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Strand Displacement Activity of the Human Immunodeficiency Virus Type 1 Reverse Transcriptase Heterodimer and Its Individual Subunits*

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By using a DNA substrate with defined gap size, we found that human immunodeficiency virus type 1 reverse transcriptase (HIV-RT) was able to perform strand displacement DNA synthesis. This activity was not affected first by calf thymus proliferating cell nuclear antigen and replication factor C and second by *Escherichia coli* single-stranded DNA-binding protein, which together allow DNA polymerase δ to perform strand displacement DNA synthesis (Podust, V., and Hübscher, U. (1993) *Nucleic Acids Res.* 21, 841–846). 3′-Azido-2′,3′-dideoxythymidine triphosphate inhibited displacement completely, indicating that DNA synthesis is required for this reaction. The HIV-RT p66 polypeptide alone could perform limited strand displacement DNA synthesis, whereas the HIV-RT p51 polypeptide was completely inactive, likely due to its inability to replicate extensively on a M13 DNA template. On the other hand the HIV-RT p51 polypeptide enhanced the strand displacement activity of the HIV-RT p66 subunit at a molar ratio of 4:1, mainly by chasing short products into longer ones. Furthermore, kinetic experiments after complementation of HIV-RT p66 with HIV-RT p51 indicated that HIV-RT p51 can restore rate and extent of strand displacement activity by HIV-RT p66 compared with the HIV-RT heterodimer p66/p51, suggesting a function of the 51-kDa polypeptide.

The human immunodeficiency virus type 1 contains a reverse transcriptase which is crucial for the replication of its RNA genome into a double-stranded DNA, which eventually is integrated in the host cell genome (see, *e.g.* Jacobo-Molina and Arnold, 1991). The reverse transcriptase consists of two polypeptides, a 66-kDa (p66) and a 51-kDa (p51) subunit (Di Marzo Veronese *et al.*, 1986). HIV-RT[1] p66 is cleaved by the viral-encoded protease generating a HIV-RT p51 and a HIV p15 polypeptide (*e.g.* reviewed in McHenry, 1989). Both subunits are found in equimolar amounts (ratio, 1:1) and both polypeptides have identical amino termini. The HIV-RT p51 lacks the carboxyl-terminal end of the HIV-RT p66 polypeptide. If both subunits were separately expressed, they displayed reverse transcriptase activity (Bathurst *et al.*, 1990), but in a heterodimer complex of HIV-RT p66/p51, the HIV-RT p51 subunit appears to be catalytically silent (El Dirani-Diab *et al.*, 1992). On the other hand it was shown that HIV-RT p66 polypeptide is able to perform DNA dependent DNA synthesis (Starnes *et al.*, 1988). Recently it had been demonstrated that examination of the individual subunits in a variety of templates under conditions optimized for each subunit revealed a significant catalytic activity for the natural HIV-RT p51 subunit (Thimmig and McHenry, 1993).

In this paper we give evidence that the HIV-RT heterodimer p66/p51 can perform strand displacement DNA synthesis, which could also be carried out in limited amounts by HIV-RT p66 alone. The HIV-RT p51 polypeptide, on the other hand, was unable to replicate extensively on natural DNA templates such as single-stranded primed M13 DNA, but could enhance the rate and extent of strand displacement activity of HIV-RT p66, suggesting a possible function for HIV-RT p51.

MATERIALS AND METHODS

Nucleic Acid Substrates

Preparation of a Substrate to Measure Strand Displacement—Single-stranded M13 (mp11) DNA was prepared according to Sambrook *et al.* (1989). The synthetic 40-mer oligodeoxynucleotide complementary to nt 7041–7080 of the M13 genome (called "primer") and the 40-mer oligodeoxynucleotide complementary to nt 6771–6810 (called "terminator") were synthesized at the service Department of the University of Zürich (Zoology) and purified by electrophoresis on a 5% denaturing PAGE. The primer was labeled with [γ-^{32}P]ATP (Amersham) and polynucleotide kinase without isotopic dilution (equal amount of oligonucleotide and labeled nucleotide in the reaction mixture) according to Sambrook *et al.* (1989). The labeled primer (2-fold molar excess over template) and unlabeled terminator (10-fold excess) were annealed to M13 DNA as described (Podust *et al.*, 1992a). For both oligonucleotides, 1 A_{260} = 33 μg/ml according to Sambrook *et al.* (1989).

Preparation of a Substrate to Measure Strand Displacement DNA Synthesis—The substrate was the same as the gapped substrate mentioned above except that the terminator instead of the primer was labeled with [γ-^{32}P]ATP. After the annealing reaction the substrate was separated from free [γ-^{32}P]ATP by adding 50 μl of buffer C (see below) to the kinase reaction, and the solution was filtered over a Sephadex G 50 column (1 × 2 cm; Pharmacia) equilibrated previously with buffer C. Samples of 100 μl were eluted by adding 600 μl of buffer C. Radioactive samples were eluted in fractions 4–6. Fractions with the highest amount of radioactivity were pooled and adjusted to 10 mM $MgCl_2$.

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[1] The abbreviations used are: HIV, human immunodeficiency virus; RT, reverse transcriptase; PCNA, proliferating cell nuclear antigen; RF-C, replication factor C; SSB, single-stranded DNA-binding protein; DTT, dithiothreitol; AZTTP, 3′-azido-2′,3′-dideoxythymidine triphosphate; nt, nucleotide(s).

Digestion of the entire substrate with 2 units of the restriction enzyme *Dra*I (Boehringer) was carried out at 37 °C for 4 h according to the manufacturer's protocol.

The homopolymers poly(rA) and poly(dA) (Pharmacia; concentrations were determined according to the manufacture's protocol) were mixed at a weight ratio 10:1 to the oligomer oligo(dT)$_{12-18}$ (Pharmacia) in 20 mM Tris-HCl (pH 8), containing 20 mM NaCl and 1 mM EDTA, heated at 75 °C for 10 min with subsequent slow cooling to room temperature.

Buffers

The following buffers were used. Buffer A: 50 mM Tris-HCl (pH 7.3), 10% (v/v) glycerol, 1 mM EDTA, 1 mM DTT, and 1 mg/ml phenylmethylsulfonyl fluoride; buffer B: 50 mM Tris-HCl (pH 7.3), 50% (v/v) glycerol, 1 mM EDTA, and 25 mM KCl; buffer C: 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA; buffer D: 50 mM Tris-HCl (pH 7.5), 50% (v/v) formamide, 20 mM EDTA, 0.03% (w/v) xylene cyanol, and 0.03% (w/v) bromphenol blue; buffer E: 20% (w/v) sucrose, 50 mM EDTA (pH 8), 1% SDS, 0.03% (w/v) xylene cyanol and 0.03% (w/v) bromphenol blue.

Enzymes and Proteins

HIV-RT heterodimer p66/p51 was overexpressed in *Escherichia coli* (gift of L. Loeb, Joseph Gottstein Memorial Cancer Research Laboratory, University of Washington) and purified based on a previous published procedure (Hafkemeyer *et al.*, 1991). The fraction containing RT activity after the phosphocellulose column was pooled, dialyzed against buffer A, and then applied to an fast protein liquid chromatography Mono Q column equilibrated with the same buffer. Enzyme was eluted from the column with a 0–0.6 M KCl gradient in buffer A. Fractions with RT activity were eluted at 200 mM KCl. Pooled fractions were dialyzed against buffer B and stored at −20 °C. HIV-RT p66 and HIV-RT p51 were purified as described (Thimmig and McHenry, 1993). All enzymes were purified to >95% purity and had a specific activity (tested on poly(rA)/oligo(dT)) of: HIV-RT heterodimer p66/p51, 2500 units/ml, 17,400 units/mg; HIV-RT p66, 17,100 units/ml, 15,000 units/mg; and HIV-RT p51, 1800 units/ml, 1400 units/mg. Calf thymus DNA polymerases α and δ were isolated as described in Podust *et al.* (1992b) and Weiser *et al.* (1991). *E. coli* SSB was purified from the overproducer RLM 727 following the procedure of Lohman *et al.* (1986). PCNA and RF-C from calf thymus were purified according to Prelich *et al.* (1987) and Podust *et al.* (1992a), respectively.

Enzymatic Assays

DNA Polymerases—RT activity was determined in a final volume of 25 µl containing 50 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 80 mM KCl, 1 mM DTT, 0.5 mM EDTA, 10 µM [^{3}H]dTTP (500 cpm/pmol), 1 µg of poly(rA)/oligo(dT) (base ratio, 10:1), and enzyme fractions to be tested. Activity measurement on single-stranded primed M13 DNA contained, in a final volume of 25 µl, 40 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, dATP, dGTP, dCTP each at 50 µM, 10 µM [^{3}H]dTTP (500 cpm/pmol), 100 ng of single-stranded primed M13 DNA, and enzyme fractions to be tested. DNA polymerase α was tested with activated calf thymus DNA and DNA polymerase δ with poly(dA)/oligo(dT) (base ratio, 10:1) in the presence of PCNA as described (Weiser *et al.*, 1991). 1 unit of DNA polymerase activity corresponds to the incorporation of 1 nmol of dNMP into acid-precipitable material in 60 min at 37 °C.

Strand Displacement Activity—The assay to measure strand displacement activity contained, in a final volume of 25 µl, the following components: 40 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.2 mg/ml bovine serum albumin, 10 mM $MgCl_2$, 1 mM ATP, dATP, dGTP, dCTP, dTTP each at 50 µM, 100 ng of gapped [^{32}P]M13 DNA (labeled primer), 100 ng of PCNA, 6 ng of RF-C, 350 ng of *E. coli* SSB, and amounts of DNA polymerases as indicated in the figure legends. The mixture was incubated for 1 h at 37 °C unless otherwise mentioned, terminated by addition of 5 µl of solution A (3.5% SDS, 0.7 mg/ml proteinase K, 20 mM EDTA (pH 8)), and incubated for another 30 min at 37 °C. Then 4 µl of 3 M sodium acetate and 40 µl of isopropyl alcohol were added. All samples were centrifuged for 30 min at 15,000 × *g*. Pellets were washed with 80% ethanol and finally dissolved in 20 µl of buffer D. The samples were heated for 5 min at 95 °C before loading onto a 5% PAGE, containing 7 M urea (17 cm × 21 cm × 0.8 mm). Electrophoresis was performed at 30 V/cm until the bromphenol blue dye reached the bottom. The gel was then fixed in 10% acetic acid containing 12% methanol, dried at 80 °C, and exposed to an x-ray film (Kodak, X-Omat S).

Strand Displacement DNA Synthesis—To measure strand displacement DNA synthesis, the following components were used in a final volume of 25 µl: 40 mM Tris-HCl (pH 7.5), 0.2 mg/ml bovine serum albumin, 1 mM DTT, 8 mM $MgCl_2$, dATP, dGTP, dCTP, dTTP each at 50 µM, 50 ng of digested gapped [^{32}P]M13 DNA (labeled terminator), and amounts of HIV-1-RT as indicated in the figure legends. The mixture was incubated for 1 h at 37 °C unless otherwise mentioned and the reaction terminated by addition of 5 µl of buffer E. Samples were then loaded on a 12% PAGE (17 cm × 21 cm × 0.8 mm) and electrophoresis performed at 30 V/cm for 60 min. Finally the gel was fixed in 10% acetic acid containing 12% methanol, dried at 80 °C, and exposed to a x-ray film (Kodak, X-Omat S).

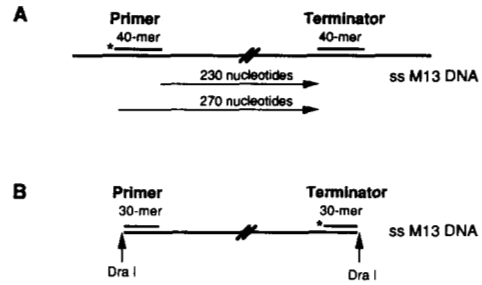

FIG. 1. **Gapped DNA substrate to measure strand displacement DNA synthesis.** The M13 DNA substrate was prepared by annealing two synthetic 40-mer oligonucleotides, thus leaving a 230-nt gap and either the primer (*A*) or the terminator (*B*) was labeled as outlined under "Materials and Methods." The substrate in *B* was additionally digested with the restriction enzyme *Dra*I to give a terminator fragment that had after the DNA synthesis reaction the same size (30 nt).

PhosphorImager

Gels with labeled separated DNA products were exposed for 3 h to a special screen for a PhophorImager (Molecular Dynamics). After exposure, the screen was scanned into the PhophorImager and the indicated regions (see Figs. 3, 5, 6, and 7) quantified by using the Image Quant program of Molecular Dynamics.

Other Methods

Protein determinations were performed according to Bradford (1976).

RESULTS

DNA Substrates Used to Study Strand Displacement DNA Synthesis—To measure strand displacement DNA synthesis, we used single-stranded M13 DNA annealed to two oligonucleotides of 40 bases in length (*substrate A* in Fig. 1). The gap between the two 40-mers was 230 nucleotides. The 40-mer at the *left* represented the primer and is for this reason called primer, whereas the 40-mer at the *right side* was used to act as a potential obstacle for DNA synthesis and is called terminator. The terminator could also be used as a primer. This did not interfere with the analysis, since primer extension assays were exclusively performed with labeled primer.

To test whether the displacement activity is dependent on DNA synthesis the same oligonucleotides as in A were used, but in this experiment the terminator instead of the primer was labeled at its 5′ end. After annealing, the substrate was digested with the restriction enzyme *Dra*I, producing a primer and terminator each of 30 nucleotides in length (*substrate B* in Fig. 1). This substrate allowed the discrimination of strand displacement activity from 5′ to 3′ exonuclease activity and also from endonucleases.

HIV-RT Heterodimer p66/p51 Can Perfom Strand Displacement—Strand displacement of HIV-RT heterodimer p66/p51 was tested on a substrate as indicated in Fig. 1*A*. From our previous observations (Podust and Hübscher, 1993) it was known that the eukaryotic DNA polymerase α is able to fill the gap of 230 nucleotides nearly completely, but has no strand displacement activity. Instead the DNA polymerase δ has the possibility in presence of the three proteins PCNA, RF-C, and *E. coli* SSB, not only to fill the gap, but also to displace DNA. Therefore the two DNA polymerases were used as positive or negative controls for strand displacement activity (Fig. 2, *lanes 1, 8,* and *9*, respectively). *Lane 8* indicates that the two proteins, PCNA and RF-C, are necessary for strand displacement

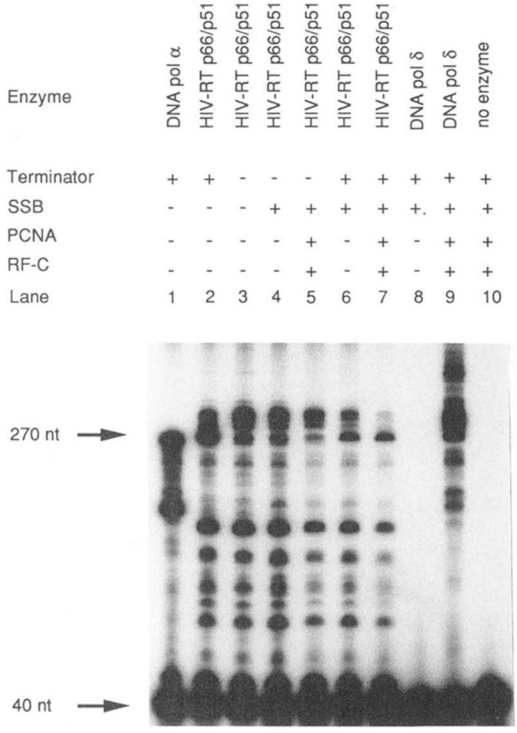

FIG. 2. **Strand displacement activity of HIV-RT heterodimer p66/p51 in the absence or presence of calf thymus PCNA, calf thymus RF-C, and *E. coli* SSB.** Reactions were carried out under conditions described under "Materials and Methods" by using either singly primed M13 DNA (*substrate A* in Fig. 1) without a terminator (*lanes 3–5*) or gapped M13 DNA with a terminator (*lanes 1, 2,* and *6–10*). Where indicated on the *top* of the autoradiogram, the following amounts of enzymes and proteins were added: DNA polymerase α (0.25 unit), DNA polymerase δ (0.15 unit), HIV-RT heterodimer p66/p51 (0.1 unit), PCNA (100 ng), RF-C (6 ng), and *E. coli* SSB (350 ng). Markers were: 40 nt correspond to the labeled primer (*lane 10*); 270-nt marker was polymerized on a gapped M13 DNA with terminator by DNA polymerase α (*lane 1*), which is known to fill this gap to near completion in the absence of PCNA, RF-C, and *E. coli* SSB (Podust and Hübscher, 1993).

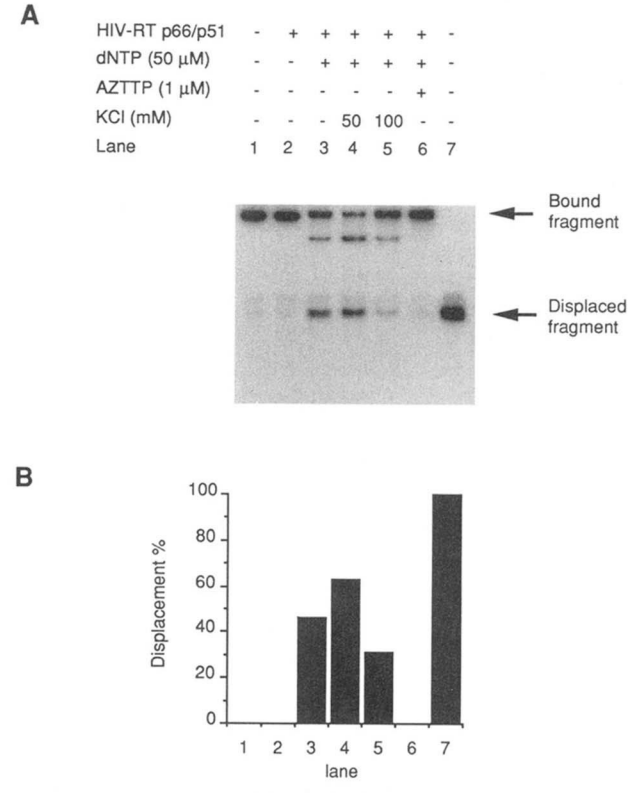

FIG. 3. **Strand displacement activity by HIV-RT heterodimer p66/p51 is dependent on DNA synthesis.** *A*, the reaction mixtures containing 0.3 unit of the HIV-RT heterodimer p66/p51 and 50 ng of the gapped substrate with the labeled terminator (*substrate B* in Fig. 1) were incubated as described under "Materials and Methods." dNTP's (50 μM), AZTTP (1 μM), and KCl (50 or 100 mM) were added where indicated on the *top* of the autoradiogram. *Lane 7*, substrate was heated at 97 °C for 5 min. *B*, quantification of the experiment shown in *A*.

by DNA polymerase δ. *Lanes 2–7* show DNA synthesis by HIV-RT heterodimer p66/p51. The length of the product is the same irrespective of whether a terminator was present or not (*lanes 2* and *3*). The fact that the enzyme stops at the same place, even in the absence of a terminator, is likely due to known termination sites at this place of the single-stranded M13 DNA, which partially inhibited DNA synthesis by HIV-RT heterodimer p66/p51 (Abbotts *et al.*, 1993). Three such sequences (NNNNNCGNNANNNA/T) occur in this region of single-stranded M13 DNA, one of these immediately before the terminator starts and the other two within 80 nucleotides of the terminator region but there are some DNA products clearly longer than 270 nucleotides. DNA synthesis by HIV-RT heterodimer p66/p51 was not inhibited in the presence of *E. coli* SSB (*lanes 4* and *6*). Simultaneous addition of *E. coli* SSB, PCNA, and RF-C, however, inhibited DNA synthesis 50% (*lane 5*) as well as strand displacement more than 90% (*lane 7*). These results suggested that HIV-RT is able to perform strand displacement in the absence of PCNA, RF-C, and *E. coli* SSB (Podust and Hübscher, 1993).

Strand Displacement by HIV-RT Heterodimer p66/p51 Is Dependent on DNA Synthesis—To see whether strand displacement is dependent on DNA synthesis, we tested the HIV-RT heterodimer p66/p51 with the substrate B shown in Fig. 1. Fig. 3, *lane 2*, shows that the HIV-RT heterodimer p66/p51 was unable in the absence of the four dNTP's to displace the 30-mer, thus excluding the possibility that the activity is a *bona fide* DNA helicase. DNA helicase activity could not be detected under different conditions tested, *e.g.* by varing nucleoside triphosphates and comparing it with various cellular DNA helicases (Thömmes *et al.*, 1992). If, however, the four dNTP's were included the terminator was displaced (*lane 3*). 50 mM KCl stimulated displacement 20% (*lane 4*), whereas 100 mM reduced the activity more than 40% (*lane 5*). In no case could labeled monophosphates be detected, indicating that the 30-mer terminator was displaced as a whole. When the DNA synthesis was inhibited with AZTTP, a specific HIV-RT chain termination inhibitor displacement is decreased more than 99% (*lane 6*), suggesting that DNA synthesis was required for strand displacement. The band below the bound fragment (*lanes 3–5*) likely represents DNA synthesis from the terminator with substrate that was not cut by *Dra*I (see also Fig. 1).

Limited Strand Displacement Synthesis Can Also Be Carried Out by HIV-RT p66—To test whether the strand displacement activity is due to the HIV-RT heterodimer p66/p51 or to one of the subunits, we investigated each of the two subunits alone. For a meaningful comparison, we first tested the activity of HIV-RT heterodimer p66/p51, HIV-RT p66, and HIV-RT p51 in a standard RT assay on poly(rA)/oligo(dT). The rationale to measure HIV-RT p51 is described by Thimmig and McHenry (1993). The same amounts of RT activity on poly(rA)/oligo(dT) (Fig. 4*A*) were then tested on single-stranded primed M13 DNA (Fig. 4*B*). Fig. 4*B* shows that if the same amount of RT activity on poly(rA)/oligo(dT) was taken, the HIV-RT heterodimer p66/p51 and, to a lesser extent, the HIV-RT p66 polypeptide could perform DNA synthesis on a natural DNA-primed DNA substrate. The HIV-RT p51, on the other hand, was inactive. Also after testing different buffers such as KPO_4, imidazol, HEPES,

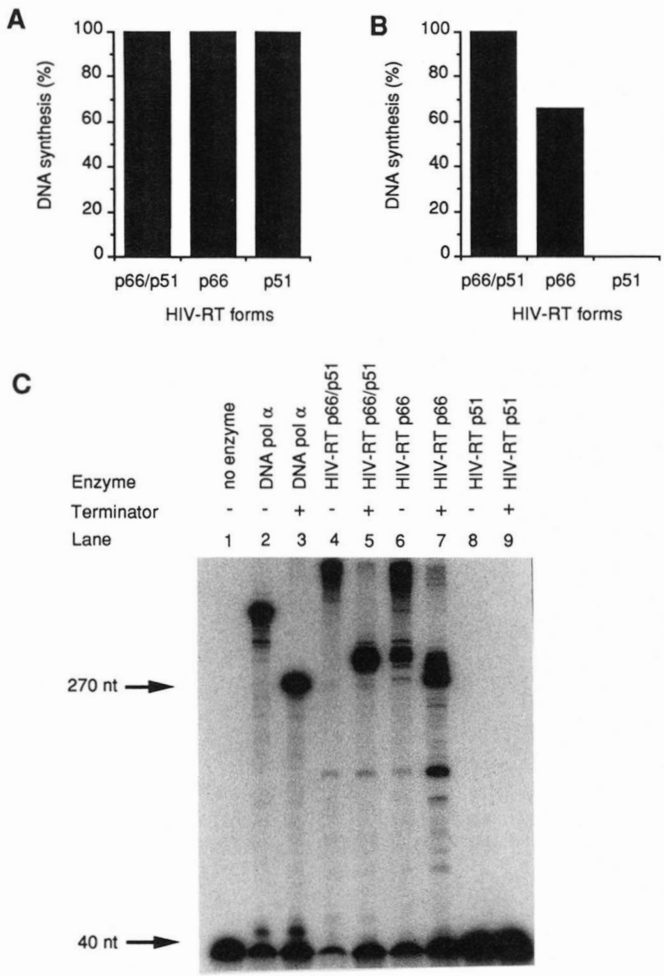

FIG. 4. **Strand displacement activity by three forms of HIV-RT.** Three forms of HIV-RT were tested: 1) the HIV-RT heterodimer p66/p51, 2) HIV-RT p66 polypeptide, and 3) HIV-RT p51 polypeptide. *A*, the three HIV-RT forms were first titrated on poly(rA)/oligo(dT) and compared with each other for activity. 100% corresponds to 530 pmol/60 min incorporation for HIV-RT heterodimer p66/p51, HIV-RT p66, and for HIV-RT p51. *B*, the same amount of enzyme as in *A* on poly(rA)/oligo(dT) was tested in a reaction mixture containing singly primed M13 DNA as described under "Materials and Methods," resulting in 88 pmol for HIV-RT heterodimer p66/p51, 59 pmol for HIV-RT p66, and <0.1 pmol for HIV-RT p51. *C*, reactions were carried out under conditions as described under "Materials and Methods" by using singly primed M13 DNA (*substrate A* in Fig. 1) without terminator (*lanes 1, 2, 4, 6*, and *8*) or gapped M13 DNA with terminator (*lanes 3, 5, 7*, and *9*). Each reaction contained the same amount of units as in *A* for HIV-RT heterodimer p66/p51, HIV-RT p66, and HIV-RT p51. Markers used were: 40 nt correspond to the labeled primer (*lane 1*); 270-nt marker was polymerized on a gapped M13 DNA with terminator by 0.5 unit of DNA polymerase α (*lane 3*).

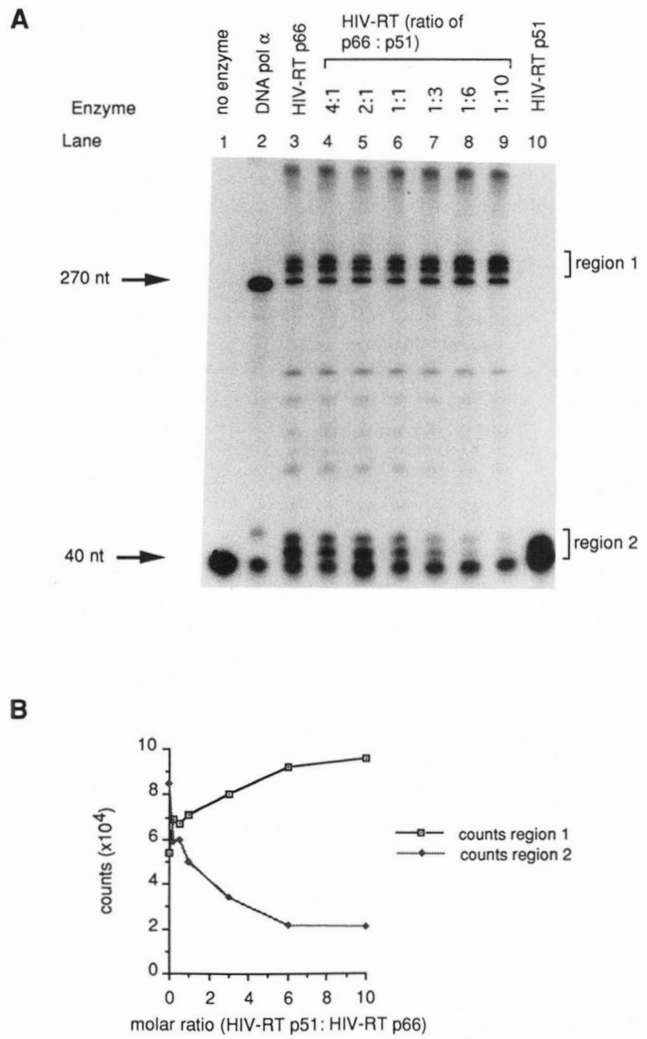

FIG. 5. **Strand displacement activity by HIV-RT p66 in the presence of increasing amounts of HIV-RT p51.** *A*, reactions were carried out under conditions as described under "Materials and Methods" by using gapped M13 DNA containing a terminator (*substrate A* in Fig. 1). Markers were: *lanes 1* and *2*, 40 nt corresponds to initial labeled primer (*lane 1*); 270-nt marker was polymerized on a gapped M13 DNA with terminator by 0.5 unit of DNA polymerase α (*lane 2*). *Lanes 3–9* contained 0.15 unit of HIV-RT p66 and were supplemented with various molar amounts of HIV-RT p51; *lane 3*, none; *lane 4*, 1:4 (HIV-RT p51:HIV-RT p66); *lane 5*, 1:2; *lane 6*, 1:1; *lane 7*, 3:1; *lane 8*, 6:1; *lane 9*, 10:1; *lane 10*, the same amount of HIV-RT p51 was used as in *lane 9*, but in the absence of HIV-RT p66. The two subunits were preincubated for 5 min at 37 °C. *B*, the two regions, indicated by *1* and *2*, were quantified by using a PhophorImager, and the counts were blotted against the different amounts of HIV-RT p51 added to 0.15 unit of HIV-RT p66.

bis-Tris, different pH values (6.5–8.5), and different salt concentrations (10–100 mM KCl), no activity of HIV-RT p51 on single-stranded primed M13 DNA could be found (data not shown). Fig. 4*C* shows the product analysis of these three HIV-RT forms on the substrate A shown in Fig. 1. As control, DNA polymerase α was used to indicate the localization of the terminator (*lane 3*: 270 nucleotides). The HIV-RT p66 alone could perform limited strand displacement compared with the HIV-RT heterodimer p66/p51 (compare *lanes 7* and *5*), whereas as expected, the HIV-RT p51 polypeptide was essentially inactive under these conditions (*lanes 8* and *9*).

HIV-RT p51 Can Influence the Strand Displacement Activity of HIV-RT p66—Next we tested whether the limited strand displacement activity of HIV-RT p66 can be reconstituted by addition of HIV-RT p51. Different ratios of molar amounts of HIV-RT p66 and HIV-RT p51 were preincubated in an assay mix (without DNA) at 37 °C for 5 min. After this time the DNA substrate was added to the protein mixture. Fig. 5*A* (*lanes 3–9*) shows different ratios of HIV-RT p66 and HIV-RT p51. There is enhancement of the bands longer than 270 nucleotides upon addition of excess HIV-RT p51 over HIV-RT p66. In contrast the products smaller than 270 nucleotides decreased by adding HIV-RT p51. HIV-RT p51 alone at the highest amount tested has, as already observed in Fig. 4*C*, virtually no DNA synthesis activity (*lane 10*). Again DNA polymerase α was used as a control (*lane 2*: 270 nucleotides). Fig. 5*B* quantifies the increase as well as the decrease of the two regions indicated in Fig. 5*A* (*regions 1* and *2*). Region 1 was diagnostic for increase of strand displacement and was calculated from all bands beyond the 270 nucleotides, whereas region 2 indicated the increase in DNA synthesis. In Fig. 6*A* the same effect was seen in a kinetic

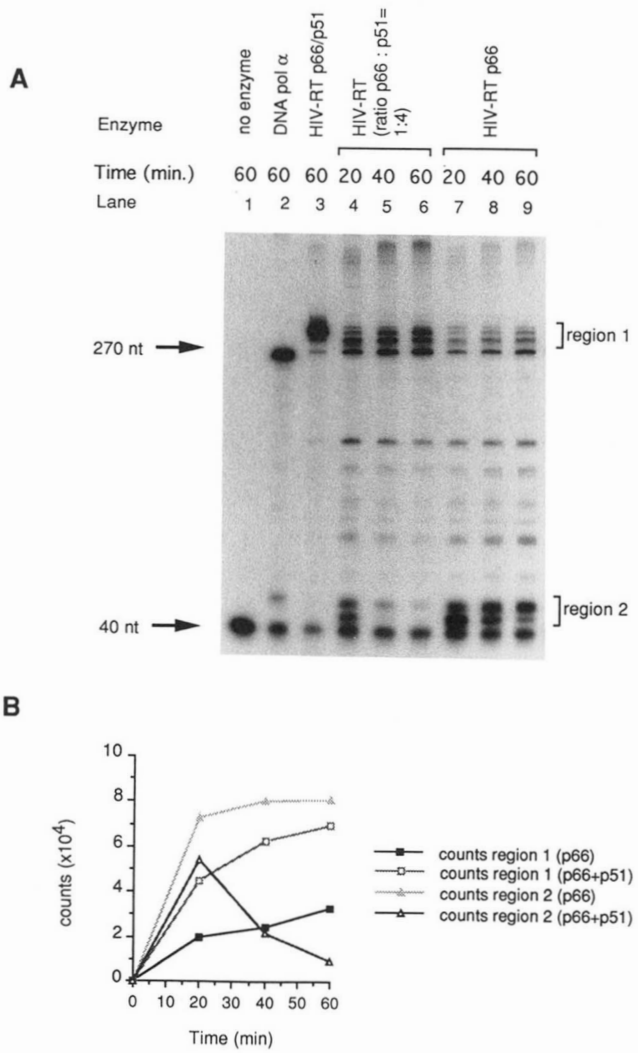

FIG. 6. **Kinetics of strand displacement activity of HIV-RT p66 in the presence and absence of HIV-RT p51.** *A*, reactions were carried out under conditions as described under "Materials and Methods" by using gapped M13 DNA containing a terminator (*substrate A* in Fig. 1). 0.15 unit of HIV-RT p66 was used for all reactions. HIV-RT p66 was preincubated in the reaction mixture for 5 min at 37 °C, either with no HIV-RT p51 or with HIV-RT p51 at a ratio of 4:1 (HIV-RT p51:HIV-RT p66) and then added to the labeled substrate. Reactions were stopped after 20, 40, and 60 min. Markers used were: 40 nt correspond to the labeled primer (*lane 1*); 270-nt marker was polymerized on a gapped M13 DNA with terminator by 0.5 unit of DNA polymerase α (*lane 2*) and the strand displacement products by 0.3 unit of HIV-RT heterodimer p66/p51 (*lane 3*, compare also Fig. 4, *lane 5*). *B*, the two regions indicated by *1* and *2* were quantified with a PhophorImager and the kinetics blotted against region 1 (*squares*) and region 2 (*triangles*) for either HIV-RT p66 alone (*closed symbols*) and the reconstituted HIV-RT heterodimer HIV-RT p66 + p51 (*open symbols*).

experiment at a ratio of 1:4 of HIV-RT p66 to HIV-RT p51 (*lanes 4–6*) and was compared with the synthesized products during the same time points of HIV-RT p66 alone (*lanes 7–9*). HIV-RT heterodimer p66/p51 (*lane 3*) and, as outlined earlier, DNA polymerase α served as controls (*lane 2*). From the quantification of the band intensities with the PhophorImager (Fig. 6*B*), it was obvious that the region above the primer (=*region 2*) decreased in presence of HIV-RT p51 (ratio, 1:4 (HIV-RT p66:HIV-RT p51)) 10 times faster than in its absence. Strand displacement was stimulated by HIV-RT p51, and it appears that both rate and extent are affected by HIV-RT p51 (see below).

Rate and Extent of Strand Displacement Can Be Restored upon Addition of HIV-RT p51 to HIV-RT p66—In a further experiment we reconstituted strand displacement of HIV-RT

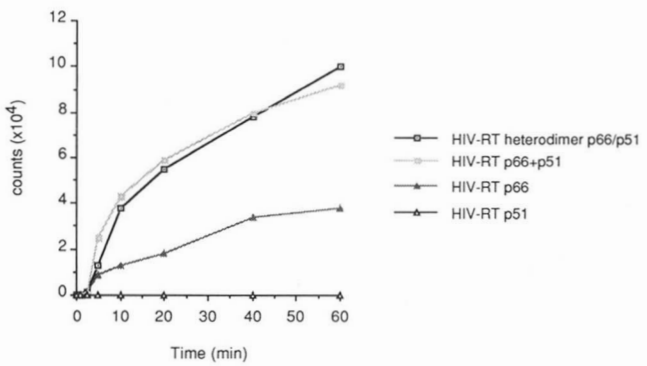

FIG. 7. **Reconstitution of strand displacement activity of HIV-RT p66 by HIV-RT p51 in excess of enzyme over template/primer.** Reactions were carried out under conditions as described under "Materials and Methods" by using gapped M13 DNA containing a terminator (*substrate A* in Fig. 1). Four kinetics were run in parallel: HIV-RT p51 (2 units), HIV-RT p66 (2 units), HIV-RT heterodimer p66/p51 (2 units), HIV-RT p66 (2 units), and HIV-RT p51 at a molar ratio 1:4 (HIV-RT p66:HIV-RT p51) that had been preincubated for 8 h at 4 °C. Aliquots were removed after 0, 1, 2.5, 5, 10, 20, 40, and 60 min, separated on a 5% PAGE, 7 M urea gel, dried, exposed, and the DNA products corresponding to region 1 in Figs. 5 and 6 were quantified by using a PhosphorImager as described under "Materials and Methods." *Open triangle*, HIV-RT p51; *closed triangle*, HIV-RT p66; *open squares*, HIV-RT heterodimer p66/p51; *closed squares*, reconstituted HIV-RT p66 +p51.

p66 with HIV-RT p51 in excess of enzyme over template/primer. Fig. 7 shows a kinetic analysis where region 1 (see Fig. 6) was quantified. As expected, HIV-RT p66 was able to partially displace the terminator, and HIV-RT p51 was completely inactive. If HIV-RT p51 was preincubated with HIV-RT p66 for 8 h at 4 °C at a molar ratio of 1:4 (HIV-RT p66:HIV-RT p51), a complete restoration of rate and extent of strand displacement activity was observed. Since the curve superimposed the corresponding one obtained with the authentic HIV-RT heterodimer p66/p51, we conclude that the HIV-RT p51 facilitates strand displacement of HIV-RT p66.

HIV-RT Heterodimer p66/p51 and HIV-RT p66 Catalyze Strand Displacement in a Single Round of DNA Synthesis—Finally, an enzyme trap experiment (DiStefano *et al.*, 1991) should support the suggestion that HIV-RT heterodimer p66/p51 and HIV-RT p66 can perform strand displacement in a single round of DNA synthesis. From Fig. 8 it is evident that the heterodimeric HIV-RT p66/p51 is more able to do so than the HIV-RT p66 alone.

DISCUSSION

In this paper we show that the HIV-RT heterodimer p66/p51 can perform strand displacement. The data for the HIV-RT heterodimer p66/p51 are in agreement with an earlier report by Huber *et al.* (1989). This activity is not affected by calf thymus PCNA and RF-C or *E. coli* SSB (or replication protein A, data not shown). In addition strand displacement was dependent on DNA synthesis. To test whether the activity is due to one of the individual subunits of HIV-RT, the HIV-RT p66 and HIV-RT p51 were each tested individually. HIV-RT p51 was unable to perform extensive DNA-dependent DNA synthesis on single-stranded primed M13 DNA, whereas on the synthetic poly(rA)/oligo(dT), activity could be detected. HIV-RT p66, on the other hand, showed DNA synthesis activity on both templates. To a lesser extent than the HIV-RT heterodimer p66/p51, the HIV-RT p66 was able to perform strand displacement. This activity is enhanced upon addition of HIV-RT p51. This enhancement of strand displacement activity could already be seen at a molar ratio of 4:1 (HIV-RT p66:HIV-RT p51). Rate and extent of strand displacement could be completely restored upon addition of HIV-RT p51 to HIV-RT p66. In sum, the function of HIV-RT p51 might be to enhance tha rate of DNA syn-

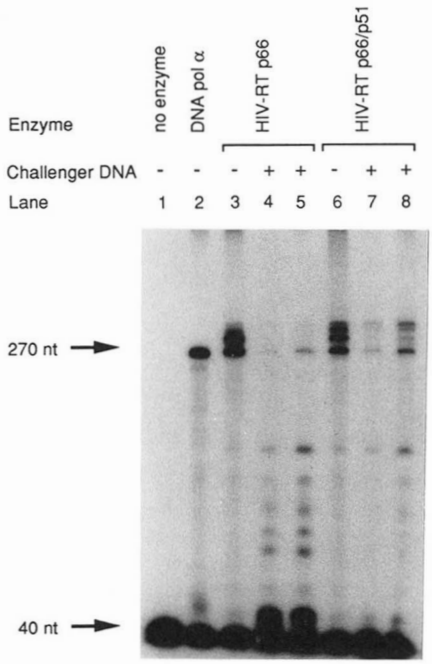

FIG. 8. **A template challenge experiment suggests that the heterodimeric HIV-RT p66/p51 and HIV-RT p66 catalyze the strand displacement in a single round of DNA synthesis.** Reactions were carried out under conditions as described under "Materials and Methods" by using gapped M13 DNA containing a terminator (*substrate A* in Fig. 1). *Lanes 1* and *2*, 40 nt corresponds to initial labeled primer (*lane 1*); 270-nt marker was polymerized on a gapped M13 DNA with terminator by 0.5 unit of DNA polymerase α (*lane 2*); *lane 3*, HIV-RT p66 (0.2 unit) was first mixed with the labeled substrate (40 fmol 3′ OH ends) for 2 min at 37 °C, followed by the addition of the four dNTPs; *lane 4*, HIV-RT p66 (0.2 unit) was mixed with the labeled substrate (40 fmol of 3′ OH ends) and the challenger DNA poly(rA)/oligo(dT) (20 pmol of 3′ OH ends, 500-fold excess) for 2 min at 37 °C, followed by the addition of the four dNTPs; *lane 5*, HIV-RT p66 (0.2 unit) was first mixed with the labeled substrate (40 fmol of 3′ OH ends) for 2 min at 37 °C, followed first by the addition of the four dNTPs with subsequent incubation for 1 min at 37 °C and second by addition of the challenger DNA poly(rA)/oligo(dT) (20 pmol of 3′ OH ends, 500-fold excess); *lanes 6–8*, same as *lanes 4–6* with the HIV-RT heterodimer p66/p51 (0.15 unit) instead of the HIV-RT p66.

thesis and the strand displacement activity of HIV-RT p66. Up to now, no function of HIV-RT p51 could be found. A possible role of HIV-RT p51 might be to act in the asymmetric dimer (Thimmig an McHenry, 1993) as an enhancer of the strand displacement activity of HIV-RT p66.

In the *E. coli* bacteriophage Ø29 it has been found that mutations near the *Exo*III box of Ø29 DNA polymerase inhibited strand displacement by this enzyme (Soengas *et al.*, 1992). A similar sequence is also found in DNA polymerase δ (Cullmann *et al.*, 1993), an enzyme possessing strand displacement activity in the presence of *E. coli* SSB, PCNA, and RF-C (Podust and Hübscher, 1993) and in HIV-RT (Ratner *et al.*, 1985). Site-directed mutagenesis experiments of these domains will be required to test the hypothesis of a general strand displacement domain in certain DNA polymerases.

The strand displacement activity of HIV-RT heterodimer p66/p51 could also play a critical role in the replication cycle of HIV 1 (for review see, *e.g.* Varmus *et al.*, 1985; McHenry, 1989). Low molecular weight DNA extraction of cells infected by HIV-1 resulted in populations of three types of DNA molecules. The first is a linear band of 9.5 kilobase pairs, probably out of the cytoplasm which seems to appear already after 4 h after infection (Kim *et al.*, 1989) and to undergo integration after 24 h (Robinson and Zinkus, 1990). Two additional populations of relaxed circular forms of 15 kilobase pairs and a supercoiled form of 6 kilobase pairs are only formed in the nucleus (Shank and Varmus, 1978; Kim *et al.*, 1989). With the strand displacement activity of HIV-RT heterodimer p66/p51, it is likely that double-stranded linear full-length HIV DNA is generated by strand displacement events of the resulting plus strand segment on the 3′ polypurine tract, as was shown in mellitin-permeabilized virions (Boone and Skalka, 1981). These linear double-stranded DNA molecules could then be transported into the nucleus, where they are integrated into the host genome.

Charneau and Clavel (1991) found a population of gapped linear molecules where the synthesis of the 5′ half of the viral plus strand is stopped near the central polypurin tract, the initiation site of the 3′ half. This stop seems to be discrete, which could be due to a limited amount of brief strand displacement of HIV-RT, whereas the gap might be caused through a mutation in this polypurine tract. A similar event was found in cauliflower mosaic virus (Pfeiffer and Hohn, 1983). The gapped molecules are then also transported to the nucleus and circularized apparently by using the repair functions of the host cell.

In conclusion, it is conceivable that the strand displacement activity of HIV-RT might be required in later stages of the HIV replication cycle, where DNA-dependent DNA synthesis occurs on double-stranded DNA.

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