Requirements for DNA Unpairing during Displacement Synthesis by HIV-1 Reverse Transcriptase*

Received for publication, August 10, 2004, and in revised form, September 28, 2004
Published, JBC Papers in Press, September 30, 2004, DOI 10.1074/jbc.M409134200

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DNA displacement synthesis by reverse transcriptase during retroviral replication is required for the production of the linear precursor to integration. The sensitivity of unpaired thymines to KMnO4 oxidation was used to probe for the extent of DNA melting by human immunodeficiency virus, type 1 (HIV-1) reverse transcriptase in front of the primer terminus in model oligonucleotide-based displacement constructs. Unpairing of the two base pairs downstream of the primer (+1 and +2 positions) requires the presence of the next correct dNTP, indicating that DNA melting only occurs after the formation of the ternary complex with the enzyme tightly clamped around the DNA. The amount or extent of DNA melting is not significantly affected by the length of the already-displaced strand or the base composition of the DNA beyond the +2 position. The P61W mutant form of HIV-1 reverse transcriptase, which is partially impaired for displacement synthesis, exhibits a reduction in the amount of melting at the +1 and +2 positions. These results demonstrate the importance of the observed melting to displacement synthesis and suggest that the unpairing reaction is mediated by an intimate association between the fingers region of the enzyme and the DNA in the closed clamp conformation of the protein.

HIV-1\(^1\) reverse transcriptase is a multifunctional enzyme with a polymerase domain that catalyzes both RNA-dependent and DNA-dependent DNA synthesis and an RNase H domain that catalyzes degradation of RNA when it is hybridized to DNA (for reviews, see Refs. 1 and 2). Viral reverse transcription is initiated from a specific tRNA primer that anneals near the 5′-end of the plus-sense RNA genome at the primer binding site. Extension of the tRNA primer followed by the first strand transfer allows the completion of the minus DNA strand and generates the substrate for the RNase H cleavage that creates the plus-strand primer at the polypurine tract. Removal of the tRNA allows a second strand transfer that occurs via complementary primer binding site regions at the 3′-ends of both plus- and minus-strands. Completion of both plus- and minus-strands to produce the long terminal repeat-flanked linear product required for integration is believed to occur through a circular intermediate in which reverse transcriptase must carry out strand displacement synthesis through a stretch of DNA ∼600 bp in length (3, 4).

Reverse transcriptases have been shown to be capable of displacing either a DNA or an RNA non-template strand ahead of the primer terminus during polymerase chain elongation \textit{in vitro} (4–10), and there is evidence for displacement synthesis during plus-strand synthesis in permeabilized virions (11–13). In addition, RNA displacement synthesis may be required for removal of stably annealed fragments of the RNA genome after minus strand DNA synthesis (14).

HIV-1 reverse transcriptase is a heterodimer, consisting of 66- and 51-kDa subunits that share the same N terminus. Proteolytic cleavage of the p66 subunit, which removes the C-terminal RNase H domain, produces the smaller p51 subunit. Both the p66/p51 heterodimer and the p66 homodimer are capable of carrying out displacement synthesis (5, 8). Five subdomains make up the p66 subunit: the RNase H subdomain, a connection subdomain, and the palm, fingers, and thumb subdomains that bear a resemblance to a right hand (15). A variety of crystal structures of HIV-1 reverse transcriptase without and with short primer-template oligonucleotides (16–20) have contributed greatly to our understanding of substrate binding and the catalytic mechanisms of the enzyme. However, no crystal structure has yet been published of HIV-1 reverse transcriptase complexed with a primer/template/non-template strand that would reveal the relationship between the polymerase and the non-template strand during displacement synthesis.

Structural studies have led to the development of models for primer/template binding involving both a template grip and a primer grip (15, 17–19) and for conformational changes in reverse transcriptase induced by dNTP binding (20). Kinetic analyses have supported the concept of an ordered polymerization mechanism with a conformational change accompanying primer/template binding, followed by an additional conformational change associated with dNTP binding that leads to a productive complex (21–25). Importantly, this latter conformational change that results in the formation of the ternary complex involving the enzyme, primer/template, and dNTP shifts the fingers domain closer to the palm to more tightly wrap the enzyme around the DNA (20). Furthermore, the structure of this ternary complex reveals that the three unpaired template residues ahead of the primer terminus are folded back away from the DNA axis and interact with the fingers domain. A cocrystal structure showing DNA bound to an N-terminal fragment of Moloney murine leukemia virus reverse transcriptase similarly implicates residues in the fingers sub-domain in template binding (26).

Recently, amino acid changes at position 61 within the fingers domain of HIV-1 reverse transcriptase have revealed a role for this residue in both template binding and displacement synthesis (27). Whereas changing Phe\(^{61}\) to tyrosine or leucine

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* This work was supported by Grant CA51605 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\(^1\) The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; DTT, dithiothreitol; WT, wild type.
increased the efficiency of displacement synthesis with some loss in processivity, a change to trypton at this position was shown to significantly reduce the displacement capability of the polymerase without affecting processivity. Model building based on the crystal structure of the enzyme with a bound dNTP (20) suggests that an interaction between Phe61 and the template base two nucleotides in front of the primer terminus (referred to as the +2 position) is involved in destabilizing the helix at this position and thereby promoting displacement synthesis.

We have shown previously that reverse transcriptases are capable of DNA displacement synthesis through several hundred base pairs at a rate 4-fold slower than that of non-displacement synthesis (4, 6, 9). Moreover, based on the similar effects of temperature on non-displacement and displacement synthesis, it seems likely that displacement synthesis occurs by an active mechanism as opposed to a strictly passive process dependent on breathing of the helix in advance of the primer terminus (6). However, a strict helicase-like mechanism (28) would appear to be ruled out by the finding that the polymerase is unable to melt the DNA helix in the presence of either ATP or dNTPs under conditions where extension is prevented (4). Thus, strand separation ahead of the advancing polymerase in the displacement mode could either be facilitated directly by protein-nucleic acid interactions and/or coupled to the translocation of the polymerase with each nucleotide added. Consistent with either of these possibilities is the observation based on DNase I footprinting results that the enzyme contacts the DNA template and non-template strands, respectively (29, 30). Further and 9 nucleotides downstream of the primer terminus (31) using WT HIV-1 reverse transcriptase from Worthington Biochemical Corp. (21.6 units/μg) as an activity standard. One unit of activity incorporates 1 nmol of labeled dTMP in 20 min at 37°C using poly(rA):oligo(dT) as the template primer. The specific activities of the purified WT and F61W reverse transcriptases were 9.1 and 5.2 units/μg, respectively.

Oligonucleotides and Preparation of Displacement Templates—
Mr13dideoxy was purchased from Sigma Genosys, and non-temp3moreTs were purchased from Operon. All other oligonucleotides (mlv13primer, non-temp3, temp3, non-temp4, temp4, non-temp3T2, temp3T2, non-temp3-gap2, non-temp3-gap1, non-temp3-OH1, non-temp3-OH3 and non-temp3-OH6) were purchased from Operon Technologies. All dideoxynucleotides were purified by denaturing PAGE. The names, lengths, and sequences of the DNA oligonucleotides are as follows: mlv13 primer (30-mer, 5'-CAGGTTGTCTCCGAGTATCGACCCGGACCGCCC-3'), mlvd13dideoxy (30-mer, 5'-CAGGGGTTCCTGCCGATCCCGGAGACCGCCC-3').

EXPERIMENTAL PROCEDURES

Enzymes, Reagents, and Buffers—Heterodimeric recombinant HIV-1 reverse transcriptase (14–24 units/μl, ~22 units/μg) was purchased from Worthington Biochemical Corp. T4 polynucleotide kinase (10,000 units/ml) and bovine serum albumin were purchased from New England Biolabs, Inc. Denaturing polyacrylamide gels (3% m/v) were made with Sequagel Sequencing System reagents purchased from National Diagnostics. 5% non-denaturing polyacrylamide gels (29:1 acrylamide:bis) were prepared in a high ionic strength buffer containing 8.3, 50 mM KCl, 6 mM MgCl2. Reverse transcriptase dilution buffer (RT buffer) was 50 mM Tris-HCl, pH 8.0, 1 mg/ml bovine serum albumin, 2 mM DTT. HZ stop solution was 0.3 M sodium acetate, pH 7.0, 1 mM EDTA, 25 μg/ml salmon sperm DNA. 6× SDS sample buffer was 50 mM Tris- HCl, pH 6.8, 35% glycerol, 10% SDS, 600 mM DTT, and 0.12% bromophenol blue.

E. coli strain containing plasmids coding for the WT and the F61W mutant forms of p66 and a His-tagged form of p51 were kindly provided by Dr. V. Prasad (27). To purify the heterodimeric HIV-1 reverse transcriptase, bacteria expressing p66 or p51 were separately grown to mid-log phase, and after induction and further growth, the bacterial cell pellets were collected and mixed. After lysis of the cells, the mixtures were incubated overnight at 4 °C prior to purification of the heterodimeric proteins as described previously (29) using poly(rA):oligo(dT) as the template primer. The specific activities of the purified WT and F61W reverse transcriptases were 9.1 and 5.2 units/μg, respectively.

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dideoxy-terminated primer were initiated in an identical manner. After a 20-min incubation at 37 °C, a 10-μl portion of each reaction was removed and combined with 2.5 μl of 50% glycerol. These samples were held on ice for ~10 min before being electrophoresed through a 5% non-denaturing polyacrylamide gel at 4 °C as previously described (30) to confirm that all of the labeled DNA was bound to the enzyme (not shown).

To the remaining 18 μl of each reaction was added 2 μl of freshly made 200 mM KMnO₄ (final concentration, 20 mM) followed by an incubation at room temperature for 2.5 min. The oxidation reaction was quenched with 2 μl of 14.4 mM 2-mercaptoethanol. 180 μl of H₂O, three times. The final dried samples were resuspended in 10 μl of 98% formamide, 10 mM EDTA containing a trace amount of xylene cyanol. Before loading on a denaturing 20% polyacrylamide gel, samples were heated at 65 °C for 1 min. Gels were dried and exposed to a PhosphorImager screen and analyzed using ImageQuant software. The same sensitivity and background parameters were used for an entire gel image. Each lane on a gel was analyzed separately, calculating the percentage of the total signal in the lane represented by each detectable band. Comparisons were made between lanes based on these calculations.

Time Course Extension Assays with Non-displacement and Displacement Constructs—The procedures and DNA oligonucleotides for carrying out extension assays have been described previously (6). Briefly a single-stranded template DNA from recombinant M13 phagemid pB-SMOLTR(+) DNA was isolated and linearized. For the displacement synthesis assays, oligonucleotide MLVUSI was annealed to the template and extended to the 5'-end of the template using Sequenase. For both non-displacement and displacement synthesis, extension was initiated from 5'-²²P-end-labeled oligonucleotide MLVPBSII under standard reaction conditions (20-μl final volume) using 5 units of either WT or F61W reverse transcriptase. Control reactions with T4 DNA polymerase, which is capable of only very limited displacement synthesis, were used to verify the structure of the displacement template. Extension products were analyzed by electrophoresis in a 6% denaturing polyacrylamide gel alongside size markers prepared as described previously (6). Gels were analyzed using a PhosphorImager and ImageQuant software.

RESULTS

Displacement Constructs—The displacement constructs used in this study are shown in Fig. 1. The basic displacement construct (I) is formed by annealing a 30-mer primer (mlv13primer), a 59-mer template strand (temp3), and a 41-mer non-template strand (non-temp3). The non-template strand contains a single-stranded flap (12 bases) at its 5'-end to simulate a displacement intermediate, but the sequence of the flap region is different from the sequence at the 3'-end of the primer to prevent branch migration. Each end of the construct has a two-base 3' overhang to prevent recognition of the 3'-ends as primer termini by reverse transcriptase. This construct was designed so that incorporation of different chain-terminating dideoxynucleotides would arrest movement of reverse transcriptase and position the primer terminus at a fixed distance from thymine residues in the downstream non-template
Because unpaired thymine bases are sensitive to KMnO₄ oxidation, this experimental design allowed us to test for DNA melting by the polymerase at positions downstream of the primer terminus. In control primer-extension experiments with 5'H₁₁₀₃₂-end-labeled mlv₁₃ primer, synthesis was found to proceed to the end of the template in the presence of all four dNTPs, whereas extension reactions carried out in the presence of chain-terminating ddNTPs produced fragments with the predicted lengths (data not shown) (30).

Variations on this basic construct were designed to examine the influence of synthesis, flap length, and sequence context on melting of the non-template strand downstream from the primer terminus. The mlv₁₃ dideoxy oligonucleotide used in constructs II and III was synthesized with a 2'H₁₁₀₃₂,₃'H₁₁₀₃₂-dideoxynucleotide at its 3'H₁₁₀₃₂-end to prevent extension while allowing for the formation of a ternary complex (reverse transcriptase, primer/template/non-template, and dNTP) and was confirmed to be unextendable by a standard primer extension assay (data not shown). Construct IV was designed to examine the effects of sequence context on the extent of melting by including a stretch of three consecutive thymines in the region downstream from the primer terminus. Construct V (see Fig. 4) was identical to the basic displacement construct except for variations in the length of the single-stranded non-template strand flaps.

Effect of Next Correct Nucleotide on DNA Melting at +1 and +2 Positions—KMnO₄ oxidation of thymine residues in regions of single-stranded DNA, followed by alkaline cleavage of the modified residues generates 5'-phosphate-terminated oligonucleotide fragments (33–36). Using model displacement constructs built from oligonucleotides containing thymine residues at various distances from the primer terminus in both the template and non-template strands, we previously used this property of unpaired thymines to show that the +1 and +2 base pairs are melted by reverse transcriptase (30). However, these assays were carried out in the presence of three dNTPs plus a ddNTP, so it is likely that the nucleotide binding pocket...
was occupied by the next correct dNTP in every case. Here we systematically investigate whether the next correct dNTP is required for the observed melting and by extension whether the melting is dependent on the protein conformational change that accompanies dNTP binding (20).

HIV-1 reverse transcriptase was incubated with displacement construct I containing a 5'-32P-end-labeled non-template strand under conditions of saturating enzyme (data not shown, see Ref. 30). Incubation in the presence of ddGTP and dATP placed the same thymine residue at position +2. The cleavage patterns after KMnO4 oxidation and alkaline hydrolysis are shown in Fig. 2. KMnO4 treatment of the labeled non-template strand alone generated the oligonucleotide fragment pattern indicative of cleavage at all of the thymines (thymines not shown) (lane 1 of Fig. 2, A and B), whereas treatment of the displacement construct in the absence of enzyme generated only those fragments resulting from cleavage in the single-stranded flap region (thymines 5, 8, 10, and 12) (lane 2 of Fig. 2, A and B). This latter result confirmed that all of the non-template strand was annealed in the displacement constructs. The uniformity in the intensity of the cleavages in the single-stranded flap region served as an internal control for the sample loading on the gel.

Consistent with our previous results, a thymine residue at position +1 is sensitive to KMnO4 when the incubation mixture contained dTTP, the next templated nucleotide plus the other three dNTPs (Fig. 2A, lane 4). However, this base is resistant to oxidation and therefore not melted in the presence of all of the dNTPs except for the next correct one (Fig. 2A, lane 5). The same dependence on the presence of the next correct dNTP was observed in reactions where thymine 16 was at position +2 relative to the primer terminus (Fig. 2B). A quantitative analysis revealed that the sensitivity of thymine residues at both the +1 and +2 positions to KMnO4 oxidation in the presence of the cognate dNTP was enhanced ~30-fold relative to the non-dNTP control. For both sets of reactions, none of the bases in the duplex region of the displacement construct (residues from thymine 16 to thymine 35) were sensitive to KMnO4 in control incubations carried out either in the absence of enzyme, or with enzyme and without dNTPs to prevent extension of the primer.

Unpairing of the DNA in Front of the Primer Terminus Does Not Require Prior Synthesis—In the assays just described, the assembly of the displacement constructs required at least one cycle of addition by reverse transcriptase to incorporate the chain-terminating dideoxynucleotide. To test the possibility that the observed melting of the DNA in front of the primer terminus required prior synthesis, we utilized a primer oligonucleotide containing a dideoxy 3' terminus (constructs II and III, Fig. 1). By using an unextendable primer and non-template strands with thymines at the +1 position (construct III) and the +2 position (construct II), we could test directly for any dependence of the melting on synthesis even in the presence of the next correct dNTP.

HIV-1 reverse transcriptase was incubated with displacement constructs II and III containing 5'-labeled non-template
strand in the presence of either the next correct dNTP or the three non-cognate dNTPs, and the sensitivity of thymine residues at the +1 and +2 positions to KMnO₄ was tested as described above. For displacement construct III, thymine 13 at the +1 position in the non-template strand was 2-fold more sensitive to KMnO₄ in the reaction containing enzyme in the absence of dNTPs than in reaction without enzyme (Fig. 3A, compare lane 3 with lane 2). The basis for this enhancement in sensitivity in this particular case is unknown, but it may reflect some slight melting at the +1 position even in the absence of the next correct dNTP. In the presence of the dTTP (the cognate dNTP), the sensitivity to KMnO₄ was increased 2.3-fold (average of three determinations, standard deviation ± 0.3) relative to the no-dNTP control (Fig. 3A, lanes 3 and 4). Sensitivity to KMnO₄ in the presence of the three non-cognate dNTPs was indistinguishable (within experimental error) from the no-dNTP control (Fig. 3A, compare lanes 3 and 5). The results for displacement construct II in which thymine 14 at the +2 position of the non-template strand were similar except there was no detectable enhancement of cleavage in the incubation without dNTPs (Fig. 3B). In this case, the KMnO₄ sensitivity of thymine 14 in the presence of the enzyme was increased 7-fold (average of two determinations, S.D. ± 2) relative to the no-dNTP control. These results show that the observed melting at the +1 and +2 positions in front of the primer terminus in the presence of the next incoming dNTP is not dependent on prior extension by the polymerase activity of reverse transcriptase and requires the cognate dNTP.

Effect of Flap Length on Melting—Our previously published results showing that the two base pairs immediately ahead of the primer terminus are melted and sensitive to KMnO₄ oxidation were based on displacement constructs in which the 5′ flap varied in length from 11 to 16 nucleotides (30). In the experiments with displacement constructs I–III described above, the flap varied from 12 to 15 nucleotides in length. Because primer extension by reverse transcriptase can start at a nick (4), the displaced flap clearly is not required to initiate the process of displacement synthesis. However, to address the possibility that an interaction between the displaced strand and the enzyme contributes to the observed melting at the +1 and +2 positions once displacement synthesis is underway, we employed a set of displacement constructs in which the 5′ flaps ranged from 0 (nicked substrate) to 15 nucleotides in length.

As can be seen in Fig. 4, the flap lengths tested in this experimental design (initial constructs labeled V-a through V-f) varied with the chain-terminating ddNTP included in the incubation. When ddGTP was used to arrest synthesis after three cycles of nucleotide addition, the thymine of interest was lo-
Similarly, a thymine at the KMnO₄, irrespective of flap length (Fig. 6, 52930 below, and the reactions were subjected to KMnO₄ oxidation and analysis.

for the constructs with the longer flaps. level of melting for a nick and a one-base flap as was observed next correct dNTP. Because of the difficulty in visualizing the and again the sensitivity was dependent on the presence of the cated at position +1 relative to the primer terminus and flap lengths of 1, 2, 4, 6, 9, and 15 nucleotides were generated. When ddCTP was used to arrest synthesis, the thymine of interest was positioned +2 from the primer terminus after two cycles of synthesis, generating flap lengths of 0 (nick), 1, 3, 5, 8, and 14 (Fig. 4).

HIV-1 reverse transcriptase was incubated with displacement constructs V-a through V-f and probed with KMnO₄ to detect unpaired bases in the non-template strand ahead of the primer terminus. Fig. 5 and 6 show the results for the +1 and +2 positions ahead of the primer terminus, respectively; the diagrams above the panels indicate schematically the lengths of the flaps in the arrested complexes. To facilitate comparisons between the different constructs, the oxidizable thymine residues are labeled along the left side of each panel with numbers to denote the distance in nucleotides of that thymine from the 5'-end of the non-template strand in construct V-f (Fig. 4). To achieve the required resolution of fragments with different lengths, the shorter labeled fragments generated from constructs V-b and V-c were run on the same gel (Figs. 5A and 6A), while the longer fragments from constructs V-d, V-e, and V-f were run on a separate gel (Figs. 5B and 6B).

As can be seen in Fig. 5, regardless of length of the flap, KMnO₄ sensitivity was only observed at the +1 position ahead of the primer terminus in the presence of the next correct nucleotide (Fig. 5, arrows in lane 5 of each gel). As before, in the absence of the cognate dNTP, no melting was observed at the +1 position for any of the constructs (Fig. 5, lane 6 of each gel). Similarly, a thymine at the +2 position was sensitive to KMnO₄, irrespective of flap length (Fig. 6, lane 5 of each gel), and again the sensitivity was dependent on the presence of the next correct dNTP. Because of the difficulty in visualizing the very short cleavage fragments, the data for construct V-a are not shown, although the data were consistent with a similar level of melting for a nick and a one-base flap as was observed for the constructs with the longer flaps.

Sequence Context and Downstream Melting—The design of displacement construct I permitted us to investigate the melting of an A/T base pair located at positions +1 to +4 downstream from the primer terminus. With the exception of the +1 construct where there is no upstream base pair, the A/T base pair is flanked by G/C base pairs. We designed displacement construct IV to test whether the melting effect of reverse transcriptase at the +2 position was limited to that position or whether it could be transmitted to downstream weaker A/T base pairs. Fig. 7A (no dNTP) shows the sequence of construct IV non-template strand containing a series of three thymines at residues 18–20. Also shown are the displacement constructs generated by arresting synthesis to place thymine 16 at the +2 position (ddCTP terminator) or thymine 18 at the +2 position (ddTTP terminator). To maintain a consistency with the +2 analysis done previously using displacement construct I, a G residue was the 5' neighbor of the thymine of interest in both of these constructs. Consistent with earlier observations, a thymine positioned at +1 or +2 from the primer terminus in the non-template strand after arrest with ddG or ddC, respectively, was sensitive KMnO₄ (Fig. 7B, lanes 6 and 7). As expected when synthesis was arrested with ddT, thymine 18 at the +2 position was melted. However, no KMnO₄ sensitivity of downstream thymines at the +3 and +4 (thymines 19 and 20) positions was detected (Fig. 7B, lane 8). These data strongly suggest that the extent of melting of the non-template strand by the reverse transcriptase is restricted to the two base pairs downstream from the primer terminus.

Reduced Melting at the +1 and +2 Positions by F61W Mutant of HIV-1 Reverse Transcriptase—Fisher et al. (27) recently showed that changing Phe₆₁ to Trp reduced the displacement synthesis capability of HIV-1 reverse transcriptase without affecting the processivity of the polymerase. To test our preparation of the F61W mutant reverse transcriptase in this regard, we compared the time course of displacement synthesis of the mutant enzyme with that of WT reverse transcriptase under conditions where the rates of non-displacement synthesis for the two enzymes were very similar (Fig. 8, lanes 2–11). For all of the time points analyzed, displacement synthesis by the WT enzyme produced substantially longer extension products than were observed for the F61W mutant enzyme (Fig. 8, compare lanes 17–21 with lanes 12–16). Furthermore, at the later time points, short extension products with lengths <100 nucleotides were only observed in the reaction with the mutant enzyme. The very limited extension observed with T4 DNA polymerase using the displacement template (Fig. 8, lane 23) verified that virtually all of the template strand was base-paired to non-template strand in the displacement construct. These results confirm the observation of Fisher et al. (27) that the F61W mutation is impaired for displacement synthesis relative to the WT enzyme.

This property of the F61W form of reverse transcriptase prompted us to examine the ability of the mutant enzyme to melt the DNA downstream of the primer terminus. The WT and F61W forms of HIV-1 reverse transcriptase were incubated with displacement construct I, which had been extended in the presence of ddGTP or ddCTP to place thymine 16 at the +1 and +2 positions beyond the primer terminus, respectively (see Fig. 2). Control gel shift experiments verified that the DNA was completely bound by both the WT and mutant enzymes under these conditions (data not shown). The KMnO₄ sensitivities of thymine 16 for the WT and mutant enzymes are shown in Fig. 9. It can be seen that the sensitivity and therefore the extent of melting at both +1 and +2 positions is reduced for the F61W reverse transcriptase as compared with the WT enzyme (Fig. 9, compare lanes 4 with 5 and lanes 6 with 7). Taking into account the background present in the no-enzyme control (Fig. 9, lane
KMnO₄ sensitivity at the +1 position was reduced by a factor of 1.6 with a standard deviation of 0.1 (n = 3 determinations) for the mutant compared with the WT enzyme. At the +2 position, the fold reduction in KMnO₄ sensitivity for the mutant enzyme was even greater (3.8 ± 0.2, n = 3). The KMnO₄ sensitivity of three thymines in the unpaired flap region of the displacement construct (positions 8, 10, and 12) provide a convenient control for the uniformity of sample loading on the gel. The WT to mutant ratio of the intensities of band 8 for the +1 analysis was 1.03 ± 0.02 (Fig. 9, lanes 4 and 5), whereas the same ratio for the +2 analysis was 1.05 ± 0.08 (Fig. 9, lanes 6 and 7). The corresponding ratios for bands 10 and 12 were also very close to unity, confirming that equal amounts of the samples were loaded on the gel. From these results it appears that the reduction in displacement capability of the F61W mutant reverse transcriptase is paralleled by a reduction in the observed melting at the +1 and +2 positions of the displacement construct.

**DISCUSSION**

The overall rate of displacement synthesis is reduced ~4-fold when compared with non-displacement synthesis on the same template DNA (4, 6, 9). This reduction in the rate of displacement synthesis could be due to one or both of the following factors. First, although exceptions have been noted, displacement synthesis is generally less processive than non-displacement synthesis (5, 6). Second, it is also possible that the process of unpairing the DNA in front of the growing primer terminus during displacement synthesis is rate-limiting for the addition of the incoming dNTP (6). In either case, a difference in the interaction between reverse transcriptase and the DNA is likely responsible for the reduction in rate when comparing displacement with non-displacement synthesis. The present studies were aimed at increasing our understanding of the nature of this difference for the two modes of synthesis.

Using an assay based on the sensitivity of unpaired thymines to oxidation by KMnO₄, we previously showed that reverse transcriptase melts the two base pairs in front of the primer terminus (30) and proposed that the observed melting reflects what occurs during the process of displacement synthesis. Although this proposal fits with our expectations concerning the requirements of displacement synthesis, the model primer-templates used for the analyses are static structures that only approximately reconstruct what occurs during actual synthesis. Importantly, our results demonstrating a reduction in KMnO₄ sensitivity of thymines at the +1 and +2 positions when the F61W mutant reverse transcriptase is bound to the DNA conclusively link the melting phenomenon to the process of displacement synthesis. Although a strict quantitative comparison is not possible, the magnitude of the effect of the F61W mutation on displacement synthesis that we observe is compatible with the 1.6- to 3.8-fold reduction in the melting at the +1 and +2 positions observed for the mutant reverse transcriptase relative to that for WT enzyme. These results clearly implicate Phe⁶¹ in the process of the melting the DNA ahead of the primer terminus during displacement synthesis, presumably through a direct interaction between the side chain of the amino acid and the DNA (27). Furthermore, because the F61W mutation does not affect the processivity of reverse transcriptase (27), these results indicate that even a relatively modest decrease in the ability of the enzyme to unpair the downstream DNA contributes to the observed reduction in the rate of displacement synthesis by the mutant enzyme. It is also possible that the need to melt the DNA during displacement synthesis contributes to the observed 3-fold reduction in the rate of displacement synthesis compared with non-displacement synthesis by the WT enzyme.

The observed reduction in the displacement synthesis capacity of the F61W mutant enzyme that we observe here is less profound than originally reported by Fisher et al. (27). We attribute this difference to the use of different templates and in
particular to the presence of a very strong pause site in the displacement template used in the original report, which may have led to an overestimate of the effect of the mutation on the displacement synthesis capability of the enzyme.

To initiate synthesis, reverse transcriptase binds to a primer/template and then undergoes a conformational change that clamps the enzyme tightly around the DNA and brings the fingers domain in close contact with the DNA downstream of the primer terminus (20). This conformational change depends on the presence of the cognate dNTP. Because our previous experiments demonstrating the melting of the base pairs at the /H11001 and /H11001 positions in displacement constructs were carried out in the presence of all four dNTPs (30), it was not possible to conclude whether the melting occurred before or after dNTP binding. Here we clearly show that there is no unpairing at either the /H11001 or /H11001 positions in the absence of the cognate dNTP and thus melting must occur after dNTP binding. This finding is consistent with the hypothesis that the conformational change accompanying dNTP binding brings the region of the protein responsible for melting the DNA in juxtaposition with the duplex immediately downstream of the primer terminus.

We previously observed that the rate of displacement synthesis initiated from a nick was slower for approximately the first 10 nucleotides and then increased thereafter (6). If we assume that melting of the helix is rate-limiting under these conditions, one possible explanation for the lag is that the displaced single strand itself, once it exceeds ~10 nucleotides in length, contributes to the destabilization of the helix in front of the primer terminus. We regard this explanation as unlikely in view of the fact that 5’ flaps have been shown in other contexts to have a stabilizing effect on the adjoining helix (37–39) and our failure to see any significant melting of the DNA in the no-enzyme controls with the longer flaps (see Figs. 5 and 6). A second possibility is that once the displaced single strand exceeds a certain minimum length, it can interact with one or more sites on the protein resulting in an enhancement in the rate of displacement synthesis. For example, perhaps the displaced strand becomes tethered to the protein in such a way as to create a tension that contributes to the melting of the duplex in front of primer terminus resulting in an increase in the rate of displacement synthesis with the longer flaps. This hypothesis predicts that the extent of unpairing at the /H11001 and /H11001 positions would correlate with the length of the displaced single strand at least up to a length of ~10. However, our results showing that KMnO₄ oxidation of thymines at the /H11001 and /H11001 positions are relatively insensitive to the length of the displaced single strand rules out this possibility. The finding that the unpaired region is not enlarged even when a string of three thymines are present at the /H11001, /H11001, and /H11001 positions (Fig. 7) also tends to rule against a model in which the melting is mediated through a generalized tension between the enzyme and the already-displaced flap.

A third possibility is that as the displaced single strand grows in length, it progressively interacts with a region of the protein to increase the processivity of the polymerase, and this effect accounts for the increase in the rate of displacement synthesis as the reaction proceeds. Direct measurements of processivity as a function of extension provide support for this hypothesis. Using both oligonucleotide-based templates as well as templates that were over 600 nucleotides in length, we
FIG. 9. DNA melting at the +1 and +2 positions by F61W mutant form of HIV-1 reverse transcriptase. The WT and the F61W mutant forms of HIV-1 reverse transcriptase were incubated with displacement construct 1 in the presence of dATP, dCTP, dTTP, and ddGTP (WT, lane 4; F61W, lane 5), or with dATP, dCTP, and ddGTP (WT, lane 6; F61W, lane 7) to place thymine at the +1 or +2 positions, respectively. The reactions were subjected to KMnO₄ oxidation and analyzed by electrophoresis in 20% denaturing polyacrylamide gels. The cleavage pattern resulting from treatment of the single-stranded nontemplate DNA with KMnO₄ is shown in lane 2. A control cleavage reaction carried out in the absence of reverse transcriptase is shown in lane 3. Lane 1 shows the untreated nontemplate DNA. Numbered positions of all oxidizable thymines are indicated to the left of lane 1.

previously observed a transition from a distributive mode of synthesis to a highly processive mode when the extension exceeds ~10 nucleotides (6). Similarly, a transition from distributive to processive synthesis was observed after non-displacement extension from tRNA⁵⁸⁰⁵⁸, the natural primer for non-template strand synthesis in HIV-1 replication (23, 40).

Our results combined with the results from other laboratories support the view that a relatively specific interaction between reverse transcriptase in the closed clamp conformation and the DNA is responsible for unpairing the non-template strand from the template during displacement synthesis. Based on the available crystal structures (15, 17–20) and on the recent results of Fisher et al. (27), it seems quite likely that a region of the fingers domain of the enzyme, including Phe⁶¹, makes the contacts that are responsible for the melting reaction. Furthermore, the findings reported here suggest that the primary points of contact on the DNA are the two base pairs located immediately in front of the primer terminus. Additional experimentation will be required to define in precise detail the features of the protein that unpair the DNA during displacement synthesis. Conceivably, the relevant region of the protein could constitute a new target for chemotherapeutic intervention in HIV-1 infections.

Acknowledgments—We thank Dr. V. Prasad for generously providing the plasmids that express the wild type and F61W mutant form of HIV-1 reverse transcriptase and Sharon Schultz and Heidiarrn Interth for critical comments during the preparation of the paper.

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