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†

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Received December 19, 2000; Revised Manuscript Received April 20, 2001

ABSTRACT: The heterodimeric human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) is composed of p66 and p51 subunits, p66 being the catalytic subunit. Our earlier investigation on the role of p51 in the catalytic process has shown that the p51 subunit facilitates the loading of the p66 subunit onto the template primer (TP). We had postulated that the $\beta7-\beta8$ loop of the p51 subunit may be involved in opening the polymerase cleft of p66 for DNA binding [Pandey, V. N., et al. (1996) *Biochemistry 35*, 2168]. We report here that deletion or alanine substitution of four residues of the $\beta7-\beta8$ loop results in severe impairment of the polymerase function of the heterodimeric enzyme. The enzyme activity was restored to the wild-type levels when the mutant p66 subunit was dimerized with the wild-type p51, suggesting that the intact $\beta7-\beta8$ loop in the p51 subunit is indispensable for the catalytic function of p66. Further, the template primer binding ability of the enzyme was significantly reduced upon deletion or alanine substitution in the $\beta7-\beta8$ loop. Interestingly, the loss of the TP binding ability of the mutant p66 was restored upon dimerization with wild-type p51. Examination of the glycerol gradient ultracentrifugation analysis revealed that while the wild-type HIV-1 RT sediments as a dimeric protein, the mutant enzymes carrying deletion or alanine substitution in both the subunits sediment predominantly as monomeric proteins, suggesting their inability to form stable dimers. In contrast, mutant p66 dimerized with wild-type p51 ($p66^{\Delta}/p51^{WT}$ and $p66^{Ala}/p51^{WT}$) sedimented at the dimeric position. Taken together, these results clearly implicate the importance of the $\beta7-\beta8$ loop of p51 in the formation of stable functional heterodimers.

Reverse transcriptase in all retroviruses is part of the *pol* gene and is encoded downstream from the *gag* gene in a large coding region (*1*). Translation of this coding region yields a *gag-pol* fusion protein which is subsequently cleaved by the viral protease to generate the mature reverse transcriptase present in the infectious virions (*2*). This multifunctional enzyme exhibiting both RNA- and DNA-dependent DNA polymerase activities as well as RNase H activity is responsible for converting the single-stranded genomic RNA into double-stranded proviral DNA and thus plays a key role in the viral life cycle (*3*−*6*). Reverse transcriptases from SIV,[1] FIV, HIV, and EIAV are heterodimers (p66/p51) containing one subunit as the full-length RT polypeptide, and the other is the proteolytically truncated form from the carboxy terminus (*7*−*10*). The p66 subunit exhibits both DNA polymerase and RNase H activity, whereas the p51 subunit lacking the carboxy-terminal RNase H domain exhibits neither of these activities (*11*, *12*). However, the emergence of TSAO (*tert*-butyldimethyl silylspiroaminooxathioledioside) resistant strains of HIV-1 has been shown to result from a Glu → Lys mutation at position 138 in the p51 subunit of HIV-1 RT, thereby implying some functional role of p51 in the catalytic competence of p66 (*13*). Studies from Le Grice's lab have suggested that p51 may be involved in the initiation of reverse transcription (*14*, *15*). Deletion of as few as eight amino acids from the C-terminus of the p51 subunit resulted in impairment of initiation of reverse transcription on natural $tRNA^{Lys3}$ (*14*). Interestingly, the $tRNA^{Lys3}$-primed activity of HIV-1 RT carrying the C-terminal deletion of 13 amino acids in p51 ($p51\Delta13/p66$) could be rescued, if $tRNA^{Lys3}$ containing five additional DNA nucleotides was used as the primer (*15*). These findings have suggested that the p51 subunit may play a role in disrupting additional tRNA−viral RNA base pairing outside the PBS region to proceed into productive (−) strand

† This research was supported by a grant from the National Cancer Institute of the National Institutes of Health (Grant CA 72821 to V.N.P.).

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[1] Abbreviations: 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, *tert*-butyldimethyl silylspiroaminooxathioledioside; 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.

10.1021/bi002872j CCC: $20.00
Published on Web 07/17/2001

DNA synthesis (*14*, *15*). It has also been shown that p51 may be involved in the strand displacement activity during the polymerase reaction (*16*). Recently, Cys → Ser mutation at position 280 in p51 was found to alter the RNase H activity of the heterodimeric enzyme (*17*). We have earlier demonstrated that the p51 subunit is involved in loading the p66 subunit onto the template primer (*18*). Our studies indicated that once the RT is bound to the TP, the p51 subunit can be physically dissociated and the TP-bound p66 alone is capable of catalyzing the polymerase reaction (*18*). In continuing with our investigation of the role of p51, we have generated subunit specific deletion and alanine substitution in the $\beta7-\beta8$ loop in the finger subdomain of HIV-1 RT. In this paper, we provide evidence to suggest that the intact $\beta7-\beta8$ loop of the p51 subunit is essential for the catalytic function of the p66 subunit. Deletion or alanine substitution in the $\beta7-\beta8$ loop of the p51 subunit results in severe impairment of the DNA polymerase activity of the enzyme, thus indicating the functional importance of this loop in the catalytic mechanism of the heterodimeric 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, and ^{32}P-labeled dNTPs and ATP were the products of Dupont/New England Nuclear Corp. The RNA and DNA oligomers used as template primers were synthesized at the Molecular Resource Facility at the University of Medicine and Dentistry of New Jersey and have the same sequence as described previously (*18*). 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 *Escherichia coli* expression strain BL-21(DE3) were obtained from Novagen. The HIV-1 RT expression clones (pET-28a-RT66, pET-3a-RT51, and pET-28a-RT51) constructed in this laboratory (*19*−*21*) 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 was a generous gift from M. A. Wainberg (*22*). This clone contains a 947 bp fragment of the HIV-1 genome (nucleotides 473−1420) which supplies the RNA corresponding to the U5-PBS region.

Alanine Substitution of Amino Acid Residues in the $\beta7-\beta8$ Loop. Four residues (positions 136−139) in the $\beta7-\beta8$ loop of the p51 and/or p66 subunit were substituted with alanine using two-step PCR amplification as follows. The 432 bp sequence containing N-terminal codons 1−144 were amplified by PCR using the upstream and downstream primers and pET-28a-RT51 as the template (*23*). The upstream primer having the sequence: TAT GGG GC**C ATA TG**C CCA TTA GCC CTA TTG contained a unique *Nde*I site which provided the ATG start codon. The downstream primer sequences corresponding to codons 144−131 were 5′-ATA TCT AAT CCC TGG ***TGC CGC AGC GGC*** AAC ACT AGG TAT GGT-3′, substituting four wild-type codons (positions 136−139) for alanine (shown in bold italics). In the second step, the 432 bp first-step PCR product was used as the upstream megaprimer. The downstream primer was from the C-terminal region of p51 and contained a unique *Sac*I site and a stop codon (TAT AGG GC**G AGC TC**T TAG AAG GTT TCT GCT CC). These were used to amplify the full-length p51 coding region (1320 bp) from the pET-28aRT51 vector. The second PCR product was restriction digested with *Nde*I and *Sac*I and cloned into the pET-28a vector. The clones were sequenced to ascertain the alanine substitutions at codons 136−139. Alanine substitution in the $\beta7-\beta8$ loop of the p66 subunit was carried out by inserting the 403 bp *Kpn*I and *Hin*dIII fragment of the pET-28a-RT66 vector (coding region of the RNase H domain) into the pET28a-RT51 clone having alanine substitutions (N136A, N137A, E138A, and T139A). Both the mutant clones were introduced into *E. coli* BL-21(DE3)pLysS for expression. Induction and purification of the enzyme protein were carried out as described previously for wild-type HIV-1 RT (*19*).

Deletion of Amino Acid Residues in the $\beta7-\beta8$ Loop. Mutants carrying deletion of four codons (positions 136−139) in the $\beta7-\beta8$ loop of the p66 or p51 subunit were constructed via the two-step PCR amplification process described above. The 432 bp sequence containing N-terminal codons 1−144 was amplified from the pKK-RT66 vector by PCR using the upstream and downstream primers. The sequence of the upstream primer was 5′-A AGG CCT GAA TTC ATG CCC ATT AGC CCT ATT-3′ and contained a unique *Eco*RI site preceding the ATG start codon. The sequence of downstream primer corresponding to codons 144−131 was 5′-G ATA TCT AAT CCC AGG CCT CTC ATT GTT AAC ACT AGG TAT G-3′. This primer carried two unique sites, namely, *Stu*I and *Hpa*I sites introduced at codons 140 and 136, respectively. In the second step of the PCR, the 432 bp first-step PCR product was used as the upstream megaprimer along with a downstream primer from the C-terminal region of p51 having the unique *Hin*dIII and *Sac*I sites and a stop codon (GT GCG GCC GA AGC TT TTA GAA GAG CTC *TTA* TAG TAC TTT CCT GAT TCC) to amplify the full-length p66 coding region from the pKK-RT66 vector. The second PCR product was restriction digested with *Eco*RI and *Hin*dIII and cloned into the pKK plasmid vector. The positive clones were screened in the *E. coli* GAM48 (A) strain and sequenced to ascertain the presence of *Hpa*I and *Stu*I sites at codons 136 and 140, respectively. The *Stu*I enzyme does not cleave if the restriction site is methylated. The *E. coli* GAM48 (A) strain is deficient in dam/dcm and, therefore, suitable for screening of positive clones. The positive clones were restriction digested with *Hpa*I and *Stu*I to remove codons 136−139, and the gel-purified vector was religated to construct pKK-RT66$^{\Delta136-139}$. This construct expresses the p66$^{\Delta136-139}$ subunit without the His tag sequences. For introducing the His tag at the N-terminus of the p51$^{\Delta136-139}$ subunit, the *Bal*I−*Hin*dIII fragment of pKK-RT66$^{\Delta136-139}$ was subcloned in the pET-28a-RT66 expression cassette. A unique *Sac*I site was introduced into pKK-RT66 at codon 440. Deletion of the $\beta7-\beta8$ loop in p51 was carried out by removing the 360 bp fragment from pKK-RT66$^{\Delta136-139}$ by restriction digestion with *Sac*I followed by ligation of the vector ends to generate pKK-RT51$^{\Delta136-139}$. The deletion mutants in the pET and pKK vectors were introduced into *E. coli* BL-21(DE3)pLysS and

E. coli JM109, respectively, for expression. Induction of the enzyme protein was carried out as described previously for wild-type HIV-1 RT (*24*, *25*). The enzyme with the His tag was purified from bacterial lysates by immobilized metal affinity chromatography (*19*), while a conventional column chromatography protocol was used to purify the non-His-tagged enzyme (*26*).

Preparation of the Heterodimeric Enzyme with Subunit Specific Alanine Substitution or Deletion. The p51 subunit with the His tag and the p66 subunit without the His tag were used to generate the heterodimers containing alanine substitution or deletion in either or both subunits. For each set of heterodimers, 260 μg of p51 was mixed with 660 μg of p66 in the binding buffer containing 40 mM Tris-HCl (pH 8.0) and 500 mM NaCl. The rationale for using a 1:3 ratio of p51 to 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 a 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 the excess p66 that was not dimerized with p51 bound to the IDA-Sepharose column. The heterodimeric RT was then eluted from the column with 250 mM imidazole in the same buffer. Fractions (0.5 mL) were collected, and an aliquot of each was analyzed by SDS−PAGE followed by Coomassie blue staining. The fractions containing approximately equal p66 and p51 band intensities 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, which yielded lower band intensity of the p66 subunit as compared to the p51 intensity, by increasing the molar ratio of p66 to p51 to 4:1 in the incubation mixture prior to application onto IDA-Sepharose.

DNA Polymerase Assay. The polymerase activities of the wild-type (WT) and mutant enzymes were assayed on three different template primers, poly(rA)•$(dT)_{18}$, U5-PBS HIV-1 RNA, and 49-mer U5-PBS DNA templates primed with the 17-mer PBS primer. Assays were carried out in a 50 μL volume containing 50 mM Tris-HCl (pH 8.0), 100 μg/mL bovine serum albumin, 2 mM $MgCl_2$, 1 mM dithiothreitol, 60 mM KCl, 200 nM TP, dNTPs (50 μM each), and 21 nM enzyme. With the homopolymeric (rA)•$(dT)_{18}$ template primer, the reaction mixture contained 50 μM ^{32}P-labeled dTTP (0.4 μCi/nmol). For heteropolymeric template primers, each of the four dNTPs (50 μM) was included with one of them being ^{32}P-labeled (0.2 μCi/nmol of dNTP). Reaction mixtures were incubated at 37 °C for 3 min, and reactions were terminated by the addition of ice-cold 5% trichloroacetic acid containing 5 mM inorganic pyrophosphate. The samples were filtered on Whatman GF/B filters. The filters 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 17-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 T_4 polynucleotide kinase according to the standard protocol (*27*). Primer extension reactions were carried out by incubating 2.5 nM template primer with 50 nM 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 dNTPs (100 μM each). Reactions were initiated by the addition of the enzyme and terminated by the addition of equal volume of Sanger's gel loading dye (*28*). The reaction products were analyzed by denaturing polyacrylamide−urea gel electrophoresis followed by phosphorimaging.

Gel Shift Assay Used To Determine the Extent of Formation of Enzyme−Template Primer Binary Complex. We used the 49-mer U5-PBS DNA template primed with the 5′-^{32}P-labeled 17-mer PBS primer for the binding studies. The gel shift incubation mixture contained 5 nM labeled TP, 1.5−1.8 μM wild-type HIV-1 RT or its mutant derivative, 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, and 10% glycerol in a total volume of 10 μL. The mixture was incubated at 37 °C for 30 min and loaded onto a 6% polyacrylamide gel in Tris-glycine buffer [25 mM Tris and 162 mM glycine (pH 8.4)]. The gel was run at 150 V and 4 °C for 4 h, and the shift in the DNA band due to the formation of the E−TP binary complex was detected by phosphorimaging (Molecular Dynamics).

Glycerol Gradient Ultracentrifugation. Fifty micrograms of the enzyme protein in Tris-NaCl buffer [50 mM Tris-HCl (pH 8.0) and 400 mM NaCl] was carefully loaded onto 5 mL of 10 to 30% glycerol gradients prepared in the same buffer. Gradients were centrifuged in an SW48 rotor at 48 000 rpm for 22 h and were fractionated from the bottom (*29*). Fractions were subjected to SDS−polyacrylamide gel electrophoresis to determine the protein peak in the fraction.

RESULTS

Deletion and Alanine Substitution in the $\beta7-\beta8$ Loop of the Individual Subunits of HIV-1 RT. The amino acid residues spanning codons 136−139 in the $\beta7-\beta8$ loop of the individual subunits of HIV-1 RT were chosen for deletion or alanine substitution. As described in Materials and Methods, deletion and alanine substitutions were performed by the two-step PCR megaprimer method using three oligonucleotide primers, one of which was the mutant primer corresponding to codons 144−131. For deletion, the mutant primer contained the unique *Hpa*I and *Stu*I sites at codons 136 and 140, respectively. Following screening of the positive clones, deletion in the $\beta7-\beta8$ loop was carried out by removing the *Stu*I and *Hpa*I fragment corresponding to codons 136−139. Alanine substitution and deletion in the $\beta7-\beta8$ loop of the individual subunits were screened by DNA sequencing. The positive recombinant clones in the pET expression vector were expressed in *E. coli* BL-21(DE3) pLysS, while pKK-based clones were expressed in *E. coli* JM109. The mutant enzymes were induced and purified from the cell lysate either by metal affinity column chromatography (*19*, *20*, *30*) or by the conventional procedure (*26*), depending upon the presence or absence of His tag sequences at the N-terminus. The enzyme preparations were found to be greater than 95% pure as judged by SDS−polyacrylamide gel electrophoresis. The level of protein expression, solubility, and yield were similar to those of the wild-type enzyme, suggesting that alteration in the $\beta7-\beta8$ loop did not cause any perturbation in the folding of the enzymes. The chromatographic parameters used for the purification of the mutant enzymes were essentially similar to those of the wild-type enzyme, although a change in the sedimentation profile upon glycerol gradient analysis was noted.

Table 1: Polymerase Activity of the Wild-Type HIV-1 RT and Its Mutant Derivatives Carrying Subunit Specific Deletion or Alanine Substitution in the $\beta7-\beta8$ Loop[a]

	percent of wild-type polymerase activity		
enzyme species	poly(rA)·$(dT)_{18}$	U5-PBS RNA/ 17-mer	U5-PBS DNA/ 17-mer
$p66^{WT}/p51^{WT}$	100 (584)	100 (97)	100 (114)
$p66^{WT}/p66^{WT}$	97	92	96
$p66^{\Delta}/p66^{\Delta}$	2.5	9.5	10.5
$p66^{\Delta}/p51^{\Delta}$	10.2	9.0	8.0
$p66^{Ala}/p66^{Ala}$	3.8	10.0	11.0
$p66^{Ala}/p51^{Ala}$	0.8	12.6	10.6
$p66^{\Delta}/p51^{WT}$	96.0	116.0	94
$p66^{\Delta}/p51^{D186A}$	104.0	98.0	96.0
$p66^{Ala}/p51^{WT}$	97.0	102.0	98.4
$p66^{Ala}/p51^{D186A}$	94.0	99.0	101.0
$p66^{WT}/p51^{\Delta}$	45.0	59.4	55.0
$p66^{WT}/p51^{Ala}$	40.0	51.0	48.0

[a] The polymerase activities of the wild-type HIV-1 RT and its mutant derivatives were determined with the indicated template primers at saturated dNTP concentrations as described in Materials and Methods. The values expressed as a percentage of the wild-type enzyme activity represent the averages of three sets of experiments. Values shown in parentheses are total picomoles of acid-insoluble dNMP incorporated into primer DNA by the wild-type enzyme in 3 min at 37 °C.

DNA Polymerase Activities of HIV-1 RT Carrying Deletion or Alanine Substitution in the $\beta7-\beta8$ Loop. The polymerase activity of HIV-1 RT and its mutant derivatives carrying deletion or alanine substitution in the $\beta7-\beta8$ loop was examined using the homopolymeric poly(rA)·$(dT)_{18}$ template primer as well as heteropolymeric natural U5-PBS HIV-1 RNA and DNA templates primed with the 17-mer PBS primer. The results shown in Table 1 indicate that the HIV-1 RT mutants carrying either deletion or alanine substitution in both the subunits display 1−10% of the wild-type polymerase activity. The polymerase activity of the mutant enzymes was restored to near-wild-type levels when the mutant p66 was dimerized with wild-type p51 ($p66^{\Delta}/51^{WT}$, $p66^{Ala}/51^{WT}$). Similar results were obtained when the mutant p66 was dimerized with p51 harboring an Asp → Ala mutation at position 186 in the catalytically crucial YMDD motif, but having an intact $\beta7-\beta8$ loop. The rationale for using the $p51^{D186A}$ mutant was to inactivate the residual catalytic activity of wild-type p51. Thus, the restoration of polymerase activity of mutant p66 dimerized with wild-type p51 or with $p51^{D186A}$ ($p66^{\Delta}/51^{WT}$, $p66^{Ala}/51^{WT}$, $p66^{\Delta}/51^{D186A}$, $p66^{Ala}/51^{D186A}$) may be attributed to the intact $\beta7-\beta8$ loop in the p51 subunit. These results imply that deletion or alanine substitution in the $\beta7-\beta8$ loop of the p66 has no impact on the polymerase activity of HIV-1 RT as judged by the wild-type activity levels observed with the mutant p66 dimerized with the p51 subunit carrying the intact $\beta7-\beta8$ loop. However, we were rather intrigued by the observation that the wild-type p66 dimerized with the mutant p51 ($p66^{WT}/51^{\Delta}$) exhibited approximately 40−60% of the wild-type polymerase activity. We speculated that the mutant p51 carrying deletion or alanine substitution in the $\beta7-\beta8$ loop may form an unstable dimer with wild-type p66. In such a case, wild-type p66 in the $p66^{WT}/51^{\Delta}$ or $p66^{WT}/51^{Ala}$ preparation may tend to dimerize with itself, thus accounting for the 50% decrease in polymerase activity.

A similar pattern of template primer utilization was also obtained when reaction products were analyzed on an 8% polyacrylamide−urea gel (Figure 1). Reactions were carried out for 30 and 60 s on RNA and DNA templates primed with the 5-^{32}P-labeled 17-mer PBS primer. As seen in Figure 1, deletion or alanine substitution in the $\beta7-\beta8$ loop of both the subunits resulted in significant impairment of the primer extension ability of the mutant enzymes. The enzyme species carrying deletion in both the subunits ($p66^{\Delta}/p66^{\Delta}$ and $p66^{\Delta}/p51^{\Delta}$) were nearly inactive on the DNA template (Figure 1A), while trace amounts of primer extension activity were noted on RNA templates (Figure 1B). The levels of reduction in the primer extension ability of $p66^{Ala}/p66^{Ala}$ and $p66^{Ala}/p51^{Ala}$ species were similar on both RNA and DNA templates (Figure 1C,D). As expected, the mutant p66 subunit dimerized with the wild-type p51 subunit ($p66^{\Delta}/51^{WT}$, $p66^{Ala}/51^{WT}$) displayed near-wild-type polymerase activity on both RNA and DNA templates. Similar results were obtained when mutant p66 was dimerized with another mutant p51 with the intact $\beta7-\beta8$ loop but carrying a mutation in the YMDD motif.

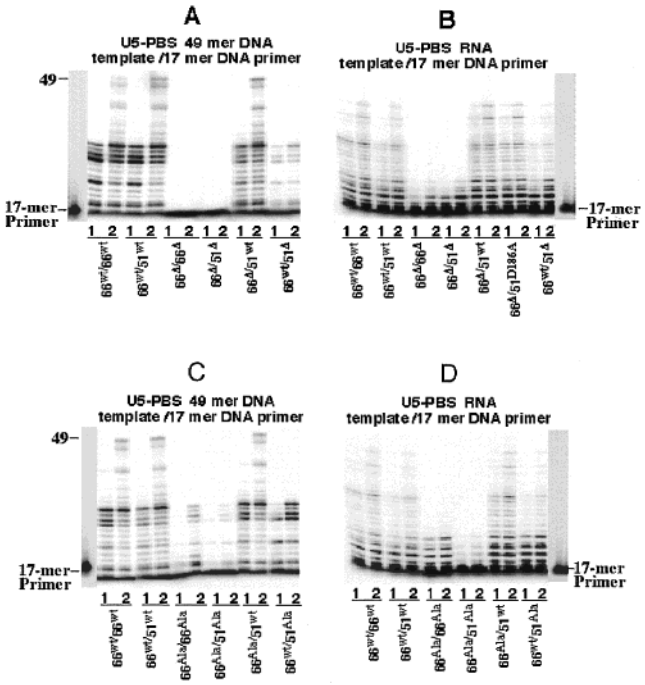

FIGURE 1: Primer extension reactions catalyzed by the wild-type HIV-1 RT and its mutant derivatives carrying deletion or alanine substitution in the $\beta7-\beta8$ loop of the individual subunit. The primer extension reactions catalyzed by the enzyme species carrying deletion (A and B) or alanine substitution (C and D) in the individual subunits were carried out on U5-PBS HIV-1 DNA and U5-PBS HIV-1 RNA templates primed with the 5′-^{32}P-labeled 17-mer PBS primer. Each set of reactions was carried out for 30 (lane 1) and 60 s (lane 2) 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−7 M urea gel and subjected to phosphorimager analysis.

These results suggest that impairment of the polymerase function of the $p66^{\Delta}/66^{\Delta}$, $p66^{\Delta}/51^{\Delta}$, $p66^{Ala}/66^{Ala}$, or $p66^{Ala}/51^{Ala}$ enzyme species is via mutation in the second subunit. However, like the results shown in Table 1, the wild-type p66 dimerized with the mutant p51 ($p66^{WT}/51^{\Delta}$, $p66^{WT}/51^{Ala}$) exhibited approximately 40−60% of the wild-type activity on both templates. In keeping with our earlier suggestion, this result may be explained by a possible dimerization defect between the wild-type p66 and the mutant p51. In this

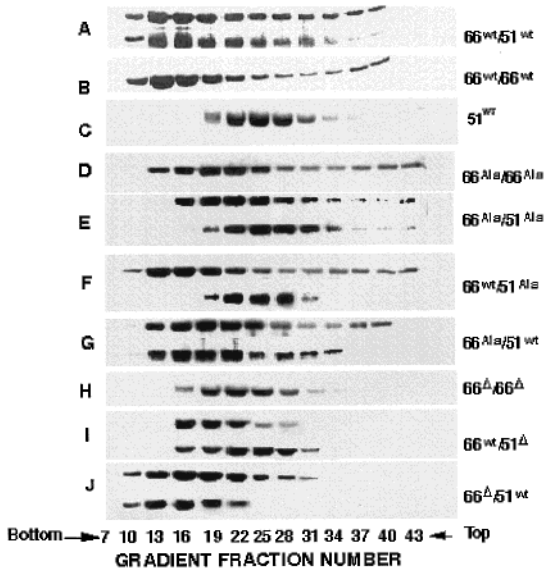

FIGURE 2: Glycerol gradient ultracentrifugation analyses of the wild-type HIV-1 RT and its mutant derivatives carrying alanine substitution or deletion in the $\beta7-\beta8$ loop of the individual subunits. The wild-type HIV-1 RT and its mutant derivatives were individually resolved via 10 to 30% linear 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 subjected to SDS−polyacrylamide gel electrophoresis. The protein peak in the fraction was visualized by Coomassie blue staining of the gel.

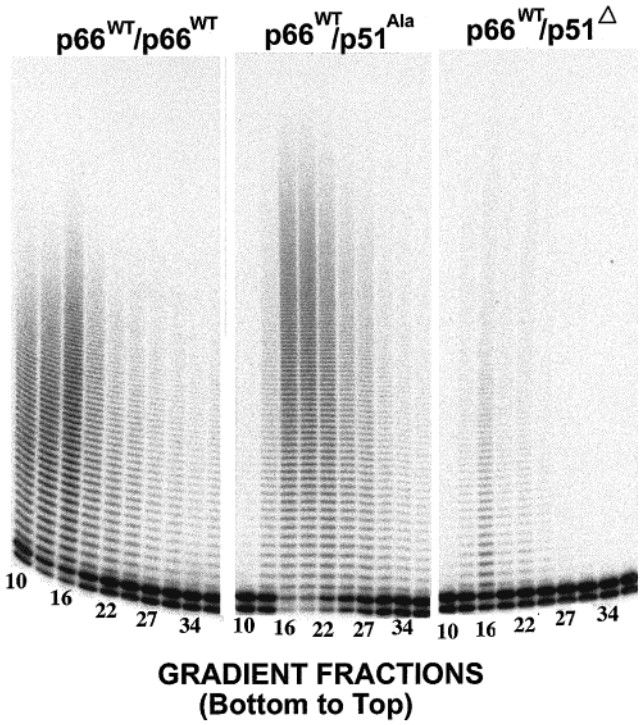

FIGURE 3: Polymerase activity profile of the glycerol gradient fractions of the wild-type p66/p66 as well as p66WT/p51$^{\Delta}$ and p66^{WT}/p51^{Ala} enzyme species. Every third fraction between fractions 10 and 37 of the glycerol gradient (Figure 2) of the wild-type p66 homodimer (p66^{WT}/p66^{WT}) as well as the p66^{WT}/p51$^{\Delta}$ and p66WT/p51^{Ala} enzyme species was diluted 10-fold and analyzed for its polymerase activity on poly(rA)·(dT)$_{18}$. The reaction mixture containing the wild-type enzyme was incubated for 30 s, while the mutants were incubated for 5 min at 37 °C.

scenario, the wild-type p66 may self-dimerize to form catalytically active enzyme species. This postulation is strengthened by the sedimentation analysis data (see Figures 2 and 3) where glycerol gradient ultracentrifugation analysis of the p66^{WT}/51$^{\Delta}$ and p66^{WT}/51^{Ala} species 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. The dimeric and monomeric conformation of mutant and wild-type enzyme species was analyzed by 10 to 30% glycerol gradient ultracentrifugation. The gradients were fractionated from the bottom, and an aliquot of each fraction was subjected to SDS−polyacrylamide gel electrophoresis followed by Coomassie blue staining. The results shown in Figure 2 indicate that both the wild-type homodimeric (p66/p66) and heterodimeric (p66/p51) enzyme species sedimented as sharp peaks between fractions 13 and 19 of the gradient (panels A and B), while the wild-type p51 species alone sedimented as a monomeric protein between fractions 22 and 28 (panel C). The sedimentation peak of the mutant p66^{Ala}/p66^{Ala} was between fractions 19 and 22 of the gradient (panel D). A clearer picture emerged from the gradient profile of p66^{Ala}/p51^{Ala}, where both p66^{Ala} and p51^{Ala} sedimented separately between fractions 19 and 25 and between fractions 22 and 28, respectively (panel E). These gradient profiles indicate that the putative enzyme species carrying alanine substitution in both the subunits may exist as unstable dimers in solution which resolve into monomers upon ultracentrifugation. As per our speculation, the mutant p51 does not dimerize readily with the wild-type p66 (p66^{WT}/p51^{Ala}). This is evident from its gradient profile, where the p66^{WT} sedimented as a homodimeric species between fractions 13 and 19 while the mutant p51^{Ala} sedimented as a monomeric species between fractions 19 and 25 (panel F). In contrast, the mutant p66 dimerized with the wild-type p51 (p66^{Ala}/p51^{WT}) sedimented as a heterodimeric species, implying that the intact $\beta7-\beta8$ loop of p51 is essential for the formation of a stable heterodimeric enzyme (panel G).

As in the sedimentation profile of the alanine substitution mutant, the enzyme species carrying deletion in both the subunits (p66$^{\Delta}$/p66Δ) sedimented at the monomeric position between fractions 19 and 25 (panel H), suggesting their unstable dimerization. Interestingly, while p66$^{\Delta}$/p51^{WT} sedimented as a heterodimeric protein (panel J), the p66^{WT}/p51$^{\Delta}$ dimer yielded two sedimentation peaks (panel I) corresponding to homodimeric p66/66 (fractions 16−19) and monomeric p51$^{\Delta}$ (fractions 22−28). This indicates that wild-type p66 in the p66^{WT}/p51$^{\Delta}$ species does not form a stable heterodimer with the mutant p51$^{\Delta}$. Analysis of the polymerase activity profile of the gradient fractions of p66^{WT}/p66^{WT}, p66^{WT}/51$^{\Delta}$, and p66^{WT}/51^{Ala} enzyme species demonstrated that the fractions between 13 and 19 corresponding to the homodimeric p66/p66 species exhibited major enzymatic activity (Figure 3). The wild-type enzyme exhibited near-equivalent activity in fractions 13 and 16. Although this does not exactly correlate with the protein band intensity seen in Figure 2, the activity profile is in agreement with the sedimentation profile of the homodimeric enzyme (p66/p66). These results support the contention that 40−60% of the polymerase activity noted with the p66^{WT}/51$^{\Delta}$ and p66^{WT}/51^{Ala} enzyme species (Figure 1) may be due to self-dimerization of the wild-type p66 in forming the catalytically

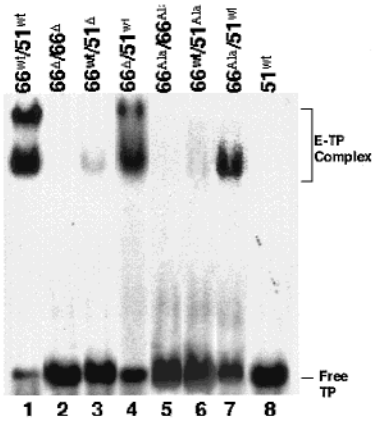

FIGURE 4: Gel shift analysis aimed at evaluation of the formation of the enzyme−TP binary complex by the wild-type HIV-1 RT and its mutant derivatives carrying alanine substitution or deletion in the $\beta7-\beta8$ loop of the individual subunits. Each enzyme protein (1.5 μM) was incubated with 5 nM 49-mer U5-PBS DNA or 5′-^{32}P 17-mer PBS primer at 37 °C for 15 min, and the E−TP complexes that formed were resolved by nondenaturing polyacrylamide gel electrophoresis as described in Materials and Methods.

active enzyme species. It may be noted that in the HIV-1 virion, RT exists as a heterodimer of p66/p51. In vitro, p66 exists as an active homodimer where one of the p66 subunits assumes the catalytic role while the second subunit assumes the role of p51. Thus, the role of the second p66 subunit in the p66/p66 homodimer is analogous to the role of p51 in the heterodimeric enzyme.

Formation of E−TP Binary Complexes by the Mutants and Wild-Type Enzyme. Glycerol gradient sedimentation analysis revealed that the loss of polymerase activity of the mutant enzymes might be due to dimerization defects. Since the open conformation of the polymerase cleft of the p66 subunit is dependent upon the formation of stable dimers with the second subunit, it is reasonable to presume that any perturbation in this dimerization process due to alteration in the $\beta7-\beta8$ loop would in turn affect the DNA binding capacity of the enzyme. To ascertain this, we analyzed the TP binding ability of these various mutant RT species by gel shift assay. The enzymes were incubated with a labeled 49−17-mer TP and analyzed by electrophoresis on a nondenaturing polyacrylamide gel (Figure 4). A variable pattern of the retarded complexes indicated qualitative differences in the binding affinities of the various enzymes for the template primer. A complete absence of any shift in the position of the labeled template primer in the case of the mutant enzymes carrying deletion or alanine substitution either in both subunits (lanes 2 and 5) or in the p51 subunit alone (lanes 3 and 6) clearly indicated a drastic reduction in their affinity for the nucleic acid. In contrast, the retarded bands seen with the complexes of RTs in which the mutant p66 carrying deletion or alanine substitution was dimerized with the wild-type p51 subunit (lanes 4 and 7) suggest that their DNA binding ability was not significantly affected. Interestingly, two distinct retarded bands were seen with the wild-type heterodimeric RT and the mutant RT in which the p66 (deletion) is dimerized with the wild-type p51 subunit (lanes 1 and 4). The faster-migrating retarded band may represent the expected species that contains one molecule of enzyme bound to the 3′-terminus of the template primer, whereas the slower-migrating retarded band may reflect complexes with one additional enzyme molecule, since this band disappeared upon addition of excess TP (results not shown). As expected, the wild-type p51 subunit alone was not able to form any detectable E−TP complex under the standard assay conditions (lane 8). Taken together, these results suggest that the intact $\beta7-\beta8$ loop of p51 is essential for the productive DNA binding function of p66.

DISCUSSION

The monomeric form of p66 is proposed to exist in a closed conformation topologically similar to p51 and assumes an open conformation upon dimerization with the second subunit (*31*). In this paper, we propose that the $\beta7-\beta8$ loop motif of p51 is one of the important regions responsible for dimerization and conferring catalytic capability to p66. Our earlier studies have shown that the p51 subunit facilitates the loading of p66 onto the template primer, after which the smaller subunit can be physically dissociated without affecting the polymerase function of the TP-bound larger subunit (*18*). We now demonstrate that the intact $\beta7-\beta8$ loop of p51 is essential for the catalytic functions of p66. Deletion or alanine substitution of four amino acids from this loop selectively in the p66 subunit had no significant impact on the dimer formation, DNA binding ability, and polymerase function of the enzyme. However, mutational changes in this loop specifically in the p51 subunit resulted in unstable dimer formation, reduction in DNA binding ability, and subsequent loss of polymerase activity of the enzyme. The importance of the $\beta7-\beta8$ loop of the p51 subunit in the dimerization process is substantiated by the sedimentation pattern of the various RT mutants carrying deletion or substitution in this loop region either in the p51 subunit alone or in both subunits. These results imply that the $\beta7-\beta8$ loop of p51 may be positioned such that any perturbation in this loop directly influences the conformation of the polymerase domain of the larger subunit.

In the three-dimensional crystal structure of HIV-1 RT (*32*, *33*), the $\beta7-\beta8$ loop of the individual subunits of HIV-1 RT is positioned differently (Figure 5A). In the p66 subunit, it is part of the finger subdomain located away from the polymerase cleft. In the p51 subunit, this loop is seen protruding prominently in the floor of the polymerase cleft of the p66 subunit. It is interesting to note that the region of p66 where the $\beta7-\beta8$ loop of p51 interacts exists as a disordered structure spanning residues 83−106 (Figure 5B). The corresponding region ($\beta6$ and αE) in murine leukemia virus reverse transcriptase (MuLV RT) exists as an ordered structure (Figure 5C). The polymerase domain of MuLV RT (*34*) and HIV-1 RT (*32*, *33*) are approximately 85% structurally similar, in contrast to their sequences being 29% identical (*34*). The $\beta6$ and αE region of MuLV RT is not at all similar in structure or sequence to the corresponding equivalent motif (residues 83−106) of HIV-1 RT. The presence of an ordered structure in the palm subdomain of MuLV RT may be structurally important in providing an open conformation to its polymerase domain. In contrast, the existence of a disordered region in the floor of the polymerase cleft of HIV-1 RT may necessitate interaction of the p51 subunit with this region for maintenance of the stability of the cleft in the open conformation.

There are six amino acid residues (SINNET) in the loop situated between two prolines at positions 133 and 140.

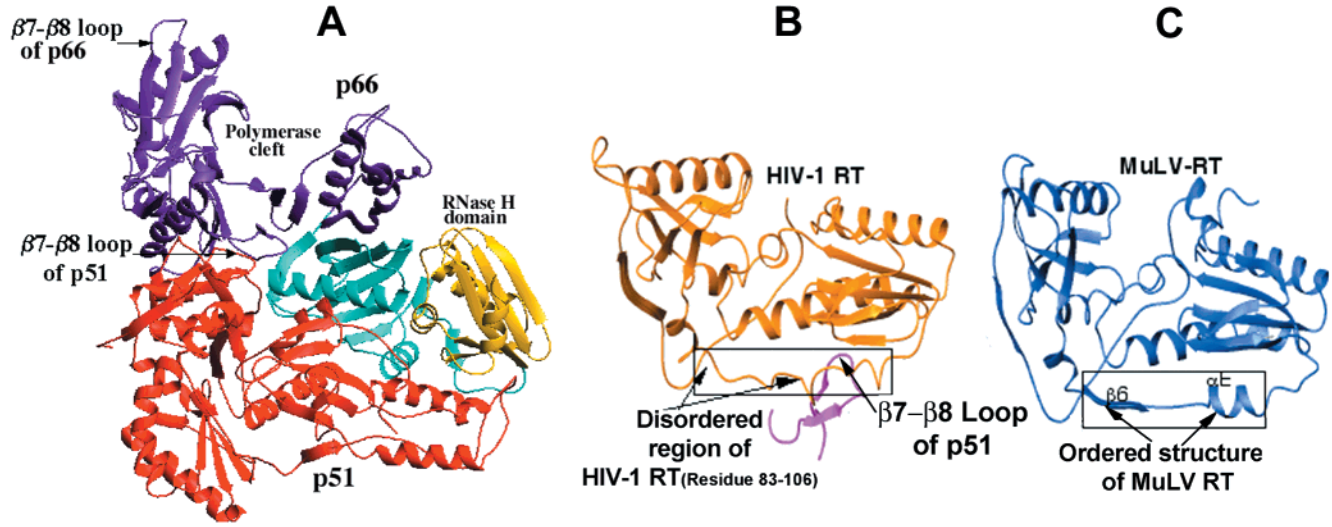

FIGURE 5: Structural organization in the floor of the polymerase cleft of p66 interacting with the $\beta7-\beta8$ loop of p51. (A) Three-dimensional crystal structure of HIV-1 RT showing the spatial location of the $\beta7-\beta8$ loop in the p66 and p51 subunits. The backbone structure of the HIV-1 RT−DNA−dNTP ternary complex was taken from Huang et al. (*33*). (B) The $\beta7-\beta8$ loop of p51 (red) is shown interacting with the disordered region in the floor of the polymerase cleft of p66 (boxed). For clarity, only the structure of the palm and finger subdomains of the catalytic p66 subunit is shown. (C) For comparison, the crystal structure of the palm and finger subdomains of a representative monomeric RT enzyme, MuLV RT (*34*), is shown in royal blue. It may be noted that the corresponding region ($\beta6\alpha E$, residues 121−146) in the floor of the polymerase cleft (boxed) of MuLV RT is an ordered structure.

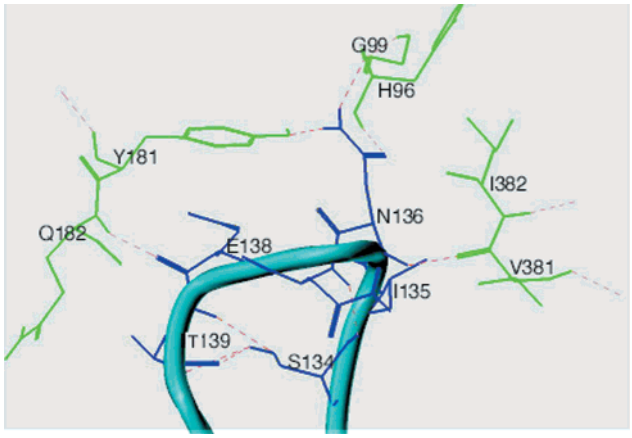

FIGURE 6: Interaction of the $\beta7-\beta8$ loop residues of p51 with the residues in the polymerase cleft of p66. The interacting residues of p66 (green) within 4 Å of the $\beta7-\beta8$ loop residues of p51 (blue) are shown. Hydrogen bonding interactions are shown as dotted lines.

Besides Ile 135, all the other amino acids of this loop have polar side chains. The integrity of the loop is maintained by hydrogen bonding between the side chains of Ser 134 and Thr 139 (Figure 6). Four of the six amino acid residues in the loop at positions 136−139 of the p51 subunit are seen interacting prominently with residues of the p66 subunit. The side chain of Asn 136 interacts with the backbone of His 96, while a backbone to backbone interaction is seen with Pro 95, Val 381, and Ile 382. The amide of Asn 137 is also within interacting distance of the side chain carbon of Pro 95 and backbone of Gly 93. Among these, Gly 93, Pro 95, and His 96 are part of the disordered region on the floor of the palm subdomain of p66 (Figure 5B). Mutational studies have shown that Gly → Ala and Pro → Gly substitutions at positions 93 and 95, respectively, of HIV-1 RT result in complete loss of polymerase function of the enzyme (*35*). Glu 138 and Thr 139 of p51 seem to provide significant interactions with a number of residues in the catalytic cleft of p66. A multiple backbone to backbone interaction of Glu 138 with Tyr 181 and Asp 186 and side chain interaction with Val 179 of p66 are seen. Among these, Asp 186 is the catalytic residue of p66 (*30*). A non-nucleoside inhibitor, TSAO, has been shown to inactivate the enzyme by interacting selectively with Glu 138 of this loop in the p51 subunit (*13*). Our earlier studies demonstrating dissociation of the heterodimeric HIV-1 RT into inactive monomers following interaction of the bulky TSAO with the $\beta7-\beta8$ loop of p51 (*18*) support the notion that this loop may play a direct role in the formation of the functionally active dimeric enzyme.

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BI002872J