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The p51 Subunit of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Is Essential in Loading the p66 Subunit on the Template Primer[†]

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ABSTRACT: Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is a dimeric enzyme consisting of p66 and p51 subunits. The functional role of the p51 subunit remains elusive since all the catalytic functions appear to be executed through the p66 subunit. We report here that the p51 subunit, in addition to providing structural support to the p66 subunit, may be involved in facilitating the loading of the p66 subunit on to the template–primer (TP). This possibility is supported by following observations: (i) Upon binding to the TP, the p51 subunit can be dissociated by acetonitrile treatment and the template–primer-bound p66 monomer alone is capable of catalyzing DNA synthesis. (ii) Photocross-linking of template–primer to HIV-1 RT is abolished by dissociation of the p51 subunit prior to the TP binding but remains unaffected after the TP binding step. (iii) The p66–TP covalent complex selectively generated by UV irradiation and separated by gel electrophoresis can incorporate a single nucleotide in situ upon its renaturation in the gel. (iv) Treatment of HIV-1 RT with (*tert*-butyldimethylsilyl)-spiroaminooxathioledioside (TSAO), an inhibitor that specifically binds to the $\beta 7\beta 8$ loop of p51, destabilizes the heterodimeric enzyme, resulting in the subsequent loss of DNA binding.

Reverse transcriptases of the immunodeficiency viruses, human, feline, and simian, are heterodimeric enzymes composed of a p66 and a p51 subunit that have a common amino terminus. The p51 subunit is the proteolytic cleavage product of p66 and lacks the RNase H domain. The heterodimeric form of the enzyme is found in the infectious virion and represents the biologically relevant and active form of the enzyme since the isolated subunits alone are functionally inactive (*1*). Three-dimensional structures of the p66/p51 heterodimeric HIV-1 RT¹ apocrystal (*2*), as well as the ligand-bound RT including the one with DNA, have provided architectural details of the enzyme protein (*3–7*). In spite of having identical amino acid sequences, the two subunits exhibit remarkably distinct folding resulting in the formation of an asymmetric dimer structure. Structure determination by X-ray crystallography has revealed that the polymerase domain of p66 folds into an open structure, while that of p51 assumes a closed conformation (*3, 8, 9*). A region

responsible for the dimer interface has been seen in the structures. Subunit-specific site-directed mutagenesis has confirmed that the polymerase activity resides selectively in the p66 subunit of the heterodimeric enzyme (*10*). Some functional implication of the p51 subunit in conferring a drug resistance phenotype has been suggested, on the basis of the clustering of the mutated amino acids in the p51 subunit (*11*). However, no conclusive proof for such a role has been obtained. A recent report on the development of TSAO resistance in HIV-1 RT has been shown to result from a Glu \rightarrow Lys mutation at position 138 in the p51 subunit, implying some functional role for p51 in the catalytic competence of p66 (*12*). The central theme of this paper is to report on the possible role of the p51 subunit of HIV-1 RT in the process of template–primer binding.

MATERIALS AND METHODS

Fast-flow chelating Sepharose (iminodiacetic-Sepharose) for immobilized metal affinity chromatography (IMAC) was purchased from Pharmacia, and ³²P-labeled dNTPs and ATP were the products of Dupont/New England Nuclear Corp. Synthetic oligomeric primers were obtained from the Molecular Resource Facility of the University of Medicine and Dentistry at New Jersey Medical School and were further purified by polyacrylamide gel electrophoresis (*13*). All other reagents were of the highest available purity grade and purchased from Fisher, Millipore Corp., Boehringer Mannheim, and Bio-Rad.

Expression and Isolation of Wild-Type HIV-1 RT, Klenow Fragment, and MuLVRT. The recombinant plasmids pKK-p66 and pKK-p51 were used for expression of the wild-type HIV-1 RT (*14*). *Escherichia coli* JM109 carrying the

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¹ Abbreviations: TSAO, (*tert*-butyldimethylsilyl)spiroaminooxathioledioside; poly(rA)·(dT)₁₈, polyriboadenylic acid annealed with (thymidylic acid)₁₈; dNTP, deoxyribonucleoside triphosphate; HIV-1 RT, human immunodeficiency virus type 1 reverse transcriptase; MuLV RT, Moloney murine leukemia virus reverse transcriptase; IMAC, immobilized metal affinity chromatography; U5-PBS RNA template, genomic RNA template corresponding to primer binding sequence region; U5-PBS DNA template, human immunodeficiency virus type 1 genomic DNA template corresponding to the primer binding sequence region; TP, template–primer; PBS, primer binding sequence.

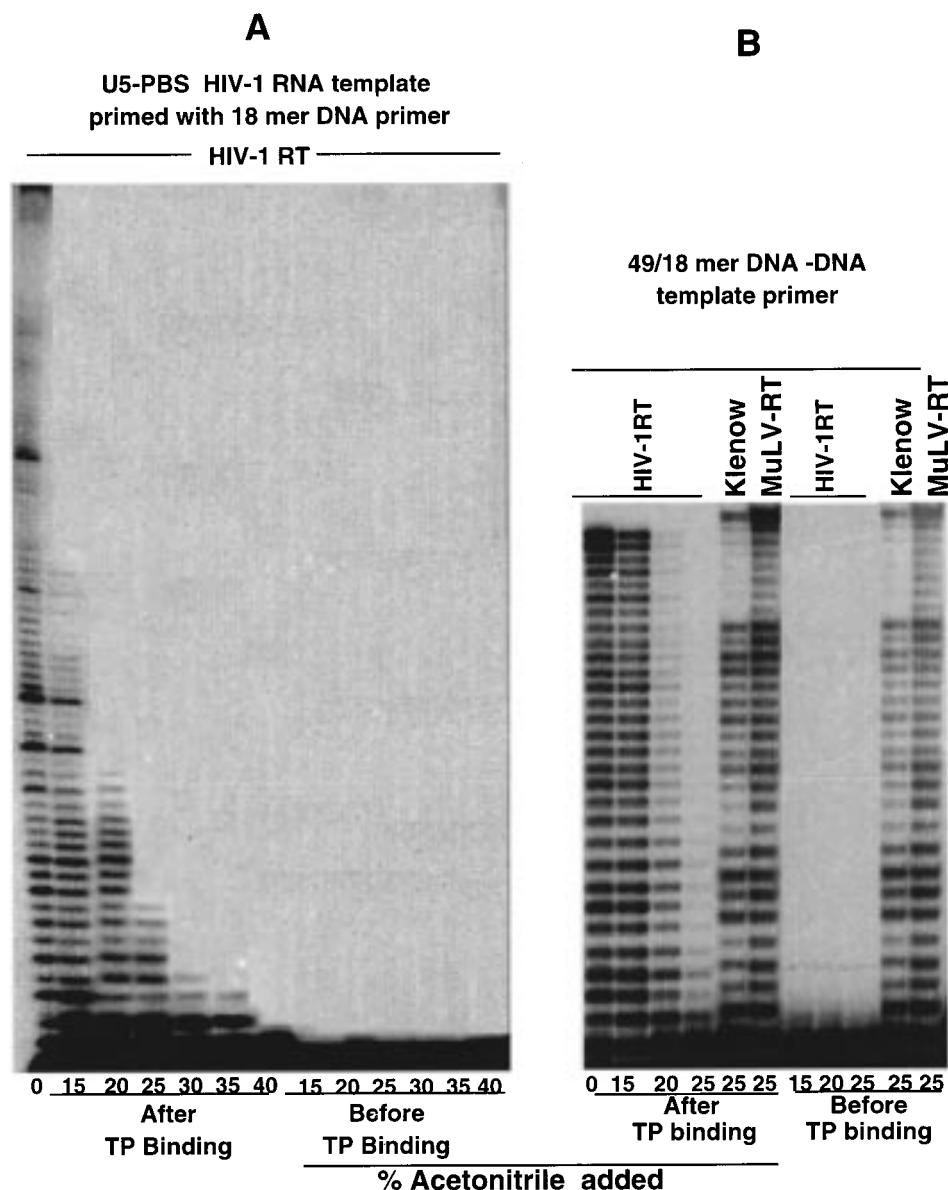


FIGURE 1: Effect of acetonitrile addition on the polymerase activity of HIV-1 RT before and after the template-primer binding step. Two sets of experiments were performed. In one set, acetonitrile was added after a brief incubation of the enzyme with the template-primer. In the second set, acetonitrile addition was made before the template-primer binding step. A typical reaction mixture (10 μ L) containing 50 mM Tris-HCl, pH 7.5, 10 mM DTT, 100 μ g of BSA/mL, 2 mM $MgCl_2$, 2.5 nM U5-PBS HIV RNA/5'- ^{32}P 18-mer PBS template-primer (panel A) or 49-mer DNA/5'- ^{32}P 18-mer PBS template-primer (panel B), and 50 ng of enzyme was incubated at 30 $^{\circ}C$ for 5 min. After addition of the indicated concentration of acetonitrile, 50 μ M dNTP was added to start the extension reaction. In experiments where acetonitrile addition was required before the template-primer binding step, the reaction was initiated by the addition of the enzyme. The reaction mixture was incubated at 37 $^{\circ}C$ for 15 min and terminated by addition of an equal volume of Sanger's gel loading solution. The samples were analyzed by 8% denaturing polyacrylamide-urea gel electrophoresis.

recombinant plasmid was grown at 37 $^{\circ}C$ in Luria broth containing ampicillin (100 μ g/mL). Induction by IPTG was performed at 0.5 OD₅₉₅ as described before (15, 16). The p66/51 heterodimeric enzyme was prepared by combining the cell pellets of the p66 and p51 clones at an appropriate ratio (16). The heterodimeric enzyme from the cell free lysate was prepared as described by Hsieh et al. (17). Klenow fragment (18, 19), and MuLV RT (20) were expressed and purified from recombinant clones according to published protocols.

RNA- and DNA-Dependent DNA Polymerase Assay. The RNA-dependent polymerase activity of RTs was monitored using U5-PBS HIV-1 RNA as template. The template RNA was synthesized by transcription of a pHIV-PBS clone (21) using T7 RNA polymerase as described before (22). For

the DNA-dependent DNA polymerase activity, a 49-mer synthetic DNA corresponding to the U5-PBS region was used as the DNA template. Both RNA and DNA templates were primed with 5'- ^{32}P labeled 18-mer PBS DNA and used in the polymerase reaction. The reaction conditions were the same as described before (22). Any variation used in a specific experiment is as described in the corresponding figure legend. Unless otherwise indicated, all reactions were carried out at 37 $^{\circ}C$.

HPLC Gel-Filtration Analysis. HPLC gel filtration of the heterodimeric and homodimeric forms of HIV-1 RT, treated or untreated with acetonitrile under various conditions, was carried out essentially as described by Wöhrle et al. (23) using two BioSep 3000 columns (300 \times 7.8 mm; Phenomenex Inc.) attached to HPLC (Varian 5500 HPLC unit). The

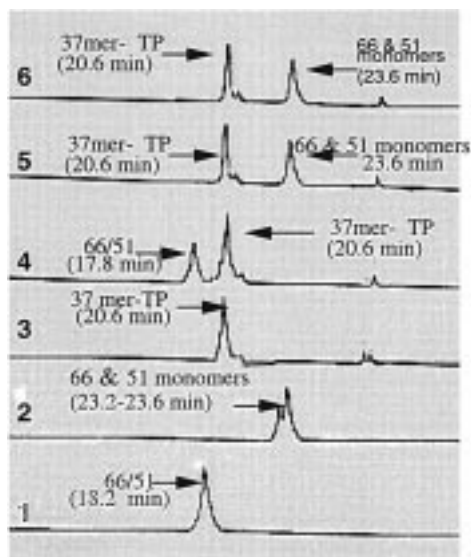


FIGURE 2: HPLC gel-filtration profile of heterodimeric HIV-1 RT treated and untreated with acetonitrile under various conditions. HPLC gel filtration of HIV-1 RT in the presence and absence of acetonitrile was carried out on two BioSep 300 columns (300 × 7.8 mm; Phenomenex Inc.) as described before (1, 23). Two hundred micrograms of HIV-1 RT was incubated separately with 20% acetonitrile in the absence or presence of template–primer and resolved on HPLC gel-filtration columns. The incubation buffer contained 20 mM Tris-HCl, pH 7.0. The gel-filtration buffer contained 20 mM Tris-HCl, pH 7.0, and 200 mM NaCl (and 20% acetonitrile when the enzyme was pretreated with acetonitrile). Row 1, control heterodimeric p66/p51 enzyme species; row 2, acetonitrile-treated p66/51 enzyme species; row 3, 37-mer self-annealing (see Chart 1) template–primer alone; row 4, p66/p51 incubated with 37-mer template–primer; row 5, p66/p51 pretreated with acetonitrile and then incubated with 37-mer TP; row 6, p66/51 preincubated with 37-mer TP and then treated with acetonitrile for 60 min.

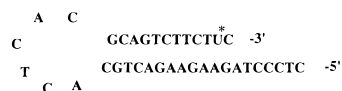
elution buffer consisted of 200 mM NaCl in 50 mM Tris-HCl, pH 7.0.

RESULTS AND DISCUSSION

It has been demonstrated that the presence of 20% acetonitrile completely dissociates the subunits of the heterodimeric HIV-1 RT into catalytically inactive monomers (1, 24). We observed that after the binding of the enzyme to the template–primer, the p51 subunit from the heterodimeric enzyme can be dissociated from the p66 at 20% acetonitrile concentration without compromising the catalytic ability of the p66 subunit bound to the template–primer. The results obtained with RNA·DNA and DNA·DNA template–primers under these conditions are shown in Figure 1. It was observed that HIV-1 RT dissociated in this manner can catalyze DNA synthesis in the presence of acetonitrile concentrations of up to 35% in the reaction mixture (Figure 1A). In contrast, addition of acetonitrile to the reaction mixture prior to TP binding at a concentration of 15% completely abolished the catalytic activity of the enzyme (Figure 1A). For comparison, we examined the effect of acetonitrile treatment before and after the TP binding step on two other DNA polymerases, namely, MuLV RT and the Klenow fragment. As shown in Figure 1B, addition of acetonitrile either before or after the TP binding step did not significantly alter the polymerase activity of these enzymes. This suggests that acetonitrile-mediated dissocia-

Chart 1

37 mer self annealing TP



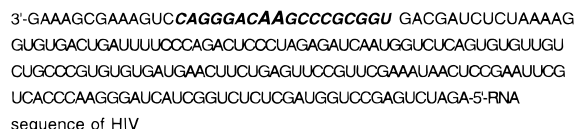
49 mer U5-PBS-DNA template



18 mer PBS primer



U5-PBS HIV-1 RNA template



tion of the p51 subunit after the template–primer binding step does not influence the catalytic ability of the p66 monomer to incorporate nucleotides, in contrast to acetonitrile-mediated dissociation seen prior to TP binding, resulting in catalytically inactive monomers. It may be argued that the p66/p51 dimer may be resistant to dissociation by acetonitrile after the TP binding step and the polymerase activity seen under these conditions may be a manifestation of this fact. Therefore, a detailed study of the effect of acetonitrile treatment on the dissociation of subunits of HIV-1 RT was undertaken.

To determine if the complete dissociation of the TP-bound subunit species of HIV-1 RT in the presence of 20% acetonitrile concentration has occurred, enzyme treated with the acetonitrile both pre- and post-TP incubation was subjected to HPLC gel filtration. The HPLC gel-filtration profile was developed to assess the separation of the subunits under various conditions (Figure 2). The native heterodimeric HIV-1 RT (p66/51) eluted at 18.2 min from the column (row 1). Pretreatment of the enzyme with 20% acetonitrile resulted in the dissociation of the p66 and p51 monomers at elution time between 23.2 and 23.6 min (row 2). The elution time of the 37-mer self-annealing TP alone (see Chart 1) was 20.6 min (row 3), and elution time of the enzyme in the presence of 37-mer–TP was 17.8 min (row 4). Acetonitrile treatment of the enzyme before the TP binding step resulted in complete dissociation of the dimers into monomers (row 5). Treatment of E–TP complex with 20% acetonitrile for 60 min also resulted in complete dissociation of both the subunits (row 6). In a parallel experiment, the enzyme (p66/51) preincubated with poly-(dA)·5'-³²P(dT)₁₈ was subjected to treatment with various concentrations of acetonitrile for 60 min under similar conditions as described above (row 6) and then supplemented with dTTP substrate to monitor the extension of the labeled primer. Interestingly, the extension assay under this condition indicated only a moderate reduction in primer extension as compared to the untreated control (Figure 3). It was estimated that 80% of the labeled primer was extended by the untreated control while approximately 65% and 30% extensions were noted with the E–TP complexes preincubated with 15% and 20% acetonitrile concentrations, respectively.

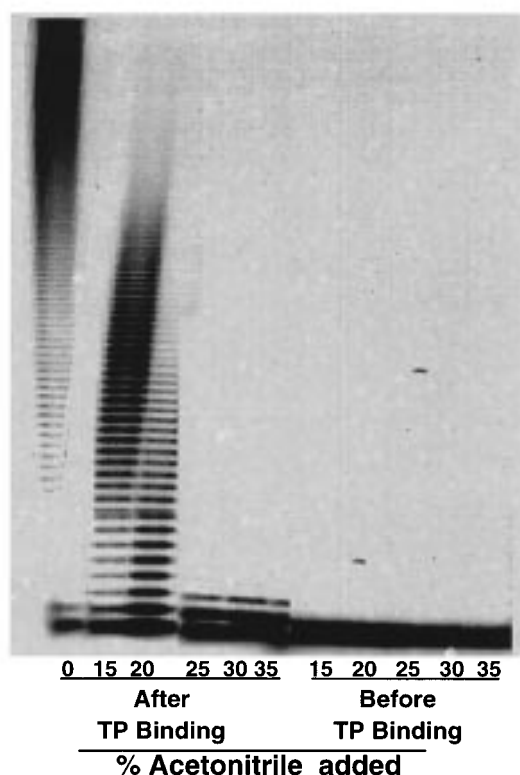


FIGURE 3: Effect of prolonged acetonitrile treatment (60 min) on the polymerase activity of HIV-1 RT before and after the template-primer binding step. Two sets of experiments were performed as described in Figure 1 except that prolonged treatments (60 min) with varying concentrations of acetonitrile were carried out in both the sets, i.e., before and after incubation of the enzyme with poly-(rA)·(dT)₁₈ template-primer. The extension reaction was carried out at 37 °C for 15 min and terminated by addition of an equal volume of Sanger's gel loading solution. The samples were analyzed by 8% denaturing polyacrylamide-urea gel electrophoresis.

It was further noted that 40–50% of the starting 18-mer oligo(dT) primer could be extended to 19-mer even up to 35% acetonitrile concentration. It has been reported that at 12% acetonitrile concentration 50% of the TP-bound heterodimeric HIV-1 RT could be dissociated into inactive monomers while complete dissociation was achieved at 16% acetonitrile (24). It is therefore unlikely that the traces of dimeric enzyme may still be present in the sample treated with 20% or higher concentrations of acetonitrile. Following the dissociation of the p51 subunit from the TP-bound heterodimeric enzyme, the p66 subunit may have assumed primer extension until it dissociates from the TP. Since the polymerase reaction mixture also contained 20% acetonitrile, the dissociated p66 cannot rebinding to the TP due to its inability to redimerize with p51 in the presence of acetonitrile.

To ascertain if p51 facilitates the binding of the TP to the enzyme, an alternative approach was used. In this case, the heterodimeric enzyme was first incubated with a self-annealing 37-mer template-primer having a bromo-dU base at the second position from the 3' terminus. Since the 3'-OH of the primer terminus is positioned in the catalytic cleft of the p66 subunit, the bdU base is expected to cross-link selectively with the p66 subunit upon irradiation of the enzyme-TP complex at 312 nm. At this wavelength, only the bdU base can be activated to cross-link with the interacting amino acid residues in the vicinity. The TP-bound heterodimer was subjected to acetonitrile treatment

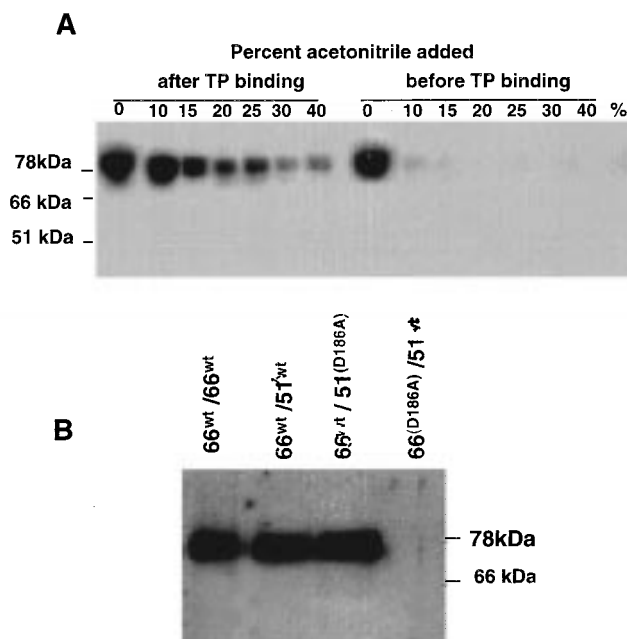


FIGURE 4: (A) Binding/photo-cross-linking of TP to p66/51 heterodimeric HIV-1 RT treated with acetonitrile before or after incubation with TP. Three micrograms of p66/51 HIV-1 RT was preincubated at 25 °C for 5 min with 5'-³²P-labeled self-annealing 37-mer TP containing photoactivatable bromo-dU base at the penultimate nucleotide from the 3' primer terminus. The enzyme-TP complex was treated for 45 min with varying concentrations of acetonitrile followed by UV irradiation at 312 nm for 3 min in a Spectrolinker (15, 16). In another set, the enzyme was pretreated with varying concentrations of acetonitrile for 45 min followed by incubation with the 5'-³²P-labeled TP. The incubation mixture was then UV-irradiated as above and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. (B) SDS-polyacrylamide gel electrophoretic separation and in situ renaturation of p66 subunit of heterodimeric HIV-1 RT (p66/51) following covalent cross-linking with the template-primer. Three micrograms of the wild type p66/51 and p66/66 enzyme were covalently cross-linked with the 37-mer self-annealing template-primer. In one set of experiments, the p51 subunit contained an Asp186Ala mutation, which would obliterate any catalytic activity due to trace levels of p51/51 homodimeric species that may be present with the p66/51 heterodimeric enzyme. In another set of experiments, the p66 subunit carrying an Asp186Ala mutation dimerized with the wild-type p51 subunit and was used as a negative control. The presence of bdU selectively ensures cross-linking at 312 nm with the catalytic subunit. The cross-linking reaction mixture contained 50 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 1 mM DTT, and 3 μg of enzyme in a final volume of 50 μL. The mixture was incubated on ice for 5 min and then irradiated at 312 nm UV for 3 min. The p66-TP covalently cross-linked species was separated from p51, un-cross-linked p66, and un-cross-linked template-primer by SDS-PAGE. After electrophoresis, the separated subunits were renatured in situ (27). The renatured gel (20 mL gel volume) was placed in a plastic tray and overlaid with a solution (20 mL) containing 2 μM [α-³²P]-dTTP (2.5 mCi/nmol), 50 mM Tris-HCl, pH 7.5, 40 mM KCl, and 6 mM magnesium acetate and incubated at 25 °C for 2 h. The gel was then washed extensively with 50 mM Tris-HCl, pH 7.5, and incubated in 5% TCA for several hours at 4 °C on a shaker to wash out radioactivity. The TCA washing step was repeated several times and the gel was finally rinsed with water, transferred to a filter paper, dried, and autoradiographed.

for 60 min to selectively dissociate the p51 subunit and then UV-irradiated at 312 nm to monitor the binding/photo-cross-linking of the TP to the enzyme. In another set of experiments, the heterodimeric HIV-1 RT was first pretreated with acetonitrile to dissociate the p51 subunit, then incubated with the template-primer, and finally subjected to UV

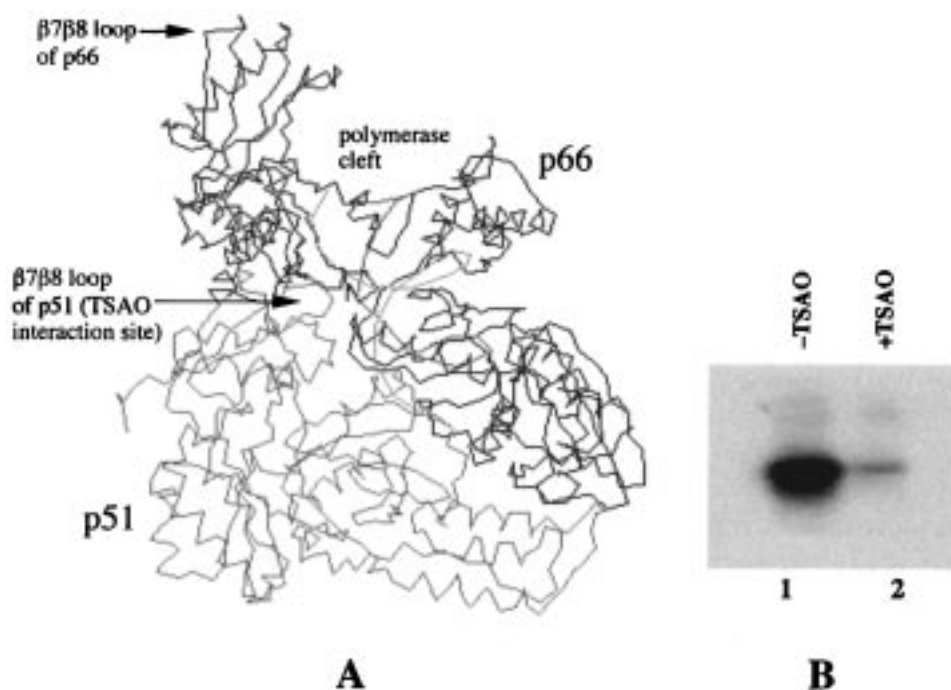


FIGURE 5: (A) Backbone structure of HIV-1 RT-DNA complex showing the position of the $\beta 7\beta 8$ loop in each subunit. The backbone structure of the HIV-1 RT-DNA complex was taken from PDB file 1HMI (7). The p66 and p51 subunits are shown in cyan and green colors, respectively. The backbone of duplex DNA is shown in light green and red colors. The $\beta 7\beta 8$ loop of p51 (TSAO binding site) is seen protruding into the floor of the polymerase cleft of p66. We propose that this interaction may be involved in opening the polymerase cleft of p66. (B) DNA binding ability of TSAO-treated HIV-1 RT. Three micrograms of p66/p51 heterodimeric HIV-1 RT pretreated with TSAO was incubated with 26/5'- ^{32}P 18-mer template-primer on ice for 5 min. The mixture was UV-irradiated at 254 nm (15) and the E-TP covalent complex was resolved by SDS-PAGE. Lane 1, control HIV-1 RT; lane 2, TSAO-treated HIV-1 RT.

irradiation. The results depicted in Figure 4A (lanes 2–7) demonstrate the effect of dissociation of p51 from the TP-bound enzyme on the binding/photo-cross-linking at varying concentrations of acetonitrile. It was observed that photo-cross-linking of TP to the enzyme could occur even upon prolonged treatment (60 min) with 40% acetonitrile. As expected, the major site of cross-linking of template-primer was contained in the p66 subunit. In contrast, dissociation of p51 prior to incubation of the enzyme with TP completely abolished the TP binding/photo-cross-linking to the enzyme in the presence of as low as 10% acetonitrile (Figure 4A, lanes 9–14). These results suggest that the p51 subunit is essential for the binding of the p66 subunit to the TP as its dissociation from the p66 subunit prior to TP binding results in complete loss of photo-cross-linking. However, the p66 subunit once bound to the TP is catalytically competent to incorporate dNTP on the bound template-primer even upon prolonged acetonitrile treatment (see Figure 3).

In the above-described experiments, both subunits were present, albeit not in dimeric form, when polymerase activity was assessed in the presence of 20% acetonitrile. To obtain further proof that the p51 subunit could be dispensed with after the DNA binding step, the photo-cross-linked p66-TP binary complex was separated by SDS-polyacrylamide gel electrophoresis and its activity was assessed after renaturation. The p66-TP covalent complex was renatured in situ and examined for its ability to incorporate a single nucleotide on the immobilized 3'-OH primer terminus. The results shown in Figure 4B clearly indicate that the template-primer-bound p66 monomer, upon renaturation, could incorporate a single nucleotide in the absence of the p51 subunit. Similar results were also obtained with the p66/p66 homodimeric enzyme where the p66 subunit cross-linked

to the TP and separated from un-cross-linked p66 subunit exhibited catalytic activity upon renaturation in the gel. The p66-TP covalent complex migrated as the 78 kDa species at a considerable distance from the 66 kDa species on SDS-PAGE; thus the possibility of in situ dimer formation with the un-cross-linked or unliganded p66 for catalysis was ruled out. Dimer formation between the two p66-TP cross-linked species is also not possible since both the p66 species would be in the DNA-bound open conformation whereas for the asymmetric dimer formation one of the two subunits would have to be in a closed conformation. Furthermore, homodimer formation of p66 or p51 with both subunits in either the open or the closed form is not possible using the same subunit interaction surfaces observed in the heterodimer (8). The covalently cross-linked TP-bound p66 molecules renatured in the gel are expected to exist in an open conformation with DNA bound in the polymerase cleft. There is another remote possibility that the radioactivity present in the renatured p66-TP complex may represent ^{32}P -dNTP bound in the substrate binding site but not incorporated on the 3'-OH primer terminus of the p66-TP cross-linked complex. This is unlikely since after incubation of the renatured gel with ^{32}P -labeled dNTP, the gel was extensively washed with 50 mM Tris-HCl, pH 7.5, followed by 5% trichloroacetic acid (TCA) containing 10 mM PPI to remove traces of ^{32}P -labeled dNTP. These washing steps ensure complete removal of the unincorporated dNTP (if any) bound or trapped with the E-TP covalent complex. Furthermore, the intensity of the radioactive band at 78 kDa position remained unaffected when the TCA denatured radioactive E-TP covalent complex was further subjected to additional washing with the Laemmli SDS electrode buffer (25), suggesting that the radioactivity in the band is due to incorporation of dNTP

by p66–TP complex and not mere trapping or binding. We have also included p66^{D186A/51^{wt}} as a control in the cross-linking-renaturation experiment. The p66^{D186A} subunit is catalytically inactive due to mutation at position 186 (16), and therefore, TP–p66^{D186A} covalent complex renatured in the gel is unable to catalyze nucleotidyltransferase reaction and the radioactive band could not be seen in the corresponding lane (Figure 4B). Binding of dNTP to HIV-1 RT in the absence of TP does not take place (26). Our results suggest that once the template–primer is bound to the p66 subunit, it does not require the participation of the other subunit for catalytic activity. The second subunit, either p51 or p66, is essential for loading of the catalytic p66 subunit of the hetero- or homodimeric RT on the template–primer for DNA synthesis.

It has been proposed that the monomeric form of p66 exists in a closed conformation topologically similar to that of p51, and its polymerase domain opens up into a large cleft upon dimerization with the p51 subunit (8). The open conformation of p66 exists as an elongated molecule with approximate dimensions of 110 Å by 45 Å. Both the polymerase and RNase H domains are aligned horizontally with respect to each other. The p66 subunit in the elongated conformation is stabilized in that position by interaction with a closed and compact p51 molecule. The specific motif in p51 that helps the p66 to adopt an open conformation is not known. An examination of the crystal structure of HIV-1 RT reveals a small groovelike region on the floor of the polymerase cleft (palm subdomain) of p66 into which the β 7 β 8 loop of the finger subdomain of p51 apparently fits and probably provides a structural support to the polymerase domain of p66. In the p66 subunit, this loop is far away from the cleft, in contrast to p51, wherein this loop is in the vicinity of the floor of the polymerase cleft (Figure 5A). This loop in p51 also contains the site for TSAO interaction (Glu 138) resulting in inactivation of the enzyme (12). An HPLC profile of TSAO-treated and untreated heterodimeric enzyme suggests that TSAO-mediated inactivation of HIV-1 RT may result from the destabilization of the p66/p51 heterodimer (data not shown). We have further shown that TSAO interaction with HIV-1 RT significantly reduces the DNA binding ability of the heterodimeric enzyme (Figure 5B). Earlier, interaction of TSAO with Glu138 in the p51 subunit was suggested to cause inhibition by steric interference due to its close proximity to the catalytic site of p66 (12, 9). These observations suggest that the β 7 β 8 loop region (residues 135–140) of p51 may be involved in conferring DNA binding ability to the p66 subunit, probably by inducing opening of the polymerase cleft. The bulky TSAO group upon binding to the β 7 β 8 loop region (Glu138) may interfere with interaction with the palm subdomain of p66, resulting in dissociation of the p66/p51 heterodimer into inactive monomers and subsequent loss of DNA binding ability. In summary, data presented here strongly suggest that, in addition to providing a strong structural support to the p66 subunit, the functional role of p51 may involve the facilitation of the binding of template–primer to the p66 subunit.

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