exhibit a double-stranded RNA-dependent RNase activity (Ben-Artzi et al. 1992), re-designated RNase H* (Hostomsky et al. 1994). However, the RNase H and RNase H* activities have been shown to share the same catalytic site (Ben-Artzi et al. 1992). Therefore, it is likely the two functions proceed by very similar mechanisms.

The 66 kDa subunit of HIV-1 RT has both DNA polymerase and RNase H activities. The RNase H domain is located at the C-terminus
of p66 (Hansen et al. 1988; Prasad and Goff 1989; Hizi et al. 1990). p51 has no RNase H activity, as the C-terminal region essential for RNase H activity has been cleaved to form p51 (see Figure 1-9). Furthermore, only dimeric HIV-1 RT (p66/p51 or p66/p66) display full RNase H activity (Restle et al. 1992). Mutagenesis studies have demonstrated that RNase H function is essential for viral infectivity (Schatz et al. 1990; Tisdale et al. 1991). The 15 kDa fragment released upon formation of p51 can be detected in virions (Hansen et al. 1987), but is unable to remain bound to substrate through several hydrolytic events, resulting in a weak, nonprocessive RNase H activity (Hansen et al. 1988). However, others have been unable to detect any significant activity in their purified, recombinant RNase H (Becerra et al. 1990; Davies et al. 1991). Interestingly, upon mixing of the 15 kDa peptide and p51, RNase H activity is restored to that of wild-type (Davies et al. 1991; Hostomsky et al. 1991), suggesting heterodimer associated RNase H is the biologically and functionally significant form.

A number of studies have demonstrated a close association between the RNase H domain and the polymerase domain, in that mutations in the RNase H domain can affect the polymerase domain and vice versa (Prasad and Goff 1989; Hizi et al. 1990; Boyer et al. 1992). In fact, the polymerase and RNase H domains share the same substrate binding site (Krug and Berger 1991), in which the active sites are separated by approximately 15 to 18 base pairs (Wöhrl and Moelling 1990; Furfine and Reardon 1991; Gopalakrishnan et al. 1992).
**Fig. 1-9: HIV-1 RT protein sequence**

The complete amino acid sequence of HIV-1 RT is listed using the one letter abbreviation of each residue. p66 contains all 560 residues, whereas p51 consists of the first 440 amino acids. The three aspartic acids which form the "catalytic triad" of HIV-1 RT (Asp$^{110}$, Asp$^{185}$ and Asp$^{186}$) are shown in bold and underlined. Conserved residues in the RNase H domain are shown in italics and underlined. Amino acids comprising the p66 primer-grip of HIV-1 RT (residues 227-235) are boxed.
DNA-AND RNA-DEPENDENT DNA POLYMERASE ACTIVITY

Recombinant p66 RT has been shown to exhibit approximately half the specific activity (polymerase activity) as compared to wild-type heterodimer (Lowe et al. 1988; Müller et al. 1989). However, recombinant p51 has been shown to have essentially no polymerase activity (Hansen et al. 1988; Tisdale et al. 1988), although low levels of activity for p51 were demonstrated at low ionic strength (Hostomsky et al. 1991). The different activities among p66/p51, p66 and p51 may be due to varying degrees of dimerization. The heterodimer is known to be more stable than either the p66 homodimer or the p51 homodimer (Becerra et al. 1991). However, upon mutagenesis of the polymerase active site of p66 followed by reconstitution into a selectively-mutated heterodimer, p51 could not compensate for mutations at the active site of p66. Furthermore, the same active site mutations in p51 did not affect enzyme activity when reconstituted with wild-type p66 (Le Grice et al. 1991; Boyer et al. 1992; Hostomsky et al. 1992). This data agrees with the aforementioned structural information, i.e. the polymerase active site in p51 is inaccessible, and the substrate binding cleft is lost (Kohlstaedt et al. 1992; Jacobo-Molina et al. 1993). Therefore, while the heterodimer (p66/p51) is clearly the enzymatically active form of the enzyme, a catalytic role for p51 in context of this heterodimer is minimal. Kinetic analyses of HIV-1 RT nucleotide incorporation have demonstrated p66/p51 has a 2-fold lower $k_{cat}$ and a 4-fold lower $K_m$ in relation to p66 homodimer.
Therefore, the $k_{\text{cat}}/K_m$ is twice as great, suggesting a definitive catalytic advantage of the heterodimer (Anderson and Coleman 1992).

**TEMPLATE-PRIMER BINDING**

The proposed crystal structure of HIV-1 RT suggests both subunits of the heterodimer are involved in template-primer binding. In an effort to determine the character and positioning of substrate within the heterodimer, various footprinting techniques have been utilized. Metzger *et al.* (1993) used hydroxyl radical footprinting to analyze HIV-1 RT complexed with a template-primer DNA and determined RT protected 18 bases of the template DNA from hydroxyl radical attack (nucleotide positions +3 to -15) and 15 bases of the primer strand (-1 to -15). Furthermore, these authors suggest a hyperaccessibility of the DNA template to hydroxyl radicals near template position -15 predicts a local conformational change in the template which may be due to the RNase H domain of p66. DNase I footprinting provides an expanded picture, suggesting HIV-1 RT covers template nucleotides +7 to -23 and primer nucleotides -1 to -25, which is consistent with the use of a larger, less invading probe (Wöhrl *et al.* 1995).

Certain regions of HIV-1 RT have been implicated in primer binding, but the reports are somewhat ambiguous. Ultraviolet cross-linking was used to covalently bind synthetic primer pd(T)$_{16}$ to p66 of HIV-1 RT, and following V8 protease hydrolysis, demonstrated binding to amino acids 195-300 (Sobol *et al.* 1991). Similarly, Basu *et al.*
localized binding of the synthetic primer p(dT)$_{15}$ to residues 288-307 of HIV-1 RT. One of several conserved regions of retroviral RT is presented in Figure 1-10 (Xlong and Eickbush 1990). This consensus sequence contains HIV-1 amino acids 224-234. Interestingly, a portion of this highly conserved region of the p66 subunit of HIV-1 RT is hypothesized to be positioned in proximity to the phosphate joining the 3' primer nucleotides and has been designated the primer-grip (Jacobo-Molina et al. 1993; Nanni et al. 1993).

**tRNA$^{Lys}_{3}$ PRIMER BINDING**

DNA synthesis catalyzed by HIV-1 RT is initiated *in vivo* from its replication primer, tRNA$^{Lys}_{3}$ (Wain-Hobson et al. 1985; Guyader et al. 1987). The 3' end of tRNA$^{Lys}_{3}$ partially unfolds and forms 18 base pairs of duplex with the viral RNA primer binding site (pbs). *In vivo*, the nucleocapsid (NC) protein may be required along with tRNA$^{Lys}_{3}$ to initiate reverse transcription from the viral genome (Barat et al. 1989). Furthermore, mutations in NC were found to affect the efficiency of reverse transcription of the viral RNA (Meric and Goff 1989), and HIV-1 NC has been shown to unwind duplex regions of tRNA (Khan and Giedroc 1992). However, either *E. coli* tRNA$^{Gln}_{2}$ or human tRNA$^{Lys}_{3}$ can be sequestered by HIV-1 RT and initiate transcription in the absence of NC as long as the template contains a primer binding site whose sequence is complementary to the 3' end of the appropriate tRNA (Kohlstaedt and Steitz 1992).
Fig. 1-10: Consensus region of retroviral reverse transcriptase implicated in template-primer binding

Amino acid sequences of various retroviral reverse transcriptases which are highly conserved and located near or as a part of the proposed primer-grip are given. The consensus sequence consists of HIV-1 residues 224-234, and the corresponding p66 HIV-1 RT secondary structural elements are shown below. Boxed residues (227, 229, and 234) have never been variant in isolated nevirapine resistant virus.
Primer-grip motifs of retroviral reverse transcriptases

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**β11b-β12 loop**

**β12**

**β12-β13 loop**

**β13**
Recombinant HIV-1 RT can also form a stable complex with free tRNA\textsuperscript{Lys}.\textsuperscript{3} (Barat et al. 1989; Weiss et al. 1992). The HIV-1 heterodimer has only one tRNA primer binding site (Barat et al. 1989; Hostomsky et al. 1992), although it is likely many different regions of RT contact the tRNA. Using synthetic tRNA\textsuperscript{Lys}.\textsuperscript{3}, Richter-Cook et al. (1992) found this has a much higher affinity for heterodimer HIV-1 RT as compared to either p66 or p51, although some of the reduced affinity of both p66 and p51 could be due to the presence of monomers which have been shown to be devoid of enzymatic activity (Müller et al. 1989; Restle et al. 1990). Cross-linking studies with HIV-1 RT/tRNA\textsuperscript{Lys}.\textsuperscript{3} complexes have suggested the anticodon domain of the tRNA interacts with both subunits of HIV-1 RT (Barat et al. 1989). Nuclease footprinting experiments employing both natural and synthetic tRNA\textsuperscript{Lys}.\textsuperscript{3} have further demonstrated HIV-1 RT contacts each loop (D, T\textsuperscript{Ψ}C, and anticodon) of natural tRNA\textsuperscript{Lys}.\textsuperscript{3} (Figure 1-11) (Wöhrle et al. 1993).

**DIMERIZATION AND IN VITRO RECONSTITUTION**

HIV-1 RT exists in vivo as a heterodimer of 66 and 51 kDa subunits. Many investigators have studied the stability of the heterodimer p66/p51 in relation to homodimer p66 and p51, in an effort to determine a possible advantage of a heterodimeric system. Becerra et al. (1991) employed analytical ultracentrifugation and gel filtration analysis to study the monomer-dimer equilibrium of each subunit combination and found the $K_A$ of the heterodimer (4.9 x 10\textsuperscript{5}
**Fig. 1-11: tRNA^{3}_{Lys.3} structure**

The sequence and predicted structure of natural tRNA^{3}_{Lys.3} is shown. Dashed lines indicate potential loop-loop interactions within the primer. Figure adapted from Wöhrl et al. (1993). Modified bases: D, dihydrouridine; Ψ, pseudouridine; S, 2-thio-5 carboxymethyl uridine methyl ester; R, N-((9-β-D-ribofuranosyl-2-methylthiopurin-6-yl) carbamoyl) threonine; T, methylribothymidine
tRNA\text{Lys,3}
to be nearly 10-fold higher than the \( K_A \) for homodimer p66 (5.1 \( \times 10^4 \) M\(^{-1}\)), whereas homodimer p51 was unable to form stable dimers under the same conditions (Becerra et al. 1991). Using circular dichroism, Anderson and Coleman (1992) have suggested dramatic changes in the conformation of the enzyme during maturation of the heterodimer, as a significant change in the conformation of p66 occurs along with the large conformational change attributed to the formation of p51.

In order to study HIV-1 RT efficiently \textit{in vitro}, a recombinant system was established which allowed the protein to be expressed in \textit{Escherichia coli} and easily purified (Le Grice and Gruninger-Leitch 1990). A purification technique employing metal chelate affinity chromatography on Ni\(^{+2}\)/Nitrilotriacetic acid (NTA) Sepharose allows the isolation of poly-histidine extended proteins (Hochuli et al. 1987). Therefore, the addition of six histidine residues to either terminus of p66 allows selective binding of this protein to the Ni\(^{+2}\)/NTA-Sepharose column. The recombinant system initially involved an expression cassette which contained both the entire HIV-1 RT gene (p66) containing a C-terminal extension of six histidines and the gene encoding the viral protease (PR) (Le Grice and Gruninger-Leitch 1990). Expressing these together in \textit{E. coli} gives p66/p51 in a 1:1 stoichiometry with DNA polymerase and RNase H activities comparable to enzyme purified from virus. The 51 kDa subunit is only retained on the column through association with p66, as the histidine extension on the C-terminus of p66 has been removed during maturation. The
heterodimer is then eluted by application of an imidazole gradient, which competes with histidine for matrix-bound nickel. In the absence of the PR gene, p66 alone is expressed. Following the discovery of the Phe\textsuperscript{440}/Tyr\textsuperscript{441} HIV-1 PR cleavage site delineating p66 and p51 (Le Grice \textit{et al.} 1989; Graves \textit{et al.} 1990), an expression cassette containing only the gene for p51 with an N-terminal six histidine extension was constructed for p51 isolation. This histidine extended p51 lysate can then be combined with the lysate of cells expressing p66 without the histidine extension and applied to the Ni\textsuperscript{2+}/NTA-Sepharose column. In this case, only p66 able to form stable contacts with p51 will be retained on the column, which allows direct determination of dimerization efficiency. These systems have already permitted reconstitution of RT subunits from different retroviruses (Howard \textit{et al.} 1991), and RT subunits which were selectively-deuterated (Lederer \textit{et al.} 1992), selectively-mutated (Le Grice \textit{et al.} 1991) and selectively-deleted (Ghosh \textit{et al.} 1995). This approach has been proven to be important in the studies of subunit interactions and function.

The projects discussed in the following chapters will use this powerful technique of \textit{in vitro} reconstitution to localize specific functions to each subunit. Specifically, selectively-deleted HIV-1 RT heterodimers were constructed and characterized in an effort to define a possible function for the p51 subunit. Furthermore, specific site-directed mutations were made within the primer-grip of the p66 subunit, and each mutant p66 was reconstituted with a wild-type
p51 to produce several selectively-mutated HIV-1 RT heterodimers. In each case, *in vitro* reconstitution was essential to study the biologically and functionally relevant form of HIV-1 RT, the heterodimer. Both studies define regions of each subunit responsible for specific functions.
CHAPTER 2

MATERIALS AND METHODS

MATERIALS

The following materials were obtained from Boehringer Mannheim: restriction enzymes, BamHI, ClaI, HinDIII, Smal, BglII; ampicillin and kanamycin; isopropyl-β-D-thiogalactopyranoside (IPTG); phenylmethanesulphonyl fluoride (PMSF); lysozyme; dithiothreitol (DTT); Coomassie blue; T4 polynucleotide kinase; DNase I; dNTPs and ddNTPs; glycogen. The restriction enzymes BglI and PstI were from New England Biolabs, Beverley, MA. Oligonucleotides were obtained from IDT (Iowa) and the Molecular Biology Core Facility (CWRU, Ohio). Routine chemicals, including Na₂HPO₄, NaH₂PO₄, NaCl, Imidazole, Tris/HCl, Tris base, MgCl₂, MgSO₄, glycerol, urea, EDTA, sodium citrate, KCl, HEPES, and Triton X-100 were from Sigma Chemicals and Fisher Scientific. Poly(rA)/oligo(dT)₁₂₋₁₈, poly(rC)/oligo(dG)₁₂₋₁₈, poly(dC)/oligo(dG)₁₂₋₁₈ and E.coli RNA polymerase were obtained from Pharmacia/LKB, Piscataway, NJ. Protein molecular weight markers (220-15 kDa) were from BRL.
Three C-terminally truncated p51 polypeptides were derived from plasmid p6HRT51 (Schatz et al. 1990), namely, (a) p6HRT51Δ13 (p66 HIV-1 RT residues Pro^1 -> Tyr^427) (b), p6HRT51Δ19 (Pro^1 -> Pro^421) and (c), p6HRT51Δ25 (Pro^1 -> Glu^415, Figure 2-1). Each coding sequence was preceded by a poly-histidine extension for purification via metal chelate chromatography of the deleted subunits and selectively-deleted heterodimers (Hochuli et al. 1987; Hochuli et al. 1988; Le Grice and Gruninger-Leitch 1990). Deletion derivatives were constructed by the polymerase chain reaction (PCR). In order to reduce possible mutations occurring through PCR, the region amplified was minimized to include only the 125 amino acid portion of RT between His^315 and the deletion end-point. This was exchanged for the equivalent region of plasmid p6HRT51 as a PflMI/HinDIII restriction fragment. p51 derivatives were sequenced to confirm deletion endpoints and authenticity of their coding sequence. Plasmids were maintained under lac-inducible control in E. coli M15::pDMI.1 (Le Grice and Gruninger-Leitch 1990) on medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. Induction of gene expression was achieved by growing the appropriate strains in antibiotic-supplemented L-broth at 37°C until A_600nm = 0.7, followed by addition of IPTG to a final concentration of 200μg/ml. After an additional 4 hrs of incubation, cultures were centrifuged 10 min at 5,000 rpm and 4°C using a Sorvall GS-3 rotor. Bacterial pellets were washed in 50mM Na_2HPO_4/NaH_2PO_4, pH 7.8 and centrifuged again for 10 min at
Fig. 2-1: Construction of p51 deletion mutations

The subdomain regions of p51 HIV-1 RT are indicated as described by Kohlstaedt et al. 1992: fingers (gray shaded), palm (striped), thumb (black) and connection (stippled). The C-terminal amino acids of p51 located within the connection subdomain are listed, as well as the end points of the C-terminal deletions in p51Δ13, p51Δ19 and p51Δ25.
6,000 rpm and 4°C using a Sorvall SS-34 rotor. Pellets were weighed and stored at -20°C until used in purification (pellets stable at -20°C indefinitely).

PURIFICATION OF p51 DERIVATIVES AND RECONSTITUTED HETERODIMER HIV-1 RT

Wild type and mutant p51 polypeptides were purified by metal chelate and ion exchange chromatography as previously described (Le Grice et al. 1991). Bacterial pellets from IPTG-induced cultures expressing either wild-type or variant His6-p51 were resuspended in 2 volumes of 50mM Na2HPO4/NaH2PO4, pH 7.8 containing 1mM PMSF. Lysozyme was added to a final concentration of 0.5 mg/ml, and the cultures incubated on ice for approximately 30 min. NaCl was then added to a final concentration of 300mM. This suspension was then incubated at room temperature for 5 min, and sonicated for six 1 min intervals separated by 1 min cooling periods. The sonicate was then centrifuged 1 hr at 4°C and 40,000 rpm. The supernatant, after being passed twice through a 21 gauge syringe needle to reduce viscosity, was applied to a 4ml Ni+2-Nitrilotriacetic acid Sepharose (NTA-Sepharose) column previously equilibrated in RTA 7.8 buffer (50mM Na2HPO4/NaH2PO4, pH 7.8, 0.3M NaCl, 10% (v/v) glycerol) at a rate of 6ml/hr. Following extensive washing of the column with 10 column volumes of RTA 7.8 buffer and 15-20 column volumes of RTA 6.0 buffer (50mM Na2HPO4/NaH2PO4, pH 6.0, 0.3M NaCl, 10% (v/v)
glycerol) to remove weakly adsorbed proteins, bound protein was eluted with a 25 ml gradient of 0-0.5M imidazole in RTA 6.0 buffer. Protein eluted from the column was initially measured by absorbance at 280nm. Each peak protein fraction was then diluted 1:1 in 1 x SDS-PAGE sample buffer (Laemmli 1970). Ten µl aliquots were then loaded onto 10% SDS-PAGE gels containing a 3.3% stacking gel (SDS-PAGE apparatus from Bio-Rad). Gels were run at 25mA per gel for approximately 30 min and then stained with Coomassie blue. Fractions containing p66 and p51 at a 1:1 stoichiometry were pooled and dialyzed in Storage Buffer D (50mM Tris/HCl pH 7.0, 25mM NaCl, 1mM EDTA and 50% glycerol).

The concentrated sample was then diluted to 5ml with RT Buffer D (50mM Tris/HCl pH 7.0, 25mM NaCl, 1mM EDTA and 10% glycerol) and applied to S-Sepharose (ion exchange column) in order to remove trace contaminants (flow rate of 6ml/hr). The column was extensively washed with RT Buffer D (10 column volumes at 12ml/hr) and heterodimer RT eluted with a 0.025-0.5M NaCl gradient in RT Buffer D. Protein was analyzed by optical density at 280nm and SDS-PAGE as above. Wild type and mutant p51 RT were dialyzed overnight in Storage Buffer D. Final preparations were approximately 95% pure, judged by Coomassie blue staining following denaturing polyacrylamide gel electrophoresis, and free of contaminating nucleases. Purified enzymes were stored at -20°C at concentrations of 1.0 mg/ml or greater. Enzymes are stable indefinitely under these conditions.
Heterodimer HIV-1 RT, containing intact p66 and a polyhistidine-extended p51 variant, was prepared as previously described (Le Grice and Gruninger-Leitch 1990; Howard et al. 1991; Lederer et al. 1992). Harvested pellets from cultures expressing mutant His6-p51 were each combined with cultures containing wild type p66, processed and applied to a Ni²⁺/NTA-Sepharose column as outlined above. Figure 2-2 presents a schematic representation of our strategy. Metal chelate chromatography yields reconstituted heterodimer and excess p51 (which binds quantitatively to the matrix). Poly-histidine-free p66 RT cannot bind to the affinity matrix and is recovered in the column effluent. Excess p51 is removed by S-Sepharose chromatography, yielding p66/p51 preparations with a 1:1 subunit stoichiometry. Reconstituted RT was stored in Storage Buffer D at -20°C at concentrations of 1.0 mg/ml or greater. Wild type p66/p51 HIV-1 RT was reconstituted in an identical fashion.

PREPARATION OF p66 PRIMER-GRIP MUTATIONS

In vitro mutagenesis (Figure 2-3) made use of the restriction endonuclease BsgI, which cleaves 5' and 3' to its recognition sequence (5'-C-G-A-(N₆)-T-G-C-3'), excising a 32 bp fragment (Kong et al. 1994). The RT coding sequence of plasmid pRT (Le Grice and Gruninger-Leitch 1990) was amplified in two portions by the polymerase chain reaction (PCR), i.e., (i) from the N-terminus to Pro²²⁶ and (ii) from Phe²²⁷ to the C-terminus. Internal PCR oligonucleotides (PCR 2 and PCR 3) were designed to contain the 3'
Fig. 2-2: Schematic representation of *in vitro* reconstitution of selectively deleted HIV-1 RT using Ni\(^{2+}\)/NTA Sepharose purification

Cultures expressing wild type p66 are mixed with those containing deleted p51 with an N-terminal poly-histidine extension. The mixed bacterial homogenates are then applied to a Ni\(^{2+}\)-nitrilotriacetic acid (NTA)-Sepharose column. The poly-histidine extended p51 will be selectively retained on the column, such that only reconstitution proficient p51 will result in the retention of p66 on the matrix. Otherwise, p66 will flow through the column and appear in the column effluent. Protein retained on the column is eluted with an imidazole gradient, and the presence of p66 in the p51-containing samples is then determined.
[i] Reconstitution proficient p51 subunit

Wild type or mutant p51

Wild type p66

Reconstitution deficient p51 subunit

[ii] p66/ p51 heterodimer

p66 + p51 subunits

Ni-NTA Sepharose

Retention of p66

Ni-NTA Sepharose

Loss of p66

[iii] Elute bound material with 0 - 0.5 M imidazole gradient

& analyze p51-containing fractions for p66
Fig. 2-3: Schematic representation of BclI cassette mutagenesis

Step 1: The nucleotide sequence encoding HIV-1 RT residues Gin$^{222}$-Met$^{230}$ is presented. Insertion of a BclI restriction site (CGANNNNTGGC) between Pro$^{226}$ and Phe$^{227}$ is described.

Step 2: Oligonucleotide pair PCR1/PCR3 amplifies the RT coding sequence such that the 5' portion of a BclI restriction site (nucleotides 'CGA') and a ClaI site were inserted downstream of Pro$^{226}$.

Step 3: Similarly, oligonucleotide pair PCR2/PCR4 incorporates a ClaI site and the 3' portion of a BclI restriction site (nucleotides 'TGC') upstream of Phe$^{227}$.

Step 4: PCR products are cleaved with ClaI and ligated into plasmid pRT to produce a new plasmid (pRT-BC) containing BclI and ClaI sites between Pro$^{226}$ and Phe$^{227}$ of HIV-1 RT. Cleavage of pRT-BC with BclI results in the excision of a 32 bp DNA fragment (cleavage sites indicated with arrows). Any oligonucleotide adaptor can then be used to replace the excised fragment.

Step 5: An adaptor altering Glu$^{224}$ to Ala is illustrated. Reconstitution of this substituted p66 with wild type p51 results in the selectively mutated heterodimer, p66E224A/p51.
1. 

```
222 223 224 225 226 227 228 229 230
Gln Lys Glu Pro Pro Phe Leu Trp Met
CAG AAA GAA CCT CCA TTC CTT TGG ATG
```

2. **PCR 1/PCR 3 Amplification**

```
ClaI  
RT 5' --- CAG AAA GAA CCT CCA CTA ATC GAT GCG GCG
        --- GTC TTT CTT GGA GGT GCT TAG CTA CGC CGC
BcgI  5'
```

3. **PCR 2/PCR 4 Amplification**

```
ClaI  
GGG GCG ATC GAT TGC TTC CTT TGG ATG --- RT 3'
        CCG CGC TAG CTA ACG AAG GAA ACC TAC --- 
Bcg I 3'
```

4. **ClaI digestion of PCR 1/PCR 3 and PCR2/PCR 4 products**

```
Gln Lys Glu Pro Pro Arg Ile Asp Cys Phe Leu Trp Met
        4
RT 5' --- CAG AAA GAA CCT CCA CGA ATC GAT TGC TTC CTT TGG ATG --- RT 3'
        4
        --- Bcg VClaI ---
```

5. **Cleavage of pRT-BC with Bcg I, ligate Glu^{224} \rightarrow Alg^{224} adaptor**

```
222 223 224 225 226 227 228 229 230
Gln Lys Ala Pro Pro Phe Leu Trp Met
RT 5' --- CAG AAA GCG CCT CCA TTC CTT TGG ATG --- RT 3'
        --- GTC TTT CGG GGA GGT AAG GAA ACC TAC ---
```

**pRT\textsuperscript{224A}**
and 5' trinucleotides of a Bcgl recognition sequence (5'-T-G-C- and -C-G-A-, respectively), together with a common hexanucleotide encoding a ClaI recognition site (-A-T-C-G-A-T-). Using this strategy, RT sequences up to residue Pro226 were amplified as a BamHI/ClaI fragment, and those from Phe227 to the C-terminus as a ClaI/HinDII fragment. The two fragments were ligated into BamHI/HinDIII-cleaved pRT, creating plasmid pRT-Bcg/Cla. Recombinant plasmids were analyzed for acquisition of a unique ClaI site, and the sequence of the RT coding region confirmed by DNA sequencing. Plasmid pRT-Bcg/Cla served as parent for generation of subsequent point mutants.

pRT-Bcg/Cla was partially digested with Bcgl (a second Bcgl site resides in the β-lactamase coding region), ligated with one of several oligonucleotide adaptors designed to sequentially alter residues Glu224 through Trp229 to Ala, and transformed into the E.coli strain M15::pDMI.1 (Le Grice and Gruninger-Leitch 1990). Due to the unusual cleavage properties of Bcgl, extraneous residues in the coding sequence of pRT-Bcg/Cla are eliminated, with the consequence that the daughter plasmid contains a single amino acid substitution in the coding sequence. The authenticity of plasmids pRTG224A - pRTW229A was again verified by DNA sequencing. As an example, Figure 2-3 illustrates construction of plasmid pRTG224A.

Plasmids pRTM230A - pRTH235A were constructed in a similar fashion. However, plasmid pRT-Bcg/Cla, which contains the additional Bcgl site, was used as the starting plasmid. Digestion of pRT-Bcg/Cla with Bcgl results in excision of the original Bcgl/Cla
adaptor fragment. A new 32 bp adaptor was then inserted which relocated the Bcgl site 9 nucleotides downstream and replaced the Clal recognition site with a unique SmaI site (producing plasmid pRT-Bcg/Sma). Digestion of pRT-Bcg/Sma with Bcgl now excises a 32 bp fragment which encodes amino acids Phe227 - Tyr232 (Figure 2-4). Three new oligonucleotide adaptors were then designed to sequentially alter Met230, Gly231 and Tyr232 to Ala, as described above. The remaining mutations, Glu233Ala, Leu234Ala and His235Ala, were constructed in the same fashion. A third pRT plasmid containing a Bcgl site was constructed which shifted the site an additional 9 nucleotides downstream. This plasmid (pRT-Bcg/Bgl) was used in conjunction with Bcgl cassette mutagenesis to produce plasmids pRT$^{E233A}$ - pRT$^{H235A}$.

PURIFICATION OF RECONSTITUTED SELECTIVELY-MUTATED HIV-1 HETEROODIMER

Selectively-mutated heterodimer HIV-1 RT was prepared by in vitro reconstitution as described (Le Grice et al. 1991; Lederer et al. 1992). Bacteria from IPTG-induced cultures containing pRT mutants were mixed with those expressing a wild type poly-histidine-extended 51 kDa subunit (derived from plasmid p6HRT51) and co-homogenized. The high speed supernatant of the bacterial homogenate was applied to Ni$^{2+}$/NTA-Sepharose and eluted with a gradient of 0 - 0.5M imidazole as outlined above. p51-containing samples were evaluated for their p66 reconstitution partner by SDS-polyacrylamide gel electrophoresis.
Fig. 2-4: Diagram of the BcoI constructs: BcoI/Cla; BcoI/Sma; BcoI/Bgl

The cleavage sites resulting from BcoI digestion of each BcoI / HIV-1 RT construct are indicated. The corresponding amino acids are listed below. Digestion of pRTBco/Cla with BcoI removes residues Glu\textsuperscript{224}-Trp\textsuperscript{229}, whereas digestion of pRTBco/Sma displaces residues Phe\textsuperscript{227}-Tyr\textsuperscript{232}, and digestion of pRTBco/Bgl removes residues Met\textsuperscript{230}-His\textsuperscript{235}. 
Primer Grip/Bcg constructs

CAG  AAA  GAA  CCT  CCA  TTC  CTT  TGG  ATG  GGT  TAT  GAA  CTC  CAT  CCT  GAT  AAA
GTC  TTT  CTT  GGA  GGT  AAG  GAA  ACC  TAC  CCA  ATA  CTT  GAG  GTA  GGA  CTA  TTT

gln  lys  glu  pro  pro  phe  leu  trp  met  gly  tyr  glu  leu  his  pro  asp  lys
222  223  224  225  226  227  228  229  230  231  232  233  234  235  236  237  238
After dialysis in Storage Buffer D, ion exchange chromatography on S-Sepharose was used to eliminate excess p51 and trace contaminants. Reconstituted RT was stored in Storage Buffer D at -20°C at concentrations of 0.2 mg/ml or higher. Heterodimer mutants p66E224A/p51 - p66H235A/p51 were prepared by these combined strategies.

IMMUNOLOGICAL ASSAYS

An immunological analysis (Western blot) was performed on column effluent samples of mutants which did not form stable heterodimers. Following SDS-PAGE electrophoresis described above, proteins were transferred onto a nylon membrane (Immobilon P, Millipore) using a Bio-Rad Trans blot apparatus (100 V for 1 hour at 4°C), according to the method of Towbin et al. (1979). Proteins were exposed to rabbit polyclonal antiserum raised against purified recombinant HIV-1 RT. Detection of immunoreactive polypeptides was accomplished using an alkaline phosphatase-coupled second antibody (Bio-Rad).

DNA POLYMERASE ASSAYS

a) Quantitative

Poly(rA)/oligo(dT)₁₂-₁₈ and poly (rC)/oligo(dG)₁₂-₁₈ template-primer combinations were used for determination of RNA-dependent DNA polymerase activity, while DNA-dependent DNA polymerase activity was determined on poly(dC)/oligo(dG)₁₂-₁₈. Assay mixtures
(30μl) contained 50mM Tris/HCl, pH 8.0, 6mM MgCl₂, 0.05% (v/v) Triton X-100, 80 mM NaCl, 5mM dithiothreitol, 0.2 unit/ml template-primer, 50μM unlabeled dNTP, 1.5 μCi ³H-TTP or ³H-GTP (DuPont/NEN) and 5-10ng enzyme. After 15 min. at 37°C, 7.5 μl aliquots were pipetted in duplicate onto Whatman DE 52 filters and dried. Filters were washed twice with 2 x SSC (SSC: 0.15M NaCl, 0.015M Na₃ citrate, pH 7.0), twice with ethanol and dried. Incorporation of radiolabeled precursor into polydeoxynucleotide was determined by liquid scintillation counting. Under these conditions, we define 1 unit of RT activity as the amount catalyzing incorporation of 1 nmole of precursor into polynucleotide in 10 min.

Under conditions described above, p51-derived DNA polymerase activity is extremely low. However, Hostovsky et al. (1991) have shown that this subunit displays considerable activity when assayed in the absence of a monovalent cation. Using this precedent, NaCl was eliminated from the assay buffer and polymerization was initiated by addition of 100ng p51.

b) Qualitative

Qualitative DNA-dependent DNA polymerase activity of the selectively-deleted heterodimers was accomplished with single stranded M13 DNA, to which a ³²P-end-labeled primer was hybridized (M13 DNA and primer courtesy of C.E. Cameron). Either selectively-deleted or wild type heterodimer (50 ng) was incubated with 125 ng of template-primer for 10 min at 37°C. Reaction products were analyzed
electrophoretically using 10% polyacrylamide/7M urea/1x TBE (50mM Tris-borate, pH 8.0, 1mM EDTA) gels run at 2000V for approximately 1.5 hr. Products were visualized by autoradiography.

In a separate assay, a synthetic 71-base DNA template with a $^{32}$P-end-labeled 36-base primer (IDT, Iowa) hybridized to its 3' terminus (Figure 2-5), was used to analyze products of "programmed" DNA-dependent DNA polymerase reactions (Wöhrl et al. 1994). This system permits analysis of "arrested" polymerization complexes, analogous to those visualized by hydroxyl radical footprinting (Metzger et al. 1993). Step-wise polymerization from the 5' $^{32}$P-labeled primer was accomplished with various elongation cocktails containing a chain-terminating ddNTP. Conditions were chosen to allow primer extension by 1, 4, 10 or 19 bases (+1 mix, 500µM ddATP; +4 mix, 50µM dATP, 500µM ddGTP; +10 mix, 50µM dATP and dGTP, 500µM ddTTP; +19 mix, 50µM dATP, dGTP, dTTP, and 500µM ddCTP). Reactions containing only dNTPs allowed complete primer extension (50µM dATP, dGTP, dTTP and dCTP). Enzyme was incubated with template-primer for 5 min at 37°C in a 7 µl mixture containing 10mM Tris/HCl pH 8.0, 80mM NaCl, 5mM DTT and 6mM MgCl$_2$, after which polymerization was initiated by addition of the appropriate dNTP/ddNTP mix. After 5 min at 37°C, DNA synthesis was terminated by addition of an equal volume of a urea-based gel loading buffer (7M urea in Tris/Borate/EDTA buffer containing 0.1% (w/v) xylene cyanol and bromophenol blue). Polymerization products were
Fig. 2-5: Template-primer used for qualitative DNA-dependent DNA polymerase analysis and DNase I footprinting

The nucleotide sequence of the 71 nt DNA template and 36 nt DNA primer used for both DNA-dependent DNA polymerase assays and DNase I footprinting analysis is shown, as well as the corresponding positions of the primer extension products. The 36 nt DNA primer is complementary to the 3' end of the template. The primer is end-labeled with $^{32}$P for DNA polymerase assays, whereas the 71 nt template is labeled with $^{32}$P on its 5' terminus for DNase I footprinting studies. The predicted conformation of the template-primer is presented below.
71 nt DNA template / 36 nt DNA primer
fractionated by high resolution gel electrophoresis and visualized by autoradiography as above.

Heparin challenge experiments involved pre-incubation of wild type or mutant RT the 71 nt template/36 nt primer (labeled at its 5' terminus with $^{32}$P) at 37°C for 5 min. Polymerization cocktail permitting primer extension by 4 nucleotides and heparin (final concentration of 2 mg/ml) were added simultaneously, and the reaction was allowed to proceed for 5 min at 37°C. DNA synthesis was terminated by the addition of an equal volume of urea based gel loading buffer, and reaction products were fractionated by high voltage electrophoresis and visualized as described above.

**DNASE I FOOTPRINTING OF REPLICATION COMPLEXES**

DNase I footprinting of replication complexes containing wild type and mutant p66/p51 RT was performed according to Wöhrl et al. (1995). Substrate was the same 71 nt DNA template described above, labeled at its 5' terminus, to which the 36 nt primer was hybridized. Template-primer was incubated with RT under conditions permitting primer extension by 4 bases ("+4" replication complexes, Metzger et al. 1993; Wöhrl et al. 1994). After 20 min at 37°C, 3.5 units of DNase I were added, and after 30 sec at room temperature the reaction was terminated by adding an equal volume of phenol/chloroform/isoamyl alcohol. Nucleic acids were precipitated in the presence of glycogen, and the dried sample was resuspended in 10 μl of urea-based gel loading buffer. Samples were fractionated by high voltage
electrophoresis and the products visualized by autoradiography. Since
the replication complexes generated increasing amounts of duplex DNA
substrate, it was necessary to prepare a control DNase I digest for the
+4 primer extension reactions. To achieve this, the appropriate
replication complex was prepared with wild type RT, then freed of
enzyme by phenol extraction. Extended DNA substrates were recovered
by ethanol precipitation and subjected to partial DNase I hydrolysis as
described above.

RNAse H ACTIVITY

Quantitative RNaseH activity was determined as previously
described (Le Grice and Gruninger-Leitch 1990). Radiolabeled
RNA/DNA hybrid was prepared by in vitro transcription of single-
stranded M13mp18 DNA with E.coli RNA polymerase. Reverse
transcriptase was incubated with substrate in 1X RNase H buffer
(50mM Tris/HCl, pH 8.0, 5mM DTT, 50mM KCl, 2mM MgCl2) at 37°C.
Samples were removed at times indicated and pipetted onto Whatman
DE 52 filters. After extensive washing with 2xSSC and ethanol, filters
were dried and radioactivity remaining in the hybrid was determined
by liquid scintillation counting.

Qualitative RNaseH activity was determined by a modification
of the method of Schatz et al. (1990). Substrate was a 90 nucleotide
(nt) RNA, prepared by in vitro transcription and labeled at its 5'
terminus with 32P, to which a 36 nt DNA oligonucleotide was
hybridized at the 3' terminus (Figure 2-6). Wild type or mutant RT
Fig. 2-6: Template-primer used for qualitative RNA-dependent DNA polymerase assays and RNase H analysis

The nucleotide sequence of the 90 nt RNA template and the 36 nt DNA primer used for RNA-dependent DNA polymerase experiments and RNase H assays is presented, along with the corresponding positions of the primer extension products. The 36 nt DNA primer is complementary to the 3' end of this RNA template. The primer is 5' \(^{32}\text{P}\) end-labeled for DNA polymerase assays, whereas the 90 nt RNA template is labeled with \(^{32}\text{P}\) at its 5' terminus for RNase H studies. The predicted conformation of the template-primer is illustrated below.
90 nt RNA template / 36 nt DNA primer

Template

5' GGCCGAAUUCGAAGCUCGGUACCCGUAAGGACUAAGGCCGCUUCURUAAUCCCUUCCCCCUCUCGGUGAUCUUUCUCUCGCCCUUGGUC 3'

Primer

5' GGUGUGAACGGACCAYAAAGGTAGGGGCAAGGG 3'
was incubated with this RNA/DNA hybrid for 1 min at 37°C in a 9 μl mixture containing 10mM Tris/HCl pH 8.0, 80mM NaCl, and 5mM DTT. Magnesium chloride was added to a final concentration of 6mM to start the reaction, and incubation continued at 37°C for the times indicated. Reactions were quenched by the addition of an equal volume of urea based loading buffer. Resulting products were fractionated by high resolution gel electrophoresis (10% polyacrylamide/7M urea/1XTBE gel; 2000V; 1.5 hr) and visualized by autoradiography.

\textbf{tRNA}^{\text{Lys}}_4 \text{ BINDING}

The interaction of selectively-mutated p66/p51 HIV-1 RT with the HIV-1 replication primer, tRNA\textsuperscript{Lys}\textsubscript{3}, was monitored by gel-mobility shift analysis as previously described (Richter-Cook \textit{et al.} 1992). Substrate for these experiments was a 5' \textsuperscript{32}P-labeled tRNA prepared by \textit{in vitro} transcription (Barat \textit{et al.} 1991), which we have shown to functionally substitute for the natural species (Wöhrle \textit{et al.} 1993). Enzyme was incubated for 15 min at room temperature with \textsuperscript{32}P-labeled tRNA\textsuperscript{Lys}\textsubscript{3} in a 5μl reaction mixture containing 25mM Hepes, pH 7.9, 0.5mM EDTA, 0.5mM MgCl\textsubscript{2}, 5mM KCl, 0.25mM DTT, 10% (v/v) glycerol. Following a 15 min incubation on ice, reaction mixtures were applied to non-denaturing 5% polyacrylamide gels, prepared in 25mM Tris/188mM glycine, 1mM EDTA, pH 8.5, and pre-run at 4°C for 1 - 2 hrs at 200 V. Electrophoresis was at 4°C for 90 min and 150V, after which gels were dried and subjected to autoradiography.
Site-directed mutagenesis was used to introduce the Trp^{229}Ala mutation into the infectious proviral clone pNI4.3 (Adachi et al. 1986). Proviral transfection of Cos7 cells was performed by standard calcium precipitation. Three days post-infection, supernatant from transfected cells was harvested and clarified by low speed centrifugation (3000 rpm, 15 min). Virus p24 core antigen (CAp24) content was determined by enzyme-linked immunosorbent assay. Virus titer was assessed by infecting 1 x 10^6 human SupT1 cells with 1 ml of clarified supernatant, or a 10-fold serial dilution (titer determined 8 days post infection). For immunological analysis, virions were collected by ultracentrifugation through a 25% sucrose cushion in 25mM Tris/HCl, pH 7.5, 50mM NaCl, 1mM EDTA (TNE). Virion pellets were disrupted in TNE containing 1% SDS, after which total protein was fractionated by SDS-polyacrylamide gel electrophoresis. Following transfer to nitrocellulose membranes, CAp24 and p66/p51 RT were detected by chemiluminescence (Amersham Corp.).
CHAPTER 3

MODULATION OF HIV-1 REVERSE TRANSCRIPTASE FUNCTION
IN SELECTIVELY-DELETED HETERODIMERS

SPECIFIC INTRODUCTION

HIV-1 RT exists in vivo as a heterodimer of two subunits, p66 and p51. p66 is known to contain the active sites for both DNA polymerase and RNase H activities. Despite the shared amino acid sequence, isolated p51 is essentially devoid of DNA polymerase activity (Hansen et al. 1988; Tisdale et al. 1988), although a low level of activity for p51 has been demonstrated at low ionic strength (Hostomsky et al. 1991). Furthermore, p51 does not contain an RNase H domain, as this domain, localized to the C-terminus of p66, is cleaved to form p51. Therefore, discerning a functional role for p51 became an interesting prospect.

The crystal structures of HIV-1 RT (Kohlstaedt et al. (1992) and Jacobo-Molina et al. (1993)) have suggested the C-terminal region of the 51 kDa subunit is in a favorable orientation to bind the replication primer tRNA\textsuperscript{Lys}.\textsuperscript{3} and may also be involved in dimerization. Therefore, in order to address the role of p51 with regards to these functions, C-terminal residues were deleted to generate mutants p51\textsuperscript{Δ13}, p51\textsuperscript{Δ19}.

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and p51Δ25, in which the respective subunits lacked 13, 19 or 25 residues. Each p51 mutant was then reconstituted with wild type p66 in an attempt to produce the selectively-deleted HIV-1 RT, p66/p51Δ13, p66/p51Δ19 and p66/p51Δ25.

RESULTS

In Vitro Reconstitution of Selectively-Deleted HIV-1 RT

As the biologically significant form of HIV-1 RT is the heterodimer, it was necessary to reconstitute each deleted p51 subunit with wild-type p66 before assessing enzyme function. As described earlier, a recombinant system allows RT expressed in E. coli to be easily purified (Le Grice and Gruninger-Leitch 1990). A purification technique employing metal chelate affinity chromatography on Ni\(^{2+}/\)NTA (Nitrilotriacetic acid)- Sepharose allows the isolation of polyhistidine extended proteins (Hochuli et al. 1987). Upon the discovery of the Phe\(^{440}/\)Tyr\(^{441}\) HIV-1 protease cleavage site delineating p66 and p51 (Le Grice et al. 1989; Graves et al. 1990), an expression cassette containing the gene for p51 preceded by six-histidines (His\(_6\)) was constructed for p51 isolation. The addition of His\(_6\) to the N-terminus of p51 allows selective binding of this protein to the Ni\(^{2+}/\)NTA-Sepharose column. Each p51 C-terminal deletion mutant (p51Δ13, p51Δ19 and p51Δ25) contained the His\(_6\) extension and was combined with poly histidine-free wild type p66 as described. In this manner, it was possible to directly access if the p51 deletion altered the ability to
form a stable heterodimer, as any p66 unable to dimerize with mutant p51 would be located in the column effluent. Assessment of dimerization was essential, as loss of any RT function due to the deletion of C-terminal residues of p51 could otherwise be attributed to a perturbation of the monomer/dimer equilibrium.

The effects of each p51 C-terminal deletion on the stability of the reconstituted heterodimer are shown in Figure 3-1. The peak protein fractions obtained from Ni⁺²/NTA-Sepharose purification were analyzed by SDS-PAGE and demonstrate that deletion of either the C-terminal 13 or 19 amino acids did not affect dimerization (both the p66 and p51 subunits are retained on the column and are co-eluted). Deletion of 25 amino acids from p51, however, severely disrupted dimer stability, such that only p51Δ25 remained bound to the Ni⁺²/NTA-Sepharose column. Western analysis confirmed the presence of wild-type p66 in the column effluent (data not shown). A significant conformational change in the deleted p51 subunit leading to the loss of essential interactions between the two subunits is the most probable explanation for our inability to obtain stable p66/p51Δ25. However, it is also possible that the deleted residues could be directly involved in dimerization. Regardless, the significant and functional form of HIV-1 RT is the heterodimer. Therefore, no further studies were conducted on the p51Δ25 mutant and larger deletions in p51 were not considered. The selectively-deleted heterodimer mutants p66/p51Δ13 and p66/p51Δ19, as well as p51Δ13 and p51Δ19
Fig. 3-1: Ni$^{2+}$/NTA-Sepharose profile of selectively deleted heterodimer HIV-1 RT

The ability of each deleted p51 subunit to reconstitute with wild type p66 to form a stable heterodimer was analyzed. Peak protein fractions (a-c), eluted from Ni$^{2+}$/NTA-Sepharose with a 0-0.5 M imidazole gradient, were fractionated by SDS-PAGE and stained with Coomassie Blue. Migration positions of p66 and His-p51 RT are indicated. The contaminating bands are nonspecifically bound bacterial proteins which are removed during the next stage of purification and are not degraded RT products (as determined by Western analysis). M, protein molecular mass markers (in kDa).
subunits, were further purified using ion exchange chromatography to obtain highly purified enzymes for functional analysis (Figure 3-2).

**DNA Polymerase Activities of Selectively-Deleted HIV-1 RT**

In order to discern any functional consequences relating to the deletion of either 13 or 19 residues from the C-terminus of p51, both RNA- and DNA-dependent DNA polymerase activities of the selectively-deleted heterodimers and p51 subunits were measured. Specific activity determinations are shown in Table 3-1. p66/p51Δ13 and p51Δ13 retained wild-type RNA-dependent DNA polymerase activity using both poly (rA)/oligo (dT)\textsubscript{12-18} and poly (rC)/oligo (dG)\textsubscript{12-18} as substrates. p66/p51Δ13 DNA-dependent DNA polymerase activity, as measured using poly (dC)/oligo (dG)\textsubscript{12-18} as template-primer, was reduced to approximately 60% of wild-type, while its p51 counterpart maintained 100% activity. In sharp contrast, the RNA- and DNA-dependent DNA polymerase activities of both p66/p51Δ19 and p51Δ19 were significantly reduced, suggesting the removal of the C-terminal 19 amino acids affects the DNA polymerase catalytic active site of p66 and/or the ability to interact with substrate, while not disrupting the ability to form a stable heterodimer.

In an effort to assay DNA polymerase activity qualitatively, wild type HIV-1 RT and the selectively-deleted mutants were allowed to extend a 15nt 5'-\textsuperscript{32}P end-labeled primer hybridized to M13 DNA. The
Fig. 3-2: S-Sepharose profile of reconstitution proficient selectively deleted heterodimer HIV-1 RT and p51 derivatives

SDS-PAGE analysis of wild type heterodimer HIV-1 RT, p66/p51Δ13, p66/p51Δ19, wt p51, p51Δ13, and p51Δ19 after ion exchange chromatography. All proteins are >95% pure. M, 98-28 kDa protein molecular mass markers.
Table 3-1: Specific DNA- and RNA-dependent DNA polymerase activities of selectively deleted heterodimer HIV-1 RT and p51 derivatives

DNA polymerase activity was measured in triplicate, the average values of which is presented. One unit of activity is defined as the amount catalyzing incorporation of 1 nmol of precursor into polydeoxynucleotide in 10 min at 37°C under the assay conditions described. RDDP, RNA-dependent DNA polymerase; DDDP, DNA-dependent DNA polymerase.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>RDDP (Units/μg) (rA)/(dT)</th>
<th>RDDP (Units/μg) (rC)/(dG)</th>
<th>DDDP (Units/μg) (dC)/(dG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p66/p51 wt</td>
<td>31.00</td>
<td>8.75</td>
<td>30.95</td>
</tr>
<tr>
<td>p66/p51Δ13</td>
<td>27.69</td>
<td>7.70</td>
<td>18.13</td>
</tr>
<tr>
<td>p66/p51Δ19</td>
<td>1.96</td>
<td>0.03</td>
<td>0.78</td>
</tr>
<tr>
<td>p51wt</td>
<td>3.76</td>
<td>1.52</td>
<td>6.94</td>
</tr>
<tr>
<td>p51Δ13</td>
<td>4.19</td>
<td>1.97</td>
<td>6.96</td>
</tr>
<tr>
<td>p51Δ19</td>
<td>0.30</td>
<td>0.16</td>
<td>2.61</td>
</tr>
</tbody>
</table>
products were then analyzed using high resolution gel electrophoresis and are shown in Figure 3-3. Both wild-type (i.e. protease-cleaved) p66/p51 and reconstituted p66/p51 efficiently extended the primer, resulting in the accumulation of high molecular weight products. p66 also produced high molecular weight cDNA, although the distribution of these products differed slightly in that an increased level of stalled products were observed. Interestingly, p66/p51Δ13 also produced a larger number of stalled intermediates, resembling the profile of p66. However, p66/p51Δ19 generated predominantly low molecular weight products. This data corresponds with the qualitative DNA polymerase data discussed above. While the removal of 13 amino acids from the C-terminus of p51 does not significantly affect DNA polymerase function, removal of six additional residues to generate p66/p51Δ19 severely disrupts DNA synthesis.

DNA- and RNA-dependent DNA polymerase activities were also measured qualitatively using a programmed primer extension assay. In each case, RT bound to a defined synthetic template-primer (described in Chapter 2) was allowed to extend the radiolabeled primer by either 4 or 19 bases, depending on the dNTP/ddNTP elongation mix used. Reaction products were analyzed by high resolution gel electrophoresis. In order to address the affinity of each selectively-deleted mutant for template-primer, each programmed primer extension reaction was also performed in the presence of heparin.
Fig. 3-3: Qualitative DNA-dependent DNA polymerase assay of selectively deleted heterodimer HIV-1 RT using a single-stranded M13 DNA template

Assays contained 50 ng of purified RT and 125 ng of M13 DNA. Products were fractionated on 10% polyacrylamide gels containing 7 M urea. Lane 1, p66/p51Δ19; lane 2, p66/p51Δ13; lane 3, wild type p66; lane 4, wild type, reconstituted p66/p51; lane 5, wild type p66/p51. Arrows indicate pause sites, and base notations are given in nucleotides.
Heparin has been previously shown to inhibit both bacterial and eukaryotic DNA-dependent RNA polymerases by forming a stable complex with the enzyme (Walter et al. 1967; Pfeffer et al. 1977). Therefore, heparin was added to our reactions to sequester free RT or RT which has disassociated from template-primer. A severely altered distribution of products in the presence of heparin would then suggest an unstable RT/substrate complex.

The results of our DNA-dependent DNA synthesis assay is presented in Figure 3-4 [A]. In the absence of heparin, wild type heterodimer HIV-1 RT catalyzed efficient primer extension, i.e. the majority of primer was extended to either position P+4 or P+19 (panel a, right lanes). Likewise, p66/p51Δ13 also formed the expected products. In agreement with earlier results, p66/p51Δ19 displayed reduced DNA-dependent DNA polymerase activity (panel c), resulting in approximately 30-40% unextended primer. Furthermore, p66/p51Δ19 was apparently undergoing frequent disassociation from the template-primer, resulting in accumulation of stalled intermediate fragments. In fact, p66/p51Δ19 did display lower affinity for template-primer than either wild type or p66/p51Δ13 when primer extension experiments were performed in the presence of heparin. Figure 3-4 [A] clearly illustrates the affinity of p66/p51Δ13 for DNA/DNA template-primer was retained (compare panels a and b, (+) heparin), whereas deletion of the C-terminal 19 residues resulted in severely reduced DNA polymerase activity in the presence of heparin.
Fig. 3-4: Qualitative DNA- and RNA-dependent polymerase assays using a programmed primer extension system and heparin challenge

DNA-dependent DNA primer extensions utilized a 5' $^{32}$P end-labeled 36 nt primer hybridized to a 71 nt DNA template as a substrate, whereas RNA-dependent DNA primer extensions used the same primer hybridized to a 90 nt RNA template. Extensions were performed using wild type HIV-1 p66/p51 RT and the selectively-deleted RT. Primer extensions to positions P+4 and P+19 are shown for each enzyme (+4, +19; (-) heparin). Lane C represents unextended 36 nt primer. Heparin challenge reactions for each enzyme are also shown (+4, +19; (+) heparin). These assays were performed in collaboration with Dr. Madhumita Ghosh.
[A] DNA-dependent DNA polymerase activity

[B] RNA-dependent DNA polymerase activity
(panel c compare (+) and (-) heparin), suggesting an altered substrate affinity.

The results of our qualitative RNA-dependent DNA polymerase assay are presented in Figure 3-4 [B]. The findings are similar to those of the DNA-dependent DNA synthesis analysis. In this assay, the 71 nt DNA template was replaced with a 90 nt RNA template (Figure 2-5), and the experiments were performed exactly as above. Wild type p66/p51 and p66/p51Δ13 have almost identical RNA-dependent DNA polymerase activities in the absence of heparin (Figure 3-4 [B] compare panels a and b), and p66/p51Δ19 is essentially devoid of RNA-dependent DNA polymerase activity, extending only a small percentage of primer to the +4 position (panel c). As the presence of heparin has such a profound effect on the RNA-dependent DNA synthesis capacity of all three enzymes, it is difficult to confidently suggest the loss of RNA-dependent DNA polymerase activity in p66/p51Δ19 was a direct result of the loss of template-primer affinity. Rather, all enzymes have a reduced affinity for this RNA/DNA substrate, resulting in the absence of extended products in the presence of heparin. However, as DNA-dependent DNA synthesis activity of p66/p51Δ19 appeared to be affected by reduced template-primer binding, the same mechanism is likely to apply.

**RNase H Activity of Selectively-Deleted HIV-1 RT**

Crystallographic analysis of HIV-1 RT have suggested the C-terminus of p51 may interact with the connection subdomain of p66,
which connects its RNase H and DNA polymerase domains (Kohlstaedt et al. 1992; Jacobo-Molina et al. 1993). Furthermore, separate investigators have reported a delicate interdependence of RNase H and polymerase activities of HIV-1 RT (Prasad and Goff 1989; Hizi et al. 1990; Boyer et al. 1992). Therefore, it was important to measure the RNase H activity of the selectively-deleted heterodimers to determine if the removal of C-terminal residues of p51 influenced p66 derived RNase H activity.

RNase H activity was initially accessed by measuring the ability of the selectively-deleted heterodimer mutants to degrade a radiolabeled RNA/DNA hybrid. The results of this analysis are shown in Figure 3-5. Clearly, the RNase H activity of p66/p51Δ13 and p66/p51Δ19 is significantly reduced (approximately 50%) in comparison to either wild-type heterodimer RT or wild-type p66 RT.

Similarly, p66/p51Δ13 and p66/p51Δ19 had significantly altered RNase H activity when measured qualitatively. In this assay, the ability to hydrolyze a specific radiolabeled 90nt RNA hybridized to a 36nt DNA primer is determined (Figure 3-6). The RNA of an RNA/DNA template-primer is not cleaved at random. Rather, alignment of the DNA polymerase active center of RT over the primer 3' OH directs specific RNase H cleavages. Under these conditions, wild type HIV-1 RT cleaves all radiolabeled RNA template to release primarily three fragments of sizes 71, 64, and 62 nt. The 71 nt hydrolysis product results from an initial endonucleolytic cleavage at template position -17 (17 bp upstream of the primer 3' terminus), and the 64 and 62 nt
Fig. 3-5: Quantitative RNase H activity of selectively deleted heterodimer HIV-1 RT

RNase H activity was determined by release of radioactivity from a radiolabeled RNA-DNA hybrid. Activities are presented as % of input counts remaining in the RNA-DNA substrate at varying incubation times. wild-type p66/p51 (open circle); wild type p66/p66 (darkened square); p66/p51Δ13 (darkened triangle); p66/51Δ19 (darkened, inverted triangle); control sample of the RNA/DNA hybrid in the absence of RT (darkened circle).
Fig. 3-6: RNase H activity of selectively deleted heterodimer HIV-1 RT using a defined heteropolymeric RNA/DNA hybrid

RNase H analysis of wild type p66/p51, p66/p51Δ13 and p66/p51Δ19 are presented at three different enzyme concentrations. An autoradiogram of the RNase H cleavage products is illustrated. Size markers were derived from a DNA sequencing ladder (lane M). Analysis was performed in collaboration with Dr. Madhumita Ghosh. Substrate (presented below) was a 5' 32P end-labeled 90 nt RNA to which a 36 nt primer was hybridized to the 3' terminus. The endonucleolytic cleavage and directional processing resulting from wild type HIV-1 RT RNase H activity are indicated.
products are most likely a result of directional processing of the cleaved template to positions -10 and -8, previously referred to as exonuclease activity (Schatz et al. 1990). Both p66/p51Δ13 and p66/p51Δ19 have reduced activity, i.e. uncleaved substrate persists even when higher levels of enzyme are used (panel c), although the results of p66/p51Δ19 are more dramatic. More significantly, the distribution of cleavage products are very different for p66/p51Δ13 and p66/p51Δ19 as compared to wild type. Rather, larger products are formed, the smallest of which is approximately 71 nt. Furthermore, subsequent cleavage events are also absent in reactions catalyzed by p66/p51Δ13 or p66/p51Δ19 (panels b and c). This result may be an indication that these larger RNA fragments may not be intermediate cleavage products. Rather, these enzymes may contain RNase H domains which have been structurally affected by the p51 C-terminal deletions, such that the RNase H active center is re-positioned over the substrate.

HIV-1 RT also degrades double-strand RNA (Ben-Artzi et al. 1992), an activity which has been re-designated RNase H* (Hostomsky et al. 1994). As the RNase H and RNase H* activities have been shown to share the same catalytic center, we assumed the RNase H and RNase H* activities would be similar and did not directly assess RNase H* activity.

**tRNA\textsuperscript{17s} Primer Binding to Selectively-Deleted HIV-1 RT**

As the crystal structure of HIV-1 RT has suggested the C-terminal region of p51 may be involved in sequestering the replication...
primer (Kohlstaedt et al. 1992), the ability of our selectively-deleted HIV-1 RT heterodimers to interact with tRNA<sub>Lys</sub>.3 and form a stable complex was addressed. Synthetic tRNA<sub>Lys</sub>.3 has previously been demonstrated to substitute for natural tRNA<sub>Lys</sub>.3 and form a stable RT/tRNA<sub>Lys</sub>.3 complex (Barat et al. 1991; Kohlstaedt and Steitz 1992; Weiss et al. 1992; Wöhrle et al. 1993). Therefore, gel-shift analyses with synthetic tRNA<sub>Lys</sub>.3 were performed with wild type p66/p51 HIV-1 RT and mutants p66/p51Δ13 and p66/p51Δ19. The enzymes were incubated with tRNA<sub>Lys</sub>.3, and the products analyzed under non-denaturing conditions. The ability to form a RT/tRNA<sub>Lys</sub>.3 complex corresponds to a shift of the radiolabeled tRNA<sub>Lys</sub>.3 and indirectly measures affinity of synthetic tRNA<sub>Lys</sub>.3 for heterodimer HIV-1 RT. The results of this analysis is shown in Figure 3-7. While wild-type heterodimer HIV-1 RT is able to bind synthetic tRNA<sub>Lys</sub>.3 at both RT/tRNA ratios tested, no detectable binding was observed at either ratio for p66/p51Δ19. p66/p51Δ13 was also severely impaired in tRNA<sub>Lys</sub>.3 binding, demonstrating only minimal affinity for tRNA<sub>Lys</sub>.3 at a 3:1 RT/tRNA ratio.

**DISCUSSION**

HIV-1 RT is a multifunctional enzyme, exhibiting RNA- and DNA-dependent DNA polymerase, RNase H, and RNase H* activities. It is also responsible for sequestering the replication primer tRNA<sub>Lys</sub>.3. Yet, both polymerase and RNase H activities have been localized to the
Fig. 3-7: Binding of the cognate replication primer, tRNA$^{Lys}_3$, to selectively deleted heterodimer HIV-1 RT

Assays contained 0.5 pmol (12ng) of radiolabeled tRNA$^{Lys}_3$ and either 0.75 pmol (90 ng) (lanes 1, 3, and 5) or 1.5 pmol (180ng) (lanes 2, 4, and 6) of heterodimer RT. Migration positions of free and RT-bound tRNA$^{Lys}_3$ are indicated.
p66 subunit. Furthermore, cross-linking studies have also implicated p66 as the subunit primarily responsible for binding the primer strand of the template-primer duplex (Sobol et al. 1991; Basu et al. 1992). Therefore, a role for the 51 kDa subunit has not been defined.

Crystallographic studies with heterodimer HIV-1 RT complexed with Nevirapine and double strand DNA have suggested the connection subdomain of p51 may form important contacts with tRNA\textsubscript{Lys.3} by interacting with the tRNA-viral RNA duplex and/or anticodon and D stem/loop structures (Kohlstaedt et al. 1992; Jacobo-Molina et al. 1993). However, the structure of a HIV-1 RT/tRNA\textsubscript{Lys.3} complex has not yet been solved to validate this hypothesis. As a consequence, we decided to investigate the contribution of the p51 C-terminus to the various functions of RT by analyzing a series of p51 mutants containing C-terminal deletions.

As the biologically and functionally significant form of HIV-1 RT is clearly the heterodimer (Di Marzo Veronese et al. 1986; Lightfoote et al. 1986), it was essential to study p51 in that context. Therefore, following construction of three C-terminal truncated p51 subunits, \textit{in vitro} reconstitution was employed to produce "selectively-deleted" heterodimers which contained wild type p66 and truncated p51.

A potential structural role for the C-terminus of p51 quickly became evident when deletion of only 25 amino acids from the C-terminus impaired its ability to form a stable heterodimer. Unfortunately, the extreme C-terminus of p51 is either disordered or too flexible to be defined by crystallographic techniques. However,
deletion of these 25 amino acids from the connection subdomain of p51 removed this entire undefined region as well as the last residue of β strand 20 (see Figure 1-5). Kohlstaedt et al. (1992) have suggested the p51 connection subdomain may interact with the connection subdomain of p66 forming contacts which could be crucial to dimerization. Therefore, it is possible that deletion of these 25 residues eliminate residues involved in forming a portion of the dimer interface. More likely, removal of these amino acids, which includes the last residue of β strand 20, may affect the tertiary structure of this subdomain by disrupting potentially important interactions between various secondary structural elements (i.e. β strand 20 may no longer interact with β strand 19, resulting in increased flexibility of α helix L). This loss of ordered structure may then result in the loss of dimerization function exhibited by p51Δ25.

As deletion of 25 amino acids from the C-terminus of p51 yielded a mutant incapable of forming a stable heterodimer (and since heterodimer RT is the biologically relevant form of the enzyme), no larger deletions were made. Interestingly, p66/p51Δ13 retained wild type RNA- and DNA-dependent DNA polymerase activity, whereas p66/p51Δ19 displayed significantly reduced DNA polymerase activities. This reduced activity was shown to be attributable, at least in part, to loss of template-primer affinity. One possible explanation for this result relies on the findings of Jacobo-Molina et al. (1992) which suggest β strand 20 of the connection subdomain of p51 interacts with the template strand, while α helix L contacts the primer
strand. Although deletion of 19 amino acids does not disrupt the 
dimer interface, it may slightly alter the positioning of $\beta$ strand 20 
and/or $\alpha$ helix L in such a way that the enzyme can no longer correctly 
interact with substrate. Removal of the C-terminal 13 amino acids 
does not affect template-primer affinity, suggesting that residues 
422-427 are either directly involved in binding template-primer or are 
necessary to retain the correct conformation required for stable 
binding.

Different modes of RNase H activity have been previously 
reported for HIV-1 RT, i.e. polymerase independent and polymerase 
dependent RNase H activities defined by Peliska and Benkovic (1992). 
In other words, RNase H activity is known to consist of a polymerase 
dependent endonucleolytic cleavage 18nt behind the primer 3' OH 
accompanied by polymerase independent directional processing of the 
RNA template from position -17 to -8 (previously defined as  
exonuclease activity by Schatz et al. (1990)). Furthermore, strand 
transfer of HIV-1 RT has been shown to be severely disrupted when 
these RNase H activities of HIV-1 RT are uncoupled (Ghosh et al. 1995). 
Therefore, as neither p66/p51A13 nor p66/p51A19 retained wild type 
RNase H activity in our quantitative assay, we characterized these 
reduced activities by directly analyzing the RNase H products derived 
from a defined heteropolymeric RNA/DNA substrate. Potentially, the 
reduced activity of the two mutants could reflect reduction in both 
RNase H activities or their uncoupling and loss of directional 
processing as seen by Ghosh et al. (1995). However, the RNase H
activity of these mutants resulted in a unique phenotype, as the
distribution of cleavage products was clearly different from wild type,
i.e. the RNase H activity of both p66/p51Δ13 and p66/p51Δ19 resulted
in accumulation of higher molecular weight products ranging from
approximately 80 to 71 nt in length. Interestingly, smaller C-terminal
deletions constructed recently (p66/p51Δ5 and p66/p51Δ9) displayed
near wild type RNase H activities, resulting in persistence of the 71 nt
intermediate, corresponding to reduced directional processing of the
substrate (M. Ghosh, personal communication). Potentially,
p66/p51Δ13, while not directly affecting template-primer affinity,
may alter the relative positions of β strand 20 and α helix L of p51 and
affect the positioning of the template-primer within the substrate
binding cleft, resulting in an RNase H endonucleolytic cleavage
between different template bases. On the other hand, as the
connection subdomains of p51 and p66 are known to interact, it is
possible the deletion of 13 or 19 amino acids from the connection
subdomain of p51 may alter its contacts with the connection domain
of p66 resulting in the repositioning of the RNase H domain with
respect to bound template-primer. The crystal structure of unliganded
HIV-1 RT (Rodgers, 1995) supports the latter hypothesis (D. Rodgers
and S. Harrison, personal communication). In any event, the fact that
deletions in p51 could affect a region found only in p66 indicates a
very intricate relationship between the two subunits.

As p66/p51Δ13 could not efficiently sequester unbound
tRNA^lys, but retained wild type DNA polymerase activity and
affinity for template-primer, binding of the replication primer (tRNA\textsubscript{Lys}.3) and the primer strand of template-primer probably involves different protein-nucleic acid interactions. Interestingly, p66/p51Δ13 is unable to initiate minus strand DNA synthesis from tRNA\textsubscript{Lys}.3 hybridized to the PBS sequence, but is capable of extending an 18 nt DNA or RNA primer hybridized to the same sequence (Eric J. Arts, personal communication). The inability of p66/p51Δ13 to use tRNA\textsubscript{Lys}.3 as a primer for minus strand DNA synthesis suggests the C-terminus of p51 may be directly interacting with a specific structural element of tRNA\textsubscript{Lys}.3. If this is true, the C-terminus may be contacting the TΨC, D, or anticodon loop of tRNA\textsubscript{Lys}.3, all of which have previously been shown to interact with HIV-1 RT (Wöhrl et al. 1993). However, it is also possible that the C-terminus of p51 is merely necessary to maintain the correct inter-subunit architecture for association with tRNA\textsubscript{Lys}.3.

**FUTURE DIRECTIONS**

Although the mechanism has not been precisely defined, the C-terminus of p51 HIV-1 RT clearly contributes to tRNA\textsubscript{Lys}.3 binding and RNase H activity. However, structural information about the extreme C-terminus is minimal; the last ordered structure is β strand 20, which is deleted only in p51Δ25. In an effort to more precisely define the role of the C-terminus of p51 HIV-1 RT, two additional deletion
mutants have already been constructed and partially characterized, namely p66/p51Δ5 and p66/p51Δ9. Both have wild type DNA polymerase and near wild type RNase H activities (similar rate of endonucleolytic cleavage, slightly reduced rate of directional processing (Ghosh, personal communication)), while tRNA\textsuperscript{Lys,3} binding is presently under investigation.

Construction and characterization of mutants p66/p51Δ10, p66/p51Δ11, and p66/p51Δ12, along with continued analysis of p66/p51Δ5 and p66/p51Δ9, could yield important information on which C-terminal residues, if any, are dispensable for tRNA\textsuperscript{Lys,3} binding. In order to specifically address the disruption of tRNA\textsuperscript{Lys,3} binding to p66/p51Δ13, RNase A and/or iodine footprinting analysis (Schatz et al. 1991; Rudinger et al. 1992; Wöhrl et al. 1993) of the selectively-deleted mutants in the presence of tRNA\textsuperscript{Lys,3} could identify which tRNA domain contacts (i.e. D-loop, ΨC loop, or anticodon) are influenced by the various deletions.

Furthermore, the nucleocapsid protein (NC) has been proposed to be involved in the annealing of tRNA\textsuperscript{Lys,3} to the viral PBS in initiation of minus strand DNA synthesis (Barat et al. 1989). NC stabilization of the RT/tRNA\textsuperscript{Lys,3}/PBS complex could be addressed by determining whether NC could compensate for the apparent loss of tRNA\textsuperscript{Lys,3} binding of p66/p51Δ13 and allow this mutant to catalyze tRNA primed (-) strand DNA synthesis.

The RNase H characteristics of p66/p51Δ10, p66/p51Δ11 and p66/p51Δ12 may elucidate the altered RNase H phenotype of
p66/p51Δ13 and p66/p51Δ19, as it is possible smaller deletions may not realign the RNase H domain. As DNA strand transfer function is dependent on RNase H activity (Peliska and Benkovic 1992), it would also be interesting to assay these selectively-deleted mutants with respect to this activity.

The results presented in this chapter suggest the p51 subunit, while not contributing directly to the catalytic properties of the heterodimer, is involved in several essential functions of RT. Obviously, the C-terminal region of p51 is a necessary element of heterodimer HIV-1 RT, and defining the precise mechanism that this region uses to communicate to the rest of the molecule should lead to a more complete assessment of p51 function.
CHAPTER 4

MUTAGENESIS OF THE PRIMER-GRIP OF p66
HIV-1 REVERSE TRANSCRIPTASE

SPECIFIC INTRODUCTION

Preliminary cross-linking studies have indicated the primer binding site of HIV-1 RT lies within residues 195-300 of the p66 subunit (Sobol et al. 1991). Furthermore, the structural model of the heterodimer HIV-1 RT/double strand DNA complex suggests the β12-β13 hairpin of p66 is in a position favorable for interaction with the 3' terminus of the primer strand and has been termed the primer-grip (Jacobo-Molina et al. 1993). The HIV-1 RT residues forming this hairpin structure are Phe<sup>227</sup>-His<sup>235</sup> (See Figures 1-5 and 1-7). Interestingly, these same amino acids are part of a highly conserved region, between residues 224-234, common to many retroviral RT (See Figure 1-10) (Xiong and Eickbush 1990). However, the identical residues in p51 are structurally undefined, that is the β12-β13 hairpin is either absent or too flexible to be discerned by x-ray crystallography (Kohlstaedt et al. 1992; Jacobo-Molina et al. 1993). Furthermore, these residues are essentially buried and unavailable to form contacts with either strand of the substrate. Due to the potential functional significance of this primer-grip region of p66 in heterodimer RT, single
amino acid substitutions were incorporated into p66 and analyzed in context of the biologically relevant heterodimer.

RESULTS

Mutagenesis and purification of the primer-grip mutants

The mutagenesis strategy used to create each primer-grip mutant was described in Figure 2-3. Using a method employing the restriction enzyme Bcgl, amino acids 224-235 were sequentially replaced with alanine. Substitution with alanine minimizes steric interference while avoiding formation of new hydrogen bonds or charge interactions (Carter and Wells 1988). As these identical residues are buried in the p51 subunit, the mutations were made only in p66. In vitro reconstitution was then used to combine mutant p66 with wild-type p51 in an attempt to form twelve selectively-mutated heterodimers (p66E224A/p51 - p66H235A/p51). The ability of each mutant p66 to form a stable heterodimer is demonstrated in Figure 4-1, a SDS-PAGE analysis of purified mutant heterodimers after Ni^{2+}/NTA-Sepharose and S-Sepharose ion exchange chromatography. With the exception of p66L234A/p51, all mutants were able to reconstitute into heterodimer. With regard to mutant p66L234A, Figure 4-2 depicts a Western analysis which demonstrates the presence of both the p66 and p51 subunit in the high speed supernatant applied to the Ni^{2+}/NTA-Sepharose column and the presence of p66L234A in the column effluent. Clearly, p66L234A was unable to form stable contacts
Fig. 4-1: S-Sepharose profile of reconstituted, HIV-1 RT primer-grip mutants

SDS-PAGE analysis of wild type heterodimer HIV-1 RT, its constituent p66 and p51 subunits, p66^{E224A}/p51 - p66^{E233A}/p51, and p66^{H235A}/p51 after metal chelate and ion exchange chromatography. All proteins are >95% pure. M, 97-28 kDa protein molecular mass markers.
Fig. 4-2: Western Analysis of L234A HIV-1 RT reconstitution

Samples from the high speed supernatant and NTA-Sepharose effluent of the combined p66L234A / His-p51 high speed supernatant were separated by SDS-polyacrylamide gel electrophoresis. Antigen detection used polyclonal antibodies against HIV-1 RT.
p66 HIV-1 RT carrying the mutation Leu234 -> Ala234 fails to reconstitute with wild type p51.
with p51, an event which could be due to structural perturbation inflicted upon the p66 subunit by the amino acid substitution. In addition, certain mutants which reconstituted into heterodimer have a reduced affinity for their p51 counterpart, reflected by the lower yield of purified heterodimer p66G231A/p51 after in vitro reconstitution (data not shown).

**DNA polymerase activities of the primer-grip mutants**

Using high resolution gel electrophoresis, it is possible to view and analyze DNA synthesis products, thereby providing a qualitative assessment of DNA- and RNA-dependent DNA polymerase activity. In this system, HIV-1 RT and mutant heterodimers are allowed to bind a specific template-primer and only extend a specific number of nucleotides, an event controlled by employing a defined dNTP/ddNTP cocktail. As described in Materials and Methods, the template-primer utilized in this experiment is composed of a 71 nucleotide DNA template and a 36 nucleotide DNA primer complementary to the 3' end of the template. Furthermore, the single stranded portion of this template-primer is capable of intramolecular base pairing (see Figure 2-4). The hairpin structure localized to the 5' end of the template allows the assessment of strand displacement activity, as a mutant DNA polymerase devoid of this activity stalls at this position on the template (Wöhr et al. 1994).

Figure 4-3 illustrates DNA-dependent DNA polymerase activity of wild-type HIV-1 RT heterodimer and primer-grip mutants.
**Fig. 4-3:** Primer extension assay and heparin challenge analysis of HIV-1 RT primer-grip mutants: DNA-dependent DNA polymerase activity

Primer extensions used a 5' $^{32}$P end-labeled 36 nt primer hybridized to a 71 nt DNA template. For each enzyme: lane 1, 4 nt extension; lane 2, 19 nt extension. Heparin challenge reactions for each enzyme are also presented (lanes 3, 4 nt extension in the presence of heparin; lanes 4, 19 nt extension in the presence of heparin; lanes C, unextended 36 nt primer). The structural elements corresponding to the altered residues are also indicated.
Primer-grip mutants
DNA-dependent DNA polymerase Activity

[A] p66/p51 wt
[B] p66R224A/p51
[C] p66P228A/p51
[D] p66P226A/p51
[E] p66P227A/p51
[F] p66L228A/p51

Gel mobility


[B12] - strand 12

[G] p66W229A/p51
[H] p66M230A/p51
[I] p66G231A/p51
[J] p66Y232A/p51
[K] p66E233A/p51
[L] p66R235A/p51

Gel mobility


[B13] - strand 13
Most mutants efficiently extend primer to positions P+4 and P+19 (lanes 1 and 2 in panels [A], [B], [C], [D], [E], [F], [K] and [L]). However, the heterodimer mutants altered in the β12-β13 loop and those immediately surrounding the loop (p66W229A/p51 - p66Y232A/p51) have a reduced ability to extend the primer to position P+19 (lane 2, panels [G]-[J]). p66W229A/p51 is the most severely compromised mutant, as no P+19 product was discernible. Rather, accumulation of products corresponding to stalling at the template hairpin structure suggests p66W229A/p51 has reduced strand displacement activity, while its ability to extend all primer to P+4 position indicates a functional DNA-dependent DNA polymerase. Similarly, p66M230A/p51, p66G231A/p51, and p66Y232A/p51 also gave rise to stalled products in the +19 reaction. However, none demonstrated the severity of p66W229A/p51, illustrated by the presence of P+19 in addition to stalled intermediates.

Clearly, DNA-dependent DNA polymerase activity was significantly altered in several of the primer-grip heterodimer mutants. Potentially, the RNA-dependent DNA polymerase activities would be similarly affected. The primer extension assay described above was modified to incorporate a 90 nucleotide RNA template to which the same 36 nucleotide DNA primer was hybridized. The single stranded portion of the RNA template was also predicted to contain extensive secondary structure (see Figure 2-5). Therefore, as for the DNA-dependent DNA polymerase assay, any mutant unable to disrupt
this secondary structure would again result in stalled intermediates, in this case near position P+3.

RNA-dependent DNA polymerase activities of wild-type heterodimer HIV-1 RT and the heterodimer primer-grip mutants are presented in Figure 4-4. All enzymes responded more severely to the secondary structure of the RNA/DNA substrate as compared to the substrate used in the DNA-dependent DNA polymerase experiments of Figure 4-3. Clearly, wild type heterodimer HIV-1 RT was unable to completely resolve secondary structure, resulting in accumulation of stalled products near the primer and approximately position P+14. However, comparison of each mutant heterodimer HIV-1 RT to wild type suggests very similar results to the DNA-dependent DNA polymerase activities. p66W229A/p51, p66M230A/p51, p66G231A/p51, and p66Y232A/p51 were severely compromised in RNA-dependent DNA polymerase activity. p66W229A/p51 failed to extend to position P+4 or P+19 (panel [G], lanes 1 and 2), accumulating only stalled products. Furthermore, a significant amount of unextended primer was evident in reactions catalyzed by p66W229A/p51, p66M230A/p51 and p66Y232A/p51. p66G231A/p51 was slightly more efficient in RNA-dependent DNA synthesis, resulting in an increased amount of extended products P+4 and P+19 (panel [H], lanes 1 and 2) as compared to p66W229A/p51, p66M230A/p51 and p66Y232A/p51.
Fig. 4-4: Primer extension assay and heparin challenge analysis of HIV-1 RT primer-grip mutants: RNA-dependent DNA polymerase activity

Primer extension and heparin challenge experiments were performed as for analysis of DNA-dependent DNA polymerase activity, with the exception that substrate was a 90 nt RNA to which a 5' $^{32}$P end-labeled 36 nt DNA was hybridized at the 3' terminus. For each enzyme: lane 1, 4 nt extension; lane 2, 19 nt extension; lane 3, 4 nt extension in the presence of heparin; lane 4, 19 nt extension in the presence of heparin. The structural elements corresponding to the altered residues are also indicated.
Primer-grip mutants
RNA-dependent DNA polymerase activity
Template-primer affinity

The reduced DNA polymerase activity exhibited by mutants p66\(^{W229A}/p51\)-p66\(^{Y232A}/p51\) could be attributed to rapid dissociation of the enzyme from the template-primer. To test this hypothesis, the stability of the RT/template-primer complex was assessed. Heparin has previously been shown to compete with template-primer for binding to both bacterial and eukaryotic DNA-dependent RNA polymerases (Walter et al. 1967; Pfeffer et al. 1977). Therefore, utilizing the primer extension assay described above, wild type or mutant RT was allowed to bind to template-primer, to which competitor heparin was added simultaneously with the programmed extension mix (+4 or +19). As heparin is present in excess, any free RT or enzyme dissociating from template-primer would presumably be sequestered and removed from the functional RT pool. Therefore, a reduction in the level of extended products in the presence of heparin suggests a decreased affinity for template-primer.

Heparin challenge experiments for both DNA- and RNA-dependent DNA synthesis are shown in Figures 4-3 and 4-4, respectively. The most dramatic results were seen in the DNA-dependent DNA polymerase system. While the addition of heparin did not significantly affect the ability of wild type HIV-1 RT to extend to position P+4 or P+19, three mutant enzymes (p66\(^{W229A}/p51\), p66\(^{M230A}/p51\), and p66\(^{Y232A}/p51\)) produced only trace amounts of extended products and failed to extend over 95% of the initial primer in
the presence of heparin, suggesting a severely reduced affinity for substrate. p66G231A/p51, p66^P226A/p51 and p66F227A/p51 also appeared to have lower affinity for this DNA/DNA template-primer as compared to wild type HIV-1 RT. However, this is not as pronounced as the effect noted for p66W229A/p51, p66M230A/p51 and p66Y232A/p51.

The results of the RNA-dependent DNA polymerase analysis in the presence of heparin were more obscure than those of the DNA-dependent system. In this case, wild type HIV-1 RT failed to extend primer to position P+4 or P+19, but generated only faint levels of fully extended and stalled products, while the majority of the enzyme rapidly dissociated from template-primer. Therefore, it is difficult to interpret the heparin challenge results of the primer-grip mutants. However, p66W229A/p51, p66M230A/p51, p66G231A/p51 and p66Y232A/p51 may exhibit reduced affinity for the RNA/DNA template-primer, in that extended products were completely absent.

**DNase I Footprinting of RT/template-primer complexes**

The affinity of RT for template-primer was also assessed by DNase I footprinting. Figure 4-5 B illustrates the template-primer utilized in these experiments. This template-primer is the same as that used in the DNA-dependent DNA polymerase primer extension assay, with the exception that the template is radiolabeled at its 5' terminus rather than the primer. Figures 4-5A and 4-6 indicate wild type HIV-1 RT was capable of shielding the template strand of the
**Fig. 4-5:**

**a)** DNase I footprinting of wild type and p66E224A/p51-p66W228A/p51 HIV-1 RT replication complexes

Substrate was a 5'-32P-end-labeled, 71 nt DNA template containing a 36 nt DNA primer complementary to the 3' end. DNase I footprints of +4 replication complexes are presented. Lanes D, DNase I digests of template DNA following a +4 primer extension reaction. Lane W, control footprint of wild type p66/p51 HIV-1 RT replication complex. Lane 1, p66E224A/p51; lane 2, p66P225A/p51; lane 3, p66P226A/p51; lane 4, p66F227A/p51; lane 5, p66L228A/p51; lane 6, p66W229A/p51. The position at which the chain-terminator ddGTP was incorporated is indicated with an arrow.

**b)** Diagram of HIV-1 RT protected regions of template-primer used for footprinting analyses

The filled box at the primer 3' terminus represents the 4-base extension. Open bars indicate template and primer bases protected from DNase I hydrolysis (Wöhrle, et al. 1995).
Fig. 4-6: DNase I footprinting of HIV-1 RT primer-grip mutants p66W229A/p51 - p66E233A/p51 and p66H235A/p51

Substrate and conditions were nearly identical to that described for Figure 4-5. Lane D, control DNase I digest of template DNA. Lane D+4, DNase I digests of template DNA following a +4 primer extension reaction. Lane W, control footprint of wild type p66/p51 HIV-1 RT replication complex. Lane 1, p66W229A/p51; lane 2, p66M230A/p51; lane 3, p66G231A/p51; lane 4, p66Y232A/p51; lane 5, p66E233A/p51; lane 6, p66H235A/p51. Template bases protected from DNase I hydrolysis are indicated to the right of the figure.
DNase I Footprinting of
p66W229A/p51-p66E233A/p51 and p66H235A/p51
template-primer duplex from DNase I cleavage. In agreement with Wöhrl et al. (1995), HIV-1 RT shields template nucleotides +7 to -23. Interestingly, p66E224A/p51- p66L228A/p51 were all capable of yielding a footprint (Figure 4-5A lanes 1-5). However, in Figure 4-6, it is clear that only p66G231A/p51 bound template-primer with an affinity comparable to wild-type (compare lanes W and 3), whereas p66W229A/p51, p66M230A/p51, p66Y232A/p51, and surprisingly p66E233A/p51 and p66H235A/p51, did not form stable complexes with template-primer. DNase I footprinting experiments were performed at several other protein concentrations (data not shown). The highest protein:template-primer ratio utilized (400ng of protein) resulted in all mutants yielding a discernible footprint, suggesting that no mutant is incapable of binding substrate. Rather, the mutants have varying degrees of affinity.

**RNase H analysis of the heterodimer primer-grip mutants**

The DNA polymerase and RNase H activities of HIV-1 RT are closely associated, as mutations made in the polymerase active center can affect RNase H function and vice versa (Prasad and Goff 1989; Hizi et al. 1990; Boyer et al. 1992). Therefore, it was necessary to assess RNase H activity of the primer-grip mutants with altered DNA polymerase efficiency. The 90 nt RNA template/36 nt DNA primer complex used for RNA-dependent DNA synthesis was utilized as a substrate for RNase H activity by relocating radiolabel to the 5' terminus of the RNA. The initial RNase H cleavage event is very
specific and dependent on the positioning of RT over the primer 3' terminus. Incubation of HIV-1 RT with this substrate yields a 71 nt fragment, resulting from an initial endonucleolytic cleavage event occurring at position -17 within the template RNA (i.e. 17 bp upstream of the primer 3' terminus), and a 62 nt fragment produced from directional processing of the cleaved RNA template to position -8. Although this directional processing event had been previously described as exonuclease activity, it is uncertain how many nucleotides are removed with each "exonucleolytic" cleavage (Schatz et al. 1990). Prolonged incubation of RT and substrate results in accumulation of primarily the 62 nt product, reflecting completed directional processing of the endonucleolytic cleavage product (i.e. the 71 nt intermediate).

The results of this RNase H assay are presented in Figure 4-7 for three different concentrations of HIV-1 RT and the primer-grip mutants. Using 100 ng of wild type HIV-1 RT and an incubation time of 5 sec, accumulation of primarily the 71 nt product was observed, together with slow accumulation of the 62 nt product resulting from directional processing. The majority of primer-grip mutants displayed this phenotype, although the rates of cleavage (endonucleolytic vs. directional processing) differed. Most significantly, p66Y232A/p51 clearly demonstrated an altered RNase H activity. Regardless of the amount of enzyme tested, p66Y232A/p51 cleaved the 90 nt template considerably slower than wild type RT or other primer-grip mutants, indicating a reduced efficiency in endonucleolytic cleavage (panels a, c
Fig. 4-7: RNase H activity of HIV-1 RT primer-grip mutants

RNase H analysis of wild type p66/p51 and the heterodimer primer-grip mutants are presented at three different enzyme concentrations and two incubation times. Substrate was a 5' $^{32}$P end-labeled 90 nt RNA to which a 36 nt primer was hybridized to the 3' terminus. Size markers were derived from a DNA sequencing ladder (lanes M and M'); Lane C, uncleaved $^{32}$P end-labeled 90 nt RNA; lane 1, wild-type HIV-1 p66/p51; lane 2, p66E224A/p51; lane 3, p66P225A/p51; lane 4, p66P226A/p51; lane 5, p66F227A/p51; lane 6, p66L228A/p51; lane 7, p66W229A/p51; lane 8, p66M230A/p51; lane 9, p66G231A/p51; lane 10, p66Y232A/p51; lane 11, p66E233A/p51; lane 12, p66H235A/p51.
and e, lane 10). However, p66Y232A/p51 appeared to be highly efficient in directional processing, as disappearance of the 90 nt template with prolonged incubation yielded solely the 62 nt fragment and no intermediate (panels b, d and f, lane 10). Similarly, p66M230A/p51 and p66G231A/p51 also demonstrated reduced endonucleolytic activity as compared to wild type and the other mutants (panels a and c, lanes 8 and 9). However, unlike p66Y232A/p51, the RNase H activity of both p66M230A/p51 and p66G231A/p51 resulted in significant accumulation of the 71 nt fragment after 5 sec incubation (panels c and e, lanes 8 and 9), although the directional processing of these mutants did appear to be more efficient than the other primer-grip mutants (panels b, d and f, lanes 8 and 9).

HIV-1 RT is also known to degrade double-strand RNA (Ben-Artzi et al. 1992), an activity re-designated RNase H* (Hostomsky et al. 1994). However, the RNase H and RNase H* activities have been shown to share the same catalytic center. Therefore, we assumed the RNase H and RNase H* activities would be similar and did not directly assess RNase H* activity.

tRNA_Lys.3 binding

As discussed in Chapter 3, binding of template-primer to RT appears to be separate and distinct from binding of the replication primer, tRNA_Lys.3. This is suggested by our finding that mutant p66/p51Δ13 retained affinity for template-primer, but lost affinity for
synthetic tRNA\textsubscript{Lys,3} (Jacques et al. 1994). Therefore, each primer-grip mutant was assessed for its ability to form a stable complex with synthetic tRNA\textsubscript{Lys,3} by gel-mobility shift analysis (Figure 4-8). Clearly, each enzyme is capable of sequestering tRNA\textsubscript{Lys,3}, resulting in shifted nucleoprotein complexes. However, the affinity of each mutant for tRNA\textsubscript{Lys,3} varies. p66\textsuperscript{E224A}/p51 and p66\textsuperscript{L228A}/p51 bind tRNA\textsubscript{Lys,3} as efficiently as wild type, while the ability of p66\textsuperscript{M230A}/p51 and p66\textsuperscript{Y232A}/p51 to form a stable complex with tRNA\textsubscript{Lys,3} appears to be significantly compromised, resulting in a reduced level of nucleoprotein complex.

**Loss of infectivity of p66\textsuperscript{W229A}/p51**

In order to assess biological significance, the W229A mutation, which results in a loss of template-primer affinity and strand displacement activity of recombinant HIV-1 RT, was incorporated into proviral DNA. In this case, the mutation would be introduced into both subunits of RT. This analysis was performed by Michele Ottmann and Jean-Luc Darlix of the Ecole Normale Superieure de Lyon. Briefly, Cos 7 cells were transfected with the mutant proviral DNA, after which the virus was harvested and analyzed for capsid (CAp24) and RT content (Figure 4-9). Both proteins were detected in the supernatant in levels comparable to wild type, suggesting the W229A mutation did not affect maturation of the gag/pol precursor. However, following infection of human Sup T1 cells, wild type virus resulted in significant growth, whereas mutant virus failed to propagate (Table 4-1).
Fig. 4-8: Binding of the cognate replication primer, tRNA\textsuperscript{Lys,3}, to HIV-1 RT primer-grip mutants

The results of gel-mobility shift experiments are presented, substrate for which was internally labeled, \textit{in vitro} synthesized tRNA\textsuperscript{Lys,3}. The molar RT:tRNA ratio used was approximately 10:1 (120ng RT : 2.5ng synthetic tRNA\textsuperscript{Lys,3}). Lane C, control, unbound tRNA\textsuperscript{Lys,3}. 
**Fig. 4-9: CAp24 and RT production of wild-type HIV-1 virus and mutant W229A HIV-1 virus**

Virus from supernatant of transfected Cos7 cells was disrupted, and virion-associated proteins were fractionated by SDS-PAGE. Antigen detection used polyclonal antibodies against CAp24 or purified HIV-1 RT p66/p51. Lane M, supernatant from a mock infection.
Table 4-1: Viral Infectivity of wild-type HIV-1 virus and W229A HIV-1 virus

The concentration of CAp24 in the clarified supernatant from transfected Cos7 cells was assessed by an enzyme-linked immunoabsorbent assay and virus titer by infection of human SupT1 cells with the supernatant.
CAp24 production by cells transfected with wild type or W229A virus and corresponding virus titers

<table>
<thead>
<tr>
<th>Virus</th>
<th>CAp24 antigen (ng/ml)</th>
<th>Virus titer (Infectious Units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>22</td>
<td>$10^4$</td>
</tr>
<tr>
<td>W229A</td>
<td>17</td>
<td>0</td>
</tr>
</tbody>
</table>
The crystal structure of the HIV-1 RT/double strand DNA complex implicates the $\beta_{12}$-$\beta_{13}$ hairpin of p66 in binding the primer strand of template-primer (Jacobob-Molina et al. 1993). This type of interaction has previously been shown to exist by Somers et al. (1993), who demonstrated a two-strand $\beta$ sheet is involved in Met receptor binding to DNA. This structure of HIV-1 RT, designated the primer-grip, is thought to position the primer strand in such a manner that its $3'$-OH is available to attack at the $\alpha$-phosphate group of an incoming dNTP. The primer-grip of p66 is comprised of amino acids Phe$^{227}$-His$^{235}$. These identical residues in p51, however, are essentially buried and removed from the substrate binding cleft. Furthermore, most of the primer-grip residues are included in a highly conserved region of retroviral RT encompassing residues 224-234 (Xiong and Eickbush 1990).

As this region of high homology appeared to have a potential function in p66 of HIV-1 RT, a series of single amino acid substitutions were incorporated into this region of p66 and reconstituted with wild type p51 in order to produce the selectively-mutated heterodimers p66$^{E224A}$/p51- p66$^{H235A}$/p51. Interestingly, the Leu$^{234}$ to Ala mutation resulted in complete loss of dimerization capacity, suggesting this highly conserved residue has a role other than positioning the primer strand of the substrate. This branched side
chain is the center residue of β strand 13 and may be necessary to stabilize the β12-β13 hairpin. Complete disruption of the β12-β13 hairpin could potentially exert a larger effect on the overall conformation of p66, resulting in a dimerization deficient subunit. The Gly^{231}Ala mutation also produced a reconstitution deficient p66. Some functional heterodimer was obtained, although the levels were significantly reduced compared to those obtained for other primer-grip mutants (<25%). Most likely, Gly^{231}, which is located in the loop connecting β12 and β13, is essential for the formation of this hairpin turn. Replacing Gly with any larger, less flexible residue may result in the inability of the peptide chain to make the tight turn necessary to form this antiparallel β sheet. Due to the significant dimerization deficiency of p66^{G231A}, the functional assessment of the recovered heterodimer p66^{G231A}/p51 presented may not be a fair characterization of the effects of the mutation on template-primer binding. Rather, it is possible the recovered heterodimer may be relatively unstable in solution, resulting in an altered monomer/dimer equilibrium. As it is difficult to reliably determine the ratio of monomer/dimer present in this preparation, discussion of the functional characterization of p66^{G231A}/p51 will be minimal. However, Gly^{231} is obviously an important residue in retaining the correct subunit architecture.

Proteins that bind DNA irrespective of nucleotide sequence are believed to recognize primarily the DNA backbone of phosphate and sugar groups. Furthermore, protein residues which directly interact
with DNA are often polar in nature, and those with multiple H-bonding side chains, namely Asn, Gin, Arg, Asp and Glu, are preferred (Creighton, 1993). The β12-β13 hairpin contains the following four polar residues: Gly\(^{231}\), Tyr\(^{232}\), Glu\(^{233}\), and His\(^{235}\). Interestingly, p66\(^{Y232A}\)/p51 displayed reduced template-primer affinity in both our heparin challenge and DNase I footprinting experiments. Similarly, p66\(^{E233A}\)/p51 and p66\(^{H235A}\)/p51 did not yield DNase I footprints, suggesting reduced affinity for substrate. However, the slight decrease in RT/template-primer stability detected by heparin challenge for these enzymes was less definitive. According to the crystal structure of the HIV-1 RT/ds DNA complex, Glu\(^{233}\) and particularly His\(^{235}\) are not in the immediate vicinity of the primer strand and may not be near enough to be involved in a direct interaction.

Met\(^{230}\), however, is immediately adjacent to the 3' terminus of the primer. Moreover, p66\(^{M230A}\)/p51, while stable as a heterodimer, is deficient as a DNA polymerase and displays weak template-primer affinity. Potentially, this residue may have an important role in stabilizing the RT/template-primer complex. The adjacent residue Trp\(^{229}\), also in the near vicinity of the 3' terminus of the primer strand, is obviously an essential residue, as substitution with Ala results in a non-infectious virus in vivo and an enzyme unable to efficiently bind template-primer or support strand displacement in vitro. However, it is unlikely Trp\(^{229}\) is directly interacting with nucleic acid as the side chain of Trp\(^{229}\) is buried and unavailable (E. Arnold, personal communication). Rather, Trp\(^{229}\) may contribute to
the architecture of the primer-grip. i.e. alteration of this residue may result in the repositioning of the primer-grip such that the primer 3'-OH is misaligned and unable to attack at the α-phosphate group of the incoming dNTP. Alternatively, Trp\textsuperscript{229} may act to correctly position Met\textsuperscript{230} for a direct interaction with nucleic acid. As alteration of Met\textsuperscript{230} also results in loss of function, this scenario is not unreasonable. Tyr\textsuperscript{232} may have a similar role. Removal of this bulky side chain may reposition the primer-grip such that protein-nucleic acid interactions within the hairpin are lost, or the β12-β13 hairpin structure is no longer positioned to lock the substrate in the orientation necessary for template-primer to form stabilizing interactions with other residues of RT. However, mutations made in the β12-β13 hairpin may result in a significant global conformational change within the subunit. The ability of most mutants to form stable heterodimers argues against this hypothesis, but can not be discounted without specific structural studies. Although the exact mechanism remains unclear, residues of the primer-grip have been experimentally demonstrated in this work to be involved in stabilizing the HIV-1 RT / template-primer complex.

HIV-1 RT has been shown to exhibit two distinct RNase H cleavage events, namely the polymerase-independent and polymerase-dependent cleavages described by Peliska and Benkovic (1992). The polymerase-dependent endonucleolytic cleavage can be uncoupled from the polymerase-independent directional processing of the RNA/DNA hybrid through step-wise C-terminal deletions of the RNase H domain.
of p66 (Ghosh et al. 1995). Interestingly, p66Y232A/p51, while having a reduced rate of RNase H cleavage of the starting substrate, appears to have an increased efficiency of directional processing, as the 71 nt endonucleolytic product is never allowed to accumulate. This suggests the loss of Tyr at position 232 of p66 RT leads to a repositioning of the primer-grip. A shift in positioning of template-primer near the RNase H active site could result in either altered cleavage or, as seen for p66Y232A/p51, a change in the rate of endonucleolytic cleavage and/or directional processing. This explanation is somewhat substantiated by the similar (although not as dramatic) RNase H cleavage rates observed for heterodimers containing substitutions in the adjacent residues, Met230 and Gly231.

p66M230A/p51 and p66Y232A/p51 also display slightly reduced affinity for synthetic tRNA\textsubscript{Lys}.3. Potentially, alteration of the primer-grip hairpin structure may exert an effect on the conformation of another part of the molecule partially involved in contacting tRNA\textsubscript{Lys}.3. As none of the mutations abolished tRNA\textsubscript{Lys}.3 binding dramatically, as seen previously for the selectively deleted heterodimers p66/p51A13 and p66/p51A19 (Jacques et al. 1994), it is unlikely this region is directly involved in sequestering the replication primer.

Smerdon et al. (1994) have suggested residues Phe227, Trp229 and Leu234 of the p66 primer-grip may form important contacts with Nevirapine. Nevirapine is known to bind in a hydrophobic pocket of HIV-1 RT formed by the palm and thumb subdomains of p66 and is in contact with 38 protein atoms. Interestingly, no Nevirapine-resistant
variants containing mutations at positions 227, 229 or 234 have been identified, suggesting these residues are essential for function. The data presented in this study partially substantiate this hypothesis, in that mutation of Trp^{229} to Ala in HIV-1 resulted in complete loss of viral infectivity. Furthermore, substituting Leu^{234} with Ala in p66 resulted in the inability to associate with p51 and form a stable heterodimer, suggesting Leu^{234} may also be an essential residue.

As the β12-β13 hairpin appears to be near the Nevirapine binding site (Kohlstaedt et al. 1992), other residues of the primer-grip may be necessary for RT/Nevirapine stability. Therefore, all of the heterodimer primer-grip mutants were assayed for Nevirapine sensitivity. The experiments were performed by J.M. Rose and C.-K. Shih of Boehringer Ingelheim and measured the effect of Nevirapine on the DNA-dependent DNA polymerase activity of RT. Two mutations, namely p66^{G231A}/p51 and p66^{Y232A}/p51 demonstrated some degree of resistance to Nevirapine, suggesting these mutants may have altered Nevirapine binding sites (data not shown). Unfortunately, p66^{W229A}/p51 and p66^{M230A}/p51 could not be tested, as they were devoid of DNA polymerase activity in this system. A more direct method of measuring Nevirapine binding to RT is necessary to clarify these results.
FUTURE DIRECTIONS

As each residue of the β12-β13 hairpin of p66 HIV-1 RT has now been sequentially converted to Ala and analyzed in context of the heterodimer, elucidating a detailed mechanism of substrate binding would involve more thoughtful amino acid substitutions. For instance, the precise role of Trp$^{229}$ in stabilizing the RT/template-primer interaction could be determined by replacing residue 229 with another bulky group, such as Phe or Tyr, to address the possibility that Trp is not essential as a specific residue, but that its "bulkiness" may be important for structural integrity of the template-primer binding site. Similarly, Trp$^{229}$ could be relocated to position 228, for example, to determine if its position is critical or if this group merely needs to be in the vicinity of template-primer. Likewise, the functional role of Tyr$^{232}$ could be similarly investigated. Met$^{230}$ is perhaps the most likely residue involved in direct interactions with the primer strand. Met$^{230}$ could be replaced with Ile, a somewhat conservative substitution structurally. Analysis of template-primer affinity of p66$^{M230I}$/p51 could discern if Met$^{230}$ is directly interacting with template-primer or merely maintaining the structure of the primer-grip.

Our characterization of the primer-grip thus far has implicated this region as an important factor in template-primer binding. A comparison of the structures of the p66 primer grip mutants using circular dichroism or 2-D NMR will be necessary in order to confirm
the mutations are not causing a gross conformational change. This structural assessment, as well as further mutational analysis within the primer-grip of p66 HIV-1 RT, will help to determine the precise mechanism of template-primer binding.


