ARTICLE
Journal of Cellular Biochemistry 109:598–605 (2010)

Journal of Cellular Biochemistry

A Single Deletion at Position 134, 135, or 136 in the Beta 7–Beta 8 Loop of the p51 Subunit of HIV-1 RT Disrupts the Formation of Heterodimeric Enzyme

Alok Upadhyay, Nootan Pandey, Chaturbhuj A. Mishra, Tanaji T. Talele, and Virendra N. Pandey*

Department of Biochemistry and Molecular Biology, Center for the Study of Emerging and Re-Emerging Pathogens, UMDNJ-New Jersey Medical School, 185-South Orange Avenue, Newark, New Jersey 07103

ABSTRACT

The human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) is a heterodimeric enzyme composed of p66 and p51 subunits. Earlier, we showed that the β7–β8 loop of p51 is crucial for polymerase activity of HIV-1 RT as either deletion or Ala substitution of amino acids in the β7–β8 loop spanning residues 136-139 in the p51 subunit impaired dimerization and, in turn, polymerase function of the enzyme (Pandey et al. [2001] Biochemistry 40: 9505–9512). In the present study, we generated subunit-specific single-deletion mutants at positions 134, 135, 136, or 137 and examined their effects on the heterodimerization, binary complex formation, and polymerase functions of the enzyme. We found that among these four residues, Ser134, Ile135, and Asn136 in the β7–β8 loop of the p51 subunit are crucial residues for dimerization and polymerase function of the enzyme, but have no impact when specifically deleted from the p66 subunit. These results demonstrate the β7–β8 loop of the p51 subunit in the formation of stable, functional heterodimeric enzyme which could be an attractive target for anti-HIV-1 drug development. J. Cell. Biochem. 109: 598–605, 2010.

KEY WORDS: HIV-1; REVERSE TRANSCRIPTION; DIMERIZATION

The HIV genome is a (+) strand single-stranded RNA approximately 10 kb long that codes for 15 proteins [Sierra et al., 2005; Basavapathruni and Anderson, 2007]. Three major coding regions, the env, pol, and gag genes, provide critical proteins for viral replication. Reverse transcriptase is encoded by an open reading frame overlapping the gag and pol genes of the viral genome [Levine et al., 1993]. Translation of this coding region yields a Gag-Pol fusion protein that is subsequently cleaved by viral protease to generate the mature asymmetric heterodimeric (p66/p51) reverse transcriptase present in the infectious virions [Mous et al., 1988; Le Grice and Gruninger-Leitch, 1990]. This multifunctional enzyme catalyzing both RNA- and DNA-dependent DNA polymerase activities, as well as RNase-H activity, is responsible for establishing infection by converting the viral RNA genome to double-stranded proviral DNA, which is then integrated into the host genome [Levine et al., 1993]. The polymerase and RNase-H activities resides in the p66 subunit, while the p51 subunit lacking the carboxy terminal RNase-H domain exhibits neither of these activities [Le Grice and Gruninger-Leitch, 1990; Le Grice et al., 1991; Hostomsky et al., 1992].

Studies from Le Grice's lab have shown that a deletion of eight amino acids from the C-terminal of the p51 subunit impaired the initiation of reverse transcription on natural $tRNA^{Lys3}$ [Jacques et al., 1994]. It has also been suggested that the smaller p51 subunit is

Abbreviations used: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; MuLV, murine leukemia virus; SIV, simian immunodeficiency virus; FIV, feline immunodeficiency virus; MMTV, mouse mammary tumor virus; EIAV, equine infectious anemia virus; TSAO, tertbutyldimethylsilylspiro-aminooxathioledioside; IMAC, immobilized metal affinity chromatography; U5-PBS RNA template, HIV-1 genomic RNA template corresponding to the primer binding sequence region; U5-PBS-DNA template, HIV-1 genomic DNA template corresponding to the PBS region; TP, template primer.

Grant sponsor: NIH/NIAID; Grant numbers: AI074477, AI042520.

Tanaji T. Talele's present address is Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. John's University, 8000 Utopia Parkway, Jamaica, NY 11439.

*Correspondence to: Dr. Virendra N. Pandey, Department of Biochemistry and Molecular Biology, Center for the Study of Emerging and Re-Emerging Pathogens, UMDNJ-New Jersey Medical School, 185-South Orange Avenue, Newark, NJ 07103. E-mail: pandey@umdnj.edu

Received 28 September 2009; Accepted 30 October 2009 • DOI 10.1002/jcb.22439 • © 2009 Wiley-Liss, Inc.
Published online 9 December 2009 in Wiley InterScience (www.interscience.wiley.com).

involved in strand-displacement activity during the polymerase reaction [Hottiger et al., 1994]. Hizi and coworkers have shown that the Cys → Ser mutation at position 280 in p51 alters the RNase-H activity of the heterodimeric enzyme [Loya et al., 1997]. Our work has demonstrated that the p51 subunit is involved in loading the p66 subunit onto the template primer [Harris et al., 1998], after which the p51 subunit can be physically dissociated without affecting the polymerase activity of the TP-bound p66 subunit [Harris et al., 1998]. Although the relevance of β7–β8 loop of the p51 for p66/p51 dimerization was clear from crystallographic studies [Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993], its implication on RT dimerization and activity was first demonstrated by our laboratory [Pandey et al., 2001]. The Ala substitutions, small deletions or duplication of this loop, impair dimerization and polymerase function of the enzyme [Pandey et al., 2001, 2002]. In order to develop new anti-HIV drugs targeting the dimer stability of HIV-1 RT, it will be necessary to understand the structural organization and positional significance of individual amino acid residues in the β7–β8 loop. In the present study, we have generated subunit specific deletion mutants of individual amino acids in the β7–β8 loop to determine which of these residues affect dimerization and polymerase function of the enzyme.

MATERIALS AND METHODS

Pfu Turbo polymerase for PCR amplification was purchased from Stratagene. Restriction endonucleases, DNA modifying enzymes and HPLC-purified dNTPs were purchased from Boehringer Mannheim. Fast-flow chelating sepharose (iminodiacetic-sepharose) for immobilized metal affinity chromatography (IMAC) was purchased from Pharmacia. [^{32}P]-labeled dNTPs and ATP were the products of Amersham Radiochemicals, GE Healthcare. The DNA oligomers used as template primers were synthesized at the Molecular Resource Facility at UMDNJ. All other reagents were of the highest available purity grade and purchased from Fisher, Millipore Corp., Boehringer Mannheim, and Bio-Rad.

PLASMID AND CLONES

The *expression* vector pET-28a and *E. coli* expression strain BL21 (DE3) were obtained from Novagen. The HIV-1 RT expression clones (pET-, pKK-RT66, and pET-28a-RT51) were used for PCR amplification and construction of the deletion mutants in the p66 and p51 subunits of HIV-1 RT. The HIV-RNA expression clone pHIV-PBS [Arts et al., 1994] containing a 947-bp fragment of HIV-1 genome (+473 to +1,420) was used to transcribe RNA corresponding to the U5-PBS region.

DELETION MUTANT CARRYING SINGLE DELETION OF INDIVIDUAL RESIDUES IN THE β7–β8 LOOP

Mutants carrying a single deletion of any one of the four amino acids at positions 134, 135, 136, and 137 in the β7–β8 loop of the p66 or p51 subunits were constructed using two primers corresponding to sense and antisense strands containing the desired deletion. The corresponding clones of p66 and p51 were amplified by high-fidelity PCR using a QuickChange^{TM,} Site-Directed Mutagenesis Kit from Stratagene. After ascertaining the mutation by DNA sequencing at the desired position, the mutant clones in pET and pKK vectors were introduced, respectively, into *E. coli* BL-21(DE3) pLysS and *E. coli JM109* for expression. Induction of the enzyme protein was done as described before [Pandey et al., 1996]. The enzyme with His-tag was purified from bacterial lysates by immobilized metal affinity chromatography [Pandey et al., 1996], while conventional column chromatography protocol was used to purify the non-His-tag enzyme [Hsieh et al., 1993].

PREPARATION OF THE HETERODIMERIC ENZYME WITH SUBUNIT SPECIFIC DELETION

The p51 subunit with His-tag and the p66 subunit without His-tag were used to generate the heterodimers containing deletions in either or both subunits. For each set of heterodimers, 260 μg of p51 was mixed with 660 μg of p66 in binding buffer containing 40 mM Tris–HCl, pH 8.0, and 500 mM NaCl. The rationale for using a 1:3 ratio of p51 and p66 was to saturate the His-tagged p51 with p66 in the column. The mixture was incubated for 16 h at 4°C and applied to Ni^{2+}-iminodiacetic-sepharose (IDA-sepharose) column (0.5 ml) pre-equilibrated with the binding buffer. The column was washed with 15 ml of the same buffer to remove excess p66 that was not dimerized with p51 bound to the IDA-sepharose column. The heterodimeric RT was eluted from the column with 250 mM imidazole in the same buffer. Fractions of 0.5 ml were collected and an aliquot of each was analyzed by SDS–PAGE, then stained with Coomasie blue. The fractions containing approximately equal band intensity of p66 and p51 were dialyzed against a buffer containing 50 mM Tris–HCl (pH 7.0), 200 mM NaCl, and 50% glycerol. We repeated the experiment in those sets that yielded lower band intensity of the p66 subunit than p51 intensity, by increasing the molar ratio of p51:p66 to 1:4 in the incubation mixture before applying it to IDA-sepharose.

DNA POLYMERASE ASSAY

Polymerase activity of the wild type (WT) and mutant enzymes were assayed on homopolymeric template-primer (poly rA.dT_{18}) as well as heteropolymeric RNA (U5-PBS HIV-1 RNA) and DNA (49-mer U5-PBS DNA) templates primed with 18-mer PBS primer. Assays were done in a 50 μl volume containing 50 mM Tris–HCl (pH 7.8); 100 μg/ml bovine serum albumin; 2 mM $MgCl_2$; 1 mM dithiothreitol; 60 mM KCl; 100 nM TP; 10 μM of each dNTP with α-[^{32}P]-dCTP (0.5 μCi/nmol); and 25 nM enzyme. Assay with poly rA.dT_{18} template primer, 20 μM [^{3}H]-TTP (1 μCi/nmol) was used as the substrate. Reactions were incubated at 37°C for 3 min and terminated by the addition of ice cold 5% trichloroacetic acid containing 5 mM inorganic pyrophosphate. The samples were filtered on Whatman GF/B filters, which were dried and counted for radioactivity in a liquid scintillation counter.

GEL ANALYSIS OF RNA- AND DNA-DEPENDENT DNA POLYMERASE ACTIVITIES

The natural U5-PBS HIV-1 RNA and DNA templates primed with 5′-^{32}P-labeled 18-mer PBS primer were used to assess the polymerase activity of the mutant and wild-type enzymes. The primers were 5′-labeled using γ-[^{32}P]-ATP and T4 polynucleotide

kinase according to the standard protocol [Ausubel et al., 1987]. Primer extension reactions were done by incubating 100 nM of the template primer with 50 nM of wild-type HIV-1 RT or its mutant derivative in a total reaction volume of 6 μl containing 25 mM Tris–HCl (pH 7.8), 10 mM DTT, 100 μg/ml BSA, 2 mM $MgCl_2$, and 10 μM of each dNTP. Reactions were initiated by the addition of the $MgCl_2$ and terminated by the addition of an equal volume of Sanger's gel loading dye [Sanger et al., 1977]. The reaction products were analyzed by denaturing polyacrylamide-urea gel electrophoresis, followed by phosphorimaging.

DISSOCIATION CONSTANT OF ENZYME–DNA BINARY COMPLEX

We used the 33-mer U5-PBS DNA template primed with the 5′-[^{32}P] labeled 21-mer PBS primer for the binding studies. Varying concentrations of the individual enzymes were incubated with 0.3 nM template-primer in a total volume of 10 μl containing 50 mM Tris–HCl (pH 7.8), 5 mM $MgCl_2$, and 0.01% BSA. An equal volume of 2x gel loading dye (0.25% bromophenol blue, 20% glycerol) was added after 10 min incubation at 4°C. The E–TP binary complexes were resolved by an electrophoretic mobility shift assay [Pandey et al., 2002] and analyzed on a phosphorImager. The percent of the labeled TP bound to the enzyme (E–TP binary complex) versus enzyme concentrations was plotted and the dissociation constant, $K_{d(DNA)}$, was determined as the enzyme concentration at which half-maximum DNA binding occurred.

GLYCEROL GRADIENT ULTRACENTRIFUGATION

To analyze monomeric and dimeric conformation of the mutant enzymes, we used glycerol gradient ultracentrifugation analysis [Misra et al., 1998; Pandey et al., 2001; Pandey et al., 2002]. In brief, 50 μg of the enzyme protein in 50 mM Tris–HCl, pH 8, and 400 mM NaCl was applied to the top of a 5-ml 10–30% linear glycerol gradient prepared in the same buffer. Gradients were centrifuged at 48,000 rpm in an SW 50.I rotor for 22 h at 4°C and fractionated from the bottom. Sixty-two fractions (80 μl each) were collected. Each fraction was measured for absorbance at OD280 using a Nanodrop spectrophotometer.

RESULTS

DNA POLYMERASE ACTIVITIES OF HIV-1 RT CARRYING A SINGLE DELETION IN THE β7–β8 LOOP

The polymerase activity of HIV-1 RT and its mutant derivatives carrying a single deletion of Ser134, Ile135, Asn136, or Asn137 in the β7–β8 loop was examined using natural U5-PBS HIV-1 RNA and DNA templates primed with 17-mer PBS primer. The HIV-1 RT mutants carrying a single deletion of either Ser134 or Ile135 in both the subunits were most affected and displayed less than 2% of the wild-type polymerase activity, while deletion of Asn136 showed 10–15% of the wild-type activity (Table I). In contrast, deletion of Asn137 had no effect on the polymerase activity of the enzyme. The polymerase activity of the mutant enzymes was restored to near wild-type levels when the p66 subunit carrying deletion of Ser134, Ile135, or Asn136 was dimerized with the wild-type p51 ($p66^{\Delta}/51^{WT}$). Similar results were obtained when the mutant p66 was dimerized with p51 having an intact β7–β8 loop, but carrying a

TABLE I. Polymerase Activity of the Wild-Type HIV-1 RT and Its Mutant Derivatives Carrying Subunit Specific Deletion in the β7–β8 Loop

Enzyme	U5-PBS DNA template/18-mer DNA primer	U5-PBS RNA template/18-mer DNA Primer
p66WT/p51WT	100 (37)	100 (31)
p66Δ134/p51Δ134	2.0 ± 0.93	1.42 ± 0.03
p66Δ135/p51Δ135	1.5 ± 0.45	0.74 ± 0.12
p66Δ136/p51Δ136	5.4 ± 2.75	5.41 ± 0.09
p66Δ137/p51Δ137	96.6 ± 3.33	87.63 ± 11.63
p66Δ134/p51WT	89.6 ± 21.17	85.2 ± 2.74
p66Δ135/p51WT	91.8 ± 8.87	80.7 ± 1.39
p66Δ136/p51WT	90.0 ± 7.94	87.43 ± 3.41
p66Δ137/p51WT	92.0 ± 4.23	87.54 ± 0.54
p66WT/p51Δ134	48.0 ± 3.89	45.13 ± 2.05
p66WT/p51Δ135	60.5 ± 2.54	42.9 ± 0.65
p66WT/p51Δ136	55.5 ± 1.5	47.46 ± 5.01
p66WT/p51Δ137	98.6 ± 16.45	90.40 ± 1.49
P66 WT/p66WT	99 ± 5.5	100 ± 2.5

The polymerase activity of the wild-type HIV-1 RT and its mutant derivatives were determined with the indicated template-primers as described in Materials and Methods Section. The values expressed as percentages of the wild-type enzyme activity represent the averages of three sets of experiments. Values shown in parentheses are picomoles of acid-insoluble dNMP incorporated per min into primer DNA by the wild-type enzyme.

D185A mutation in the catalytically crucial YMDD motif to inactivate the residual catalytic activity of the p51. This ensures that restoration of the polymerase activity of the p66 deletion mutant dimerized with the wild-type p51 can be attributed to the intact β7–β8 loop in the p51 subunit. These results imply that single deletion of Ser134, Ile135, or Asn136 in the β7–β8 loop of p51 is deleterious to the function of HIV-1 RT, but has no impact on the enzyme function if deletion is made in the larger p66 subunit These results also indicate that Asn137 is completely dispensable for the enzyme function, as its deletion in the β7–β8 loop of subunits had no effect on the enzyme activity.

Further, we found that HIV-1 RT ($p66WT/p51^{\Delta}$) carrying single deletion specifically in the p51 subunit at the 134, 135, or 136 positions exhibited approximately 42–55% of wild-type polymerase activity. The observed polymerase activity with $p66^{WT}/p51^{\Delta}$ mutants could be due to readily forming p66/p66 homodimer in the presence of dimer-defective p51 carrying the deletion in the β7–β8 loop. In so, the p66 in the $p66^{wt}/51^{\Delta}$ preparation may homodimerize, in which case only 50% of the total p66 may assume catalytically active open conformation. This may well account for the approximately 50% reduction in polymerase activity observed with $p66^{wt}/51^{\Delta}$, mutant species.

A similar pattern of template-primer use was obtained when reaction products were analyzed on an 8% polyacrylamide-urea gel (Fig. 1). Reactions were done for 3 min on RNA and DNA templates primed with 5-^{32}P labeled 18-mer PBS primer. As shown in Figure 1, deletion of Ser134, Ile135, or Asn 136 in the β7–β8 loop of both subunits resulted in nearly inactive enzyme. In contrast, deletion of Asn137 had no effect on the polymerase activity of the enzyme.

Reduction in the primer extension ability of $p66^{\Delta}/p51^{\Delta}$species was similar on both RNA and DNA templates (Fig. 1). As expected, $p66^{\Delta}$ /$p51^{WT}$ species carrying single deletion of Ser134, Ile135, or Asn136 specifically in the p66 subunit displayed near wild-type

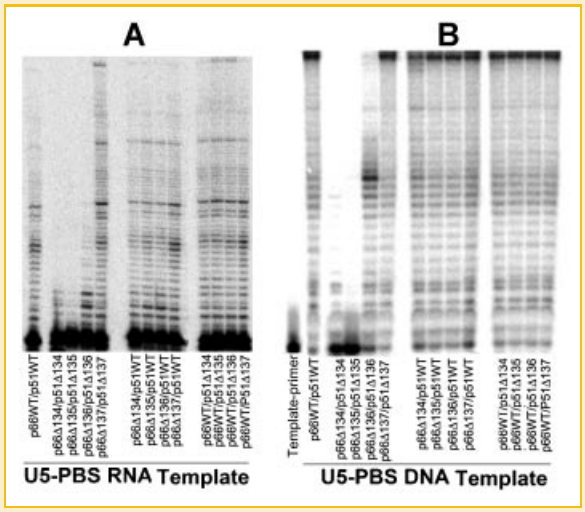

Fig. 1. Primer extension reactions catalyzed by the wild-type HIV-1 RT and its mutant derivatives carrying a single deletion in the beta 7–beta 8 loop of the individual subunit. The primer extension reactions catalyzed by enzyme species carrying the indicated deletion in individual subunits were carried out on U5-PBS HIV-1 DNA and U5-PBS HIV-1 RNA templates primed with 5′^{32}P labeled 18-mer PBS primer. Each set of reactions was done for 3 min at 25°C and quenched by the addition of an equal volume of Sanger's gel loading dye. The reaction products were resolved on an 8% polyacrylamide-7M urea gel and subjected to phosphorImager analysis.

primer extension activity on both RNA and DNA templates. These results clearly suggest that Se134, Ile135, and Asn136 are crucial in the β7–β8 loop of p51, since deletion of any of these amino acids results in loss of the enzyme's polymerase activity. As expected, wild-type p66 dimerized with p51 carrying a deletion at any of the three positions (134–136) was not significantly affected, probably due to self-dimerization (p66/p66). This contention was strengthened by our sedimentation analysis (Fig. 2C), in which glycerol gradient ultracentrifugation analysis of p66^{WT}/51$^{\Delta}$ yielded two protein peaks corresponding to homodimeric wild-type p66/p66 and monomeric p51.

GLYCEROL GRADIENT ULTRACENTRIFUGATION ANALYSIS OF WILD-TYPE AND MUTANT HIV-1 RT

We used 10–30% glycerol gradient ultracentrifugation to identify monomeric and dimeric conformations of mutant enzymes by analyzing their sedimentation profiles [Misra et al., 1998]. The gradients were fractionated from the bottom and measured for OD280 to determine the sedimentation peak. Wild-type heterodimeric (p66/p51) enzyme sedimented as a sharp peak between the 19-21 fractions of the gradient (Fig. 2, row 1), while wild-type p51 sedimented as a monomeric protein between fractions 39-41 (Fig. 2, row 2).

Sedimentation profiles of the enzyme species carrying a single deletion at position 134, 135, or 136 in both subunits (p66$^{\Delta}$/p51$^{\Delta}$) displayed two sedimentation peaks that did not correspond to the sedimentation profile of the wild type-heterodimeric species. One of these peaks corresponded to p51 monomer, suggesting that the other peak might correspond to p66 monomer (Fig. 2A, rows 3 and 4). In contrast, the gradient profile of enzyme species carrying a deletion at position 137 in both subunits yielded a relatively broad sedimentation profile with a major peak corresponding to the heterodimeric enzyme; a minor peak was also discernible at the p51 position (Fig. 2A, row 6) suggesting that a deletion at position 137 on the dimer stability is less deleterious than is a deletion at position134, 135, or 136. These gradient profiles indicate that the putative enzyme species carrying a deletion at positions 134, 135, or 136 in the β7–β8 loop of both the subunits may exist as unstable dimers in solution, which resolve into monomers upon ultracentrifugation. We postulated that the p51 with a single deletion at position 134, 135, or 136 may not dimerize readily with the wild-type p66 (p66^{wt}/p51$^{\Delta}$). This is evident from its gradient profile, in which the p66^{wt} sedimented as a homodimeric species, while mutant p51 sedimented separately as a monomeric species (Fig. 2B, rows 4–6). In contrast, the deletion mutants of p66 dimerized with the wild-type p51 (p66$^{\Delta}$/p51^{WT}) sedimented as heterodimeric species, implying that the intact β7–β8 loop of p51 is essential for the formation of a stable heterodimeric enzyme (Fig. 2C, rows 3–6).

Analysis of the polymerase activity profile of the gradient fractions of p66^{WT}/p66^{WT} and p66^{WT}/51$^{\Delta}$ enzyme species demonstrated that the fractions corresponding to the homodimeric p66/p66 species exhibited major enzymatic activity (Fig. 3). These results support our contention that the 40–60% of polymerase activity noted with the p66^{WT}/51$^{\Delta}$ enzyme species (Table I) is due to self-dimerization of the wild-type p66 to form the catalytically active enzyme species. In the HIV-1 virion, RT exists as a heterodimer of p66/p51; in vitro, p66 exists as an active homodimer in which one of the p66 subunits assumes the catalytic role, while the other subunit assumes the role of p51. Thus, the role of the second p66 subunit in p66/p66 homodimer is analogous to the role of p51 in the heterodimeric enzyme.

FORMATION OF E–TP BINARY COMPLEXES BY MUTANTS AND THE WILD-TYPE ENZYME

Sedimentation analyses have indicated that deletion in the β7–β8 loop in both subunits destabilizes heterodimeric conformation of the enzyme, resulting in the loss of polymerase activity of the enzyme. In contrast, deletion selectively in the p66 subunit has no effect on enzyme activity when dimerized with the wild-type p51. Since the open conformation of the polymerase cleft of the p66 subunit is dependent on the formation of stable dimers, any perturbation in the dimerization process may significantly affect the formation of enzyme–DNA binary complex. We therefore analyzed the DNA binding ability of these mutant RT species by gel-shift assay. The enzymes were incubated with a labeled 51-mer self annealing template primer and analyzed by electrophoresis on a nondenaturing polyacrylamide gel (Fig. 4). This showed that a deletion at position 134 or 135 in both subunits has the most deleterious effect on the DNA binding ability of the enzyme (lanes 6 and 7), while a significant to moderate reduction was noted with the other two deletion mutants (lanes 8 and 9). As expected, when p66 deletion mutants were dimerized with wild-type p51, the DNA binding ability of the enzyme was unaffected (lanes 2–5). Similarly when wild-type p66 was dimerized with the p51 deletion mutants, their DNA binding affinity was unaltered (lanes 10–13). These results suggest that

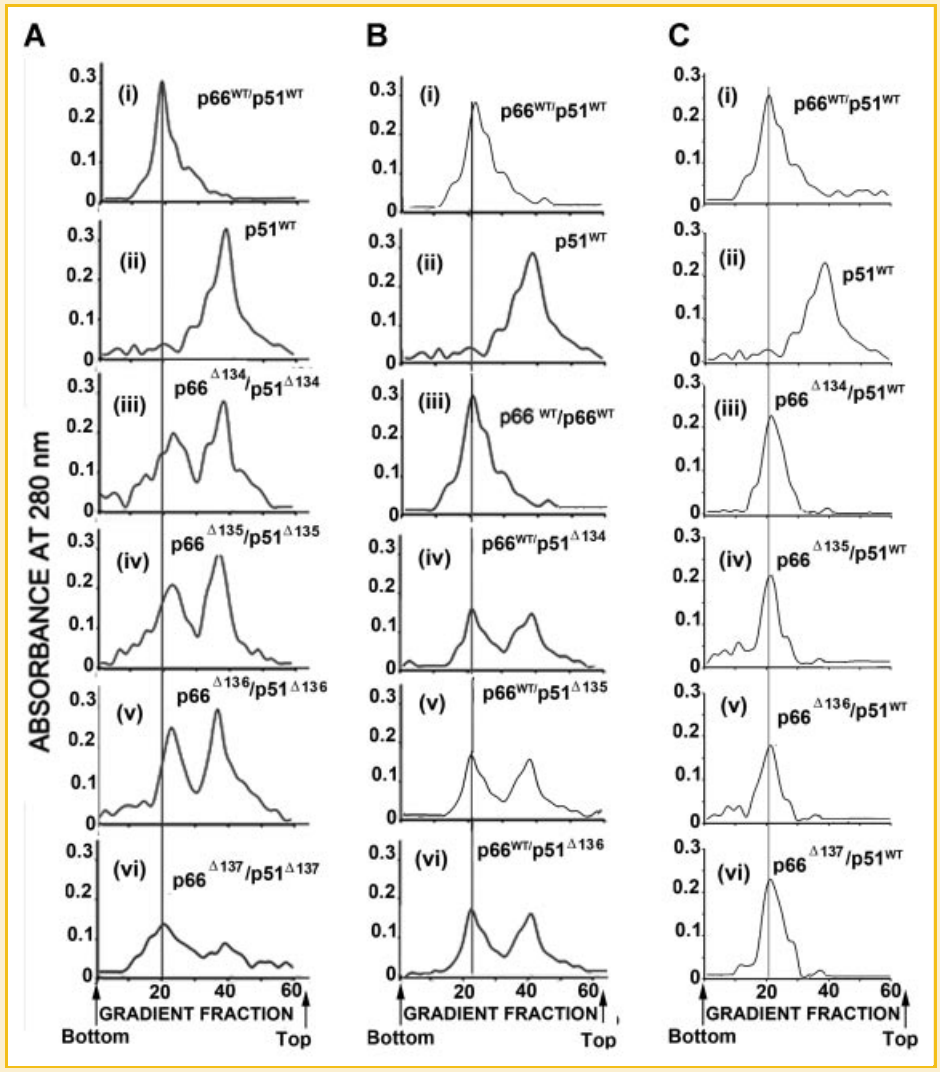

Fig. 2. Glycerol gradient ultracentrifugation analyses of the wild-type HIV-1 RT and its mutant derivatives carrying deletion in the beta 7–beta 8 loop of the individual subunits. The wild- type HIV-1 RT and its mutant derivatives were individually resolved by a 10%–30% linear glycerol gradient ultracentrifugation analysis at 48,000 rpm in an SW 50.1 rotor for 20–24 h. Gradients were fractionated from the bottom and measured for absorbance at OD_{280}. A: Both subunits carrying the indicated single deletion; (B) deletion specifically in the p51 subunit; (C) deletion specifically in the p66 subunit.

residue 134 and 135 in the β7–β8 loop of p51 are most crucial for the dimerization and productive DNA binding function of the enzyme. The $K_{d(DNA)}$ determined for these mutants carrying a single deletion at positions 134, 135 or 136 in both the subunits indicated 14- to 24-fold decrease in the DNA binding affinity while only 3-fold reduction was observed with the 137 deletion mutant (Table II).

DISCUSSION

The catalytically inactive p51 subunit of HIV-1 RT exists in closed conformation, which is derived from the p66 subunit by proteolytic removal of the RNase H domain. The polymerase cleft of the p51 subunit is filled with the connection subdomain, so that the fingers and thumb regions of the polymerase domain are pushed wide apart to support the open conformation of the p66 subunit, which is an unusually elongated molecule of approximately 110 Å × 45 Å in which polymerase and RNase H domains are arranged side by side and joined by the connection subdomain [Kohlstaedt et al., 1992]. The open and elongated p66 is held in place by a compact p51 molecule, which provides firm structural support for both of its polymerase and RNase-H domains. The finger and thumb subdomains of p51 interact, respectively, with the floor of the polymerase cleft and RNase H domain of p66. The specific motifs in p51 that help p66 to adopt an open conformation have not yet been clearly defined. Examination of the crystal structure of HIV-1 RT shows a small groove-like region under the floor of the polymerase cleft of p66 into which the β7–β8 loop of p51 seems to intrude (Fig. 5). Six amino acids span residues 134–139 on the β7–β8 loop. These are Ser 134, Ile 135, Asn 136, Asn 137, Glu138, and Thr139; these are flanked on each side by Pro 133 and Pro 140. The loop in p66 is far away from the catalytic cleft while, in the p51 subunit, the

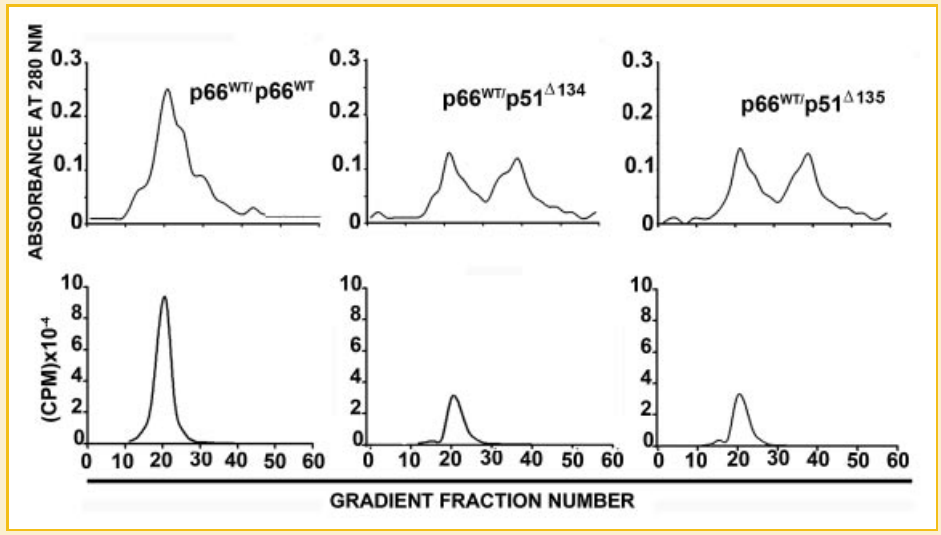

Fig. 3. Polymerase activity profile of the glycerol gradient fractions of the wild-type p66/p66, as well as the p66WT/p51$^{\Delta}$enzyme species. Every third fraction between 10 and 36 of the glycerol gradient (Fig. 2B) of the wild-type p66 homodimer (p66^{WT}/p66^{WT}), as well as p66^{WT}/p51$^{\Delta}$ enzyme species, was diluted 10-fold and analyzed for polymerase activity on poly (rA).$(dT)_{18}$ as described in Materials and Methods Section.

corresponding loop is placed in a groove-like structure under the floor (palm) of the polymerase domain of p66. Because of this difference in the relative arrangement of RT subdomains between p66 and p51, a mutation in one subunit is structurally and functionally nonequivalent to the same mutation in the other subunit.

Earlier, we showed that the p51 subunit is required for loading of the p66 subunit onto the template primer, after which it can be dissociated without affecting the polymerase activity of the TP bound p66 [Harris et al., 1998]. Based on the three-dimensional structure of HIV-1 RT [Kohlstaedt et al., 1992], we postulated that the positioning of the β7–β8 loop of p51 under the floor of the polymerase domain of p66 may induce the latter to assume an open conformation [Harris et al., 1998]. This postulation was supported by the fact that interaction of a nonnucleoside inhibitor, TSAO, specifically with the β7–β8 loop of the p51 subunit, destabilizes the heterodimeric enzyme into inactive monomers with subsequent loss of DNA binding ability [Harris et al., 1998]. We have further shown that HIV-1 RT carrying an alanine substitution or a deletion of four amino acids in its β7–β8 loop failed to form stable heterodimers, leading to drastically reduced template primer binding and polymerase reaction of the enzyme [Pandey et al., 2001]. Further, we found that insertion of six amino acids in the β7–β8 loop of p51

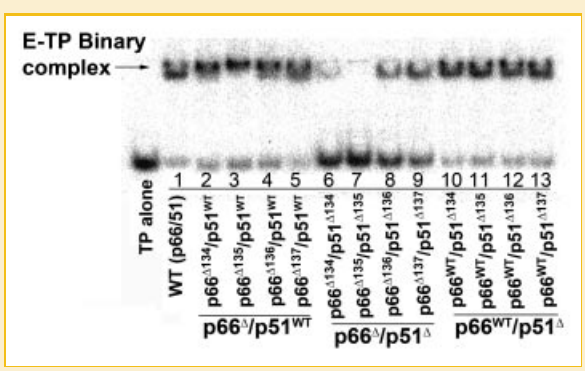

Fig. 4. Gel shift analysis of the enzyme–TP binary complex of the wild-type HIV-1 RT and its mutant derivatives carrying the single indicated deletion in the β7–β8 loop of the individual subunits. Each enzyme protein (1.5 nM) was incubated with 5 nM of -^{32}P-labeled 51-mer SATP at 37°C for 15 min. The E–TP complexes formed were resolved by nondenaturing polyacrylamide gel electrophoresis as described in Materials and Methods Section.

TABLE II. Values of $K_{d[DNA]}$ for Wild-Type HIV-1 RT and Its Mutant Derivatives*

Enzyme	Dissociation constant of the E–TP binary complex	
	$K_{d[DNA]}$ (nM)	Mutant/WT ratio
p66^{WT}/p51^{WT}	1.9	–
p66^{WT}/p66^{WT}	2.3	–
p66 $^{\Delta134}$/p51$^{\Delta134}$	47.9	25.2
p66 $^{\Delta134}$/p51^{WT}	2.1	1.10
p66 ^{WT}/p51$^{\Delta134}$	2.6[a]	1.36
p66 $^{\Delta135}$/p51$^{\Delta135}$	48.3	25.4
p66 $^{\Delta135}$/p51^{WT}	2.3	1.21
p66 ^{WT}/p51$^{\Delta135}$	2.7[a]	1.42
p66 $^{\Delta136}$/p51^{WT}	2.2	1.15
p66 $^{\Delta136}$/p51$^{\Delta136}$	27.6	14.52
p66 ^{WT}/p51$^{\Delta136}$	2.5	1.31
p66 $^{\Delta137}$/p51$^{\Delta137}$	5.7	2.91
p66 $^{\Delta137}$/p51^{WT}	1.9	1.0
p66^{WT}/p51$^{\Delta137}$	2.4	1.26

*The $K_{d[DNA]}$ in the binary complex for the wild-type HIV-1 RT and the individual mutants were determined by gel mobility shift assay using a 33-mer template annealed with 5′-^{32}P-labeled 21-mer DNA -primer. The percent of template-primer associated in the binary complexes was quantified using Image Quant software.

[a]The homodimeric p66^{WT}/p66^{WT} may form when deletion in the p51 affects the dimerization.

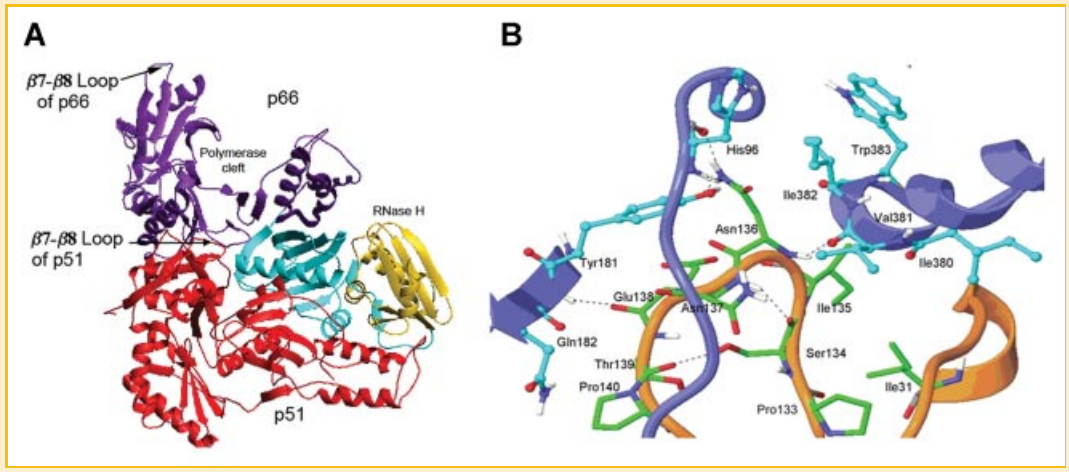

Fig. 5. A: Three-dimensional crystal structure of HIV-1 RT showing the spatial location of the β7–β8 loop in the p66 and p51 subunits. The backbone structure of the HIV-1 RT-DNA-dNTP ternary complex was taken from Huang et al. [1998]. B: Interaction of the β7–β8 loop residues of p51 with the residues in the polymerase cleft of p66. The interacting regions of the p51 subunit (including β7–β8 loop) are represented by the orange ribbon; p66 regions are represented by the blue ribbon. The interacting residues of p66 (carbon atoms in cyan, oxygen atoms in red, and nitrogen atoms in blue) in a ball-and-stick model with the β7–β8 loop residues of p51 (carbon atoms in green, oxygen atoms in red, and nitrogen atoms in blue) as a stick model are shown. Hydrogen bonding interactions are indicated by dotted black lines. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

resulted in a loss of DNA binding function due to unstable dimerization, but had no influence when inserted selectively in the p66 subunit [Pandey et al., 2002]. However, complete restoration of the DNA binding ability and polymerase activity was achieved when mutant p66 was dimerized with the wild-type p51. As previously explained, this result is not surprising because mutation in p51 is structurally and functionally nonequivalent to the same mutation in the p66 subunit.

We did further investigation involving step-wise single deletion of amino acid residues at position 134, 135, 136, or 137 in this loop to identify specific amino acids and their positions in the loop that are essential for the dimerization process. Among these, we identified Ser134, Ile135, and Asn136 as the crucial residues; deletion of any one of them from the p51 subunit completely abrogated the dimerization process of the enzyme. In contrast, deletion of Asn 137 had no significant effect on the dimerization and polymerase function of the enzyme. The side chain hydroxyl group of Ser134 was found to form a hydrogen bond with the backbone carbonyl oxygen atom of Thr139 (Ser134 –OH···O=C– Thr139, 2.1 Å, 141.1°), whereas the backbone carbonyl oxygen atom of Ser134 formed a hydrogen bond with the backbone –NH group of Asn137 (Ser134 –C=O···HN– Asn137, 2.24 Å, 153.7°) (Fig. 5). These findings clearly define the importance of intra-subunit residue-residue interactions in maintaining the secondary structure of the β7–β8 loop of the p51 subunit.

We found that Ile135 of the p51 subunit was stabilized through van der Waals interaction with the hydrophobic side chains of p66 subunit residues such as Ile380, Val381, Ile382, and Trp383, as well as the hydrophobic side chain of Ile31 of the p51 subunit. Thus, deletion of Ile135 may result in a p51 subunit that is unable to protrude into the polymerase cleft of p66 due to highly disordered structure of the β7–β8 loop. The residue Asn136 is engaged in four hydrogen bonding interactions with the p66 subunit residues: Asn136 side chain –C=O···HN His96, 2.11 Å, 168.3°; Asn136 $-CONH_2\cdots$OH Tyr181, 2.05 Å, 172.0°; Asn136 $-CONH_2\cdots$O=C– His96, 1.82 Å, 167.4°; Asn136 –NH···O=C– Val381, 2.01 Å, 141.2°. Overall, the deletion of Ser134 from the p51 subunit is solely due to the loss of intra-subunit interactions, whereas deletion of Ile135 and Asn136 is mainly due to loss of inter-subunit interactions. This suggests that both inter- and intra-subunit interactions participate in maintaining the overall integrity of the β7–β8 loop. Interestingly, Pro95, which is located at interacting distance from the backbone atoms of Asn136, could also be important in maintaining loop integrity.

Mutational studies have shown that the Pro95Ala mutation inactivates the enzyme [Boyer et al., 1994], while any conservative or nonconservative substitution at position 136 destabilizes the dimeric conformation and inactivates the polymerase activity of the enzyme [Balzarini et al., 2005]. Ser136Ala mutation specifically in the p51 subunit of transpackaged heterodimeric HIV-1 RT significantly reduced HIV-1 infectivity in cell culture [Mulky et al., 2007]. Mutational studies at positions 137 (Ans137) and 140 (Pro140) in the β7–β8 loop of p51 and at position 95 (Pro95) in the p66 subunit have demonstrated the significance of these positions or residues in the dimerization and polymerase function of the enzyme [Auwerx et al., 2005]. It is surprising that mutational changes at position 137 in the p51 subunit affect dimerization of the enzyme, while its deletion from the loop in the p51 subunit causes no significant perturbation in the dimeric conformation of the enzyme. It is possible that, following the deletion of Asn137, the β7–β8 loops undergoes rearrangement of its conformation, establishing similar contacts with the p66 subunit. Besides the β7–β8 loop, there may be other important regions that govern dimer stability of the enzyme. Two such residues may be Trp24 and Phe61, respectively located on the β1–α9 loop and β3. Gly substitutions at these positions specifically in the p51 subunit have been shown to destabilize the

dimeric conformation of the enzyme [Depollier et al., 2005]. Thus, targeting dimer stability or disruption of dimerization of HIV-1 RT provides an attractive approach for intervention in HIV-1 replication.

ACKNOWLEDGMENTS

This research was partly supported by grants from the NIH/NIAID (AI074477 and AI042520 to V.N.P.).

REFERENCES

Arts EJ, Li X, Gu Z, Kleiman L, Parniak MA, Wainberg MA. 1994. Comparison of deoxyoligonucleotide and tRNA(Lys-3) as primers in an endogenous human immunodeficiency virus-1 in vitro reverse transcription/template-switching reaction. J Biol Chem 269:14672–14680.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JS, Smith JA, Struhl K. 1987. Current protocols in molecular biology. NY: Greene Publishing Associates and Wiley-Intersciences, John Wiley & Sons.

Auwerx J, Van Nieuwenhove J, Rodriguez-Barrios F, de Castro S, Velazquez S, Ceccherini-Silberstein F, De Clercq E, Camarasa MJ, Perno CF, Gago F, Balzarini J. 2005. The N137 and P140 amino acids in the p51 and the P95 amino acid in the p66 subunit of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase are instrumental to maintain catalytic activity and to design new classes of anti-HIV-1 drugs. FEBS Lett 579:2294–2300.

Balzarini J, Auwerx J, Rodriguez-Barrios F, Chedad A, Farkas V, Ceccherini-Silberstein F, Garcia-Aparicio C, Velazquez S, De Clercq E, Perno CF, Camarasa MJ, Gago F. 2005. The amino acid Asn136 in HIV-1 reverse transcriptase (RT) maintains efficient association of both RT subunits and enables the rational design of novel RT inhibitors. Mol Pharmacol 68:49–60.

Basavapathruni A, Anderson KS. 2007. Reverse transcription of the HIV-1 pandemic. FASEB J 21:3795–3808.

Boyer PL, Tantillo C, Jacobo-Molina A, Nanni RG, Ding J, Arnold E, Hughes SH. 1994. Sensitivity of wild-type human immunodeficiency virus type 1 reverse transcriptase to dideoxynucleotides depends on template length; the sensitivity of drug-resistant mutants does not. Proc Natl Acad Sci USA 91:4882–4886.

Depollier J, Hourdou ML, Aldrian-Herrada G, Rothwell P, Restle T, Divita G. 2005. Insight into the mechanism of a peptide inhibitor of HIV reverse transcriptase dimerization. Biochemistry 44:1909–1918.

Harris D, Lee R, Misra HS, Pandey PK, Pandey VN. 1998. The p51 subunit of human immunodeficiency virus type 1 reverse transcriptase is essential in loading the p66 subunit on the template primer. Biochemistry 37:5903–5908.

Hostomsky Z, Hostomska Z, Fu TB, Taylor J. 1992. Reverse transcriptase of human immunodeficiency virus type 1: Functionality of subunits of the heterodimer in DNA synthesis. J Virol 66:3179–3182.

Hottiger M, Podust VN, Thimmig RL, McHenry C, Hubscher U. 1994. Strand displacement activity of the human immunodeficiency virus type 1 reverse transcriptase heterodimer and its individual subunits. J Biol Chem 269:986–991.

Hsieh JC, Zinnen S, Modrich P. 1993. Kinetic mechanism of the DNA-dependent DNA polymerase activity of human immunodeficiency virus reverse transcriptase. J Biol Chem 268:24607–24613.

Huang H, Chopra R, Verdine GL, Harrison SC. 1998. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: Implications for drug resistance. Science 282:1669–1675.

Jacobo-Molina A, Ding J, Nanni RG, Clark AD, Jr., Lu X, Tantillo C, Williams RL, Kamer G, Ferris AL, Clark P, et al. 1993. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA. Proc Natl Acad Sci USA 90:6320–6324.

Jacques PS, Wohrl BM, Howard KJ, Le Grice SF. 1994. Modulation of HIV-1 reverse transcriptase function in ''selectively deleted'' p66/p51 heterodimers. J Biol Chem 269:1388–1393.

Kohlstaedt LA, Wang J, Friedman JM, Rice PA, Steitz TA. 1992. Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science 256:1783–1790.

Le Grice SF, Gruninger-Leitch F. 1990. Rapid purification of homodimer and heterodimer HIV-1 reverse transcriptase by metal chelate affinity chromatography. Eur J Biochem 187:307–314.

Le Grice SF, Naas T, Wohlgensinger B, Schatz O. 1991. Subunit-selective mutagenesis indicates minimal polymerase activity in heterodimer-associated p51 HIV-1 reverse transcriptase. EMBO J 10:3905–3911.

Levine JG, Hatfield D, Oroszlan S, Rein A. 1993. Mechanisms of translational suppression used in the biosynthesis of reverse transcriptase. In: Skalka AM, Goff SP, editors. Reverse transcriptase. Plainview, NY: Cold Spring Harbor Laboratory Press.

Loya S, Gao HQ, Avidan O, Boyer PL, Hughes SH, Hizi A. 1997. Subunit-specific mutagenesis of the cysteine 280 residue of the reverse transcriptase of human immunodeficiency virus type 1: Effects on sensitivity to a specific inhibitor of the RNase H activity. J Virol 71:5668–5672.

Misra HS, Pandey PK, Pandey VN. 1998. An enzymatically active chimeric HIV-1 reverse transcriptase (RT) with the RNase-H domain of murine leukemia virus RT exists as a monomer. J Biol Chem 273:9785–9789.

Mous J, Heimer EP, Le Grice SF. 1988. Processing protease and reverse transcriptase from human immunodeficiency virus type I polyprotein in Escherichia coli. J Virol 62:1433–1436.

Mulky A, Vu BC, Conway JA, Hughes SH, Kappes JC. 2007. Analysis of amino acids in the beta7-beta8 loop of human immunodeficiency virus type 1 reverse transcriptase for their role in virus replication. J Mol Biol 365:1368–1378.

Pandey VN, Kaushik N, Rege N, Sarafianos SG, Yadav PN, Modak MJ. 1996. Role of methionine 184 of human immunodeficiency virus type-1 reverse transcriptase in the polymerase function and fidelity of DNA synthesis. Biochemistry 35:2168–2179.

Pandey PK, Kaushik N, Talele TT, Yadav PN, Pandey VN. 2001. The beta7-beta8 loop of the p51 subunit in the heterodimeric (p66/p51) human immunodeficiency virus type 1 reverse transcriptase is essential for the catalytic function of the p66 subunit. Biochemistry 40:9505–9512.

Pandey PK, Kaushik N, Singh K, Sharma B, Upadhyay AK, Kumar S, Harris D, Pandey VN. 2002. Insertion of a small peptide of six amino acids into the beta7-beta8 loop of the p51 subunit of HIV-1 reverse transcriptase perturbs the heterodimer and affects its activities. BMC Biochem 3:18.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467.

Sierra S, Kupfer B, Kaiser R. 2005. Basics of the virology of HIV-1 and its replication. J Clin Virol 34:233–244.