Interaction of the Reverse Transcriptase of Human Immunodeficiency Virus Type 1 with DNA†

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Received April 18, 1994; Revised Manuscript Received July 28, 1994®

ABSTRACT: During DNA synthesis, the binding of human immunodeficiency virus (HIV) reverse transcriptase (RT) to the template–primer precedes its binding to nucleotide triphosphates. The interaction of oligonucleotide DNA with HIV-1 RT was investigated by using a gel retardation assay. Both homodimeric (p66/p66) and heterodimeric (p66/p51) isoforms of HIV-1 RT were capable of binding the DNA oligomers. Thus, all further studies on the interaction of HIV-1 RT with DNA were done with heterodimeric RT. We have studied the conditions for optimal binding. The formation of the RT–DNA complex was primer-independent, and the extent of DNA binding was indistinguishable for both single-stranded and double-stranded DNA (either blunt-ended or recessed). The DNA binding activity of the RT was found to be dependent on oligonucleotide length. HIV-1 RT binds DNA with no apparent sequence specificity. Hence, this enzyme belongs to the sequence nonspecific DNA binding proteins. The interaction was found to be independent of DNA synthesis. The formation of the RT–DNA complex was not influenced by the presence of either template-complementary or noncomplementary dNTPs, indicating that neither DNA polymerization nor binding of the RT to the dNTP affects the stability of the complex. The gel retardation assay was utilized to examine also the effect of various HIV-1 RT inhibitors (i.e., AZT-TP, ddTTP, TIBO, and 3,5,8-trihydroxy-4-quinolone) on the enzyme–DNA interaction. The results indicate differences in the modes of action of these compounds. While there was a complete destabilization of the RT–DNA complex in the presence of 3,5,8-trihydroxy-4-quinolone, the addition of AZT-TP, ddTTP, or TIBO had no apparent effect on the stability of the complex. Most effective anti-HIV compounds are inhibitors of HIV RT; hence the interaction of the enzyme with DNA might constitute a discrete step which can serve as a target for interference by novel specific anti-HIV RT drugs.

Human immunodeficiency virus type 1 (HIV-1),[1] the causative agent of acquired immunodeficiency syndrome (AIDS), was found to exhibit an intensive genetic heterogeneity, which affects viral pathogenesis and resistance to drug therapy (Barre-Sinoussi et al., 1983; Gallo et al., 1983; Nowak, 1990). The replication of HIV, similar to that of other retroviruses, is initiated by the reverse transcription of the plus strand RNA genome into the double-stranded proviral DNA (Weiss et al., 1985; Varmus & Brown, 1989). This process is catalyzed by one enzyme, the virus-encoded reverse transcriptase (RT). The enzyme is multifunctional, exhibiting three enzymatic activities in a single molecule: (a) reverse transcription of viral RNA into a minus DNA strand by an RNA-dependent DNA polymerase activity, (b) hydrolysis of the RNA moiety from an RNA–DNA heteroduplex by an inherent ribonuclease H (RNase H) activity, and (c) copying of the minus strand DNA into a plus strand DNA by a DNA-dependent DNA polymerase function (Goff, 1990; Hizi et al., 1991). The native RT enzyme isolated from HIV-1 cores is a heterodimer composed of a 66-kDa (p66) subunit and a 51-kDa (p51) subunit, which is a carboxyl terminal truncated form of p66. The p66 subunit exhibits both the DNA polymerase and the RNase H activity, whereas the p51 subunit contains only the polymerase domain (DiMarzo Veronese et al., 1986; Hizi et al., 1988). Similar to other DNA polymerases, RT catalyzes DNA synthesis *via* an ordered mechanism in which template–primer binding by the enzyme precedes binding to the 2′-deoxynucleoside 5′-triphosphate substrates (Bryant et al., 1983; Majumdar et al., 1988). In a series of complex events that occur during the proviral double-stranded DNA synthesis from retroviral genomic RNA, a variety of different nucleic acid structures are generated. Thus, tRNA–RNA, RNA–DNA, and DNA–DNA hybrid structures, as well as single-stranded RNA and DNA, are present simultaneously (Varmus & Brown, 1989). The possibility exists, therefore, that the RT would display selective preference for recognition and binding of the various nucleic acid structures.

The relatively low fidelity of HIV-1 RT during both RNA → DNA and DNA → DNA replication steps was implicated as a major factor that contributes to the genetic variability of the HIV (Bakhanashvili & Hizi, 1992a,b, 1993; Ji & Loeb, 1992; Perrino et al., 1989; Roberts et al., 1988; Yu & Goodman, 1992). The parameters known to be important for determining the fidelity of DNA synthesis include base hydrogen bonding, base stacking, and interactions of the polymerase with double-stranded template–primer nucleotides. Recent studies have indicated another way in which HIV-1 might generate variation during reverse transcription (Peliska & Benkovic, 1992): namely, the DNA polymerase activity of HIV-1 RT incorporates additional non-template-directed nucleotides into the DNA strand of a blunt-end heteroduplex, resulting in a base misincorporation upon DNA strand transfer. Taken

† This research was supported by grant from the Israeli Cancer Association (Grant 940094-B).

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® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; AIDS, acquired immunodeficiency syndrome; AZT, 3′-azido-2′,3′-dideoxythymidine; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-one and -thione; PAGE, polyacrylamide gel electrophoresis; PCNA, proliferating cell nuclear antigen; ds DNA, double-stranded DNA; ss DNA, single-stranded DNA; HSV-1, herpes simplex virus type 1; TBE, Tris–borate/EDTA buffer.

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Chart 1: DNA Oligomers Used in HIV-1 RT Binding Experiments[a]

Oligo 1. 5' AAT GAA AGA CCC CAC CTG TAG GTT GGA TCC TTA CCC GTC AGC GGG GGT CTT TCA 3'

Oligo 2. 3' A CTT TCT GGG GTG GAC ATC CAA CCT AGG AAG GGG CAG TCG CCC CCA GAA AGT AA 5'

Oligo 3. 5' AAT GAA AGA CCC CAC CTG TAG GTT GGA TCC TTA CCC GTC AGC GGG GGT CTT TCA TT 3'

Oligo 4. 3' TTA CTT TCT GGG GTG GAC ATC CAA CCT AGG AAT GGG CAG TCG CCC CCA GAA AGT AA 5'

Oligo 5. 5' GCTCAAGGAACTTCTAAATGGCCTAGGATTTTCTACCCCAGATGAGAAGTTCCAAAAAGACCCTCCATTCCT 3'

Oligo 6. 5' ATTGGATCCAGCCCTTCCAGT 3'

[a] Shown are the nucleotide sequences of synthetic oligonucleotide substrates used in this study.

together, a characterization of the binding properties of the RT for the different DNA structures and the stabilities of the RT–nucleic acid complexes should lead to a better understanding of the interaction of HIV-1 RT with various nucleic acid structures.

In the current study we have investigated the direct interaction of HIV-1 RT with various DNA structures using a polyacrylamide gel electrophoresis (PAGE) band mobility shift assay. We observed that HIV-1 RT binds efficiently both single-stranded and double-stranded oligonucleotides. Furthermore, the gel retardation assay developed was used to study the effect of HIV-1 RT inhibitors on the enzyme–DNA interactions.

MATERIALS AND METHODS

Enzymes. p66/p66 and p66/p51, recombinant variants of wild-type HIV-1 RT, were expressed by us in *Escherichia coli* from the BH-10 proviral clone of HIV (Hizi et al., 1988). The proteins, purified according to Clark et al. (1990), showed a very high purification profile (more than 99%).

Template–Primers. The synthetic template–primer DNA substrates that were used for characterization of interactions with HIV-1 RT are listed in Chart 1. Oligonucleotide sequences were modified sequences of the LTR of HIV-1. The oligonucleotides were 5'-end-labeled by T4 polynucleotide kinase (USB) with [^{32}P]ATP according to the manufacturer's instructions.

PAGE Band Mobility Shift Assays. Complex formation between the oligonucleotide DNA and HIV-1 RT was characterized by the electrophoretic retardation of DNA as a result of its association with the enzyme. The binding reaction mixtures (final volume, 12.5 μL)—10 mM HEPES–KOH, pH 8.0; 50 mM ammonium sulfate; 0.25 mM dithiothreitol; 100 μg/mL bovine serum albumin; 10 mM KCl; 1.2 pmol HIV-1 RT; and the indicated oligonucleotide substrate concentrations—were incubated for 10 min at 32 °C. The protein–oligonucleotide mixtures were electrophoresed through a 5% polyacrylamide gel in 1 × TBE at 4 °C under 10 V/cm for 2–3 h. After electrophoresis, gels were dried and subjected to autoradiography. Complex formation between the RT and the labeled oligonucleotide probes was quantified by measuring radioactivity in cut bands of the gel, which was dried on a DE-81 filter paper (Whatman).

RESULTS

Gel Retardation Analysis of the Binding Activity Of HIV-1 RT. A prerequisite for DNA polymerization is the interaction of the DNA polymerase with its DNA substrate. To gain an understanding of the DNA binding properties of HIV-1 RT, we have used a nondenaturing PAGE band mobility shift assay to examine the interaction of this enzyme with short model oligonucleotides. HIV-1 RT was incubated with an

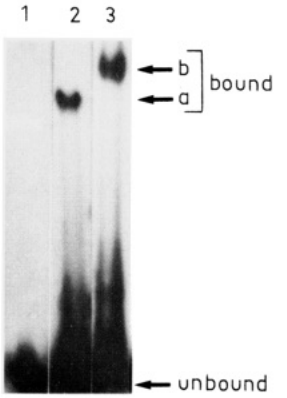

FIGURE 1: Complex formation between HIV-1 RT isoforms and oligonucleotide substrate. PAGE band mobility shift assays were performed as described under Materials and Methods. Incubations with 5'-end-labeled 54-bp duplex oligonucleotide DNA (oligo 1/oligo 2) contained no added proteins (lane 1), heterodimeric (p66/p51) HIV-1 RT (lane 2), or homodimeric (p66/p66) HIV-1 RT (lane 3). Marked by arrows are the migration positions of the heterodimeric HIV-1 RT–oligonucleotide complex (a), the homodimeric HIV-1 RT-oligonucleotide complex (b), and the unbound oligonucleotide (unbound).

oligonucleotide duplex (oligo 1/oligo 2) of 54 bp with recessed 2-nt 3' ends, in which one strand (oligo 1) was radioactively labeled at the 5' end (see Chart 1). The results demonstrate the appearance of a single labeled species migrating substantially more slowly relative to unbound DNA (Figure 1). Given the high degree of purity of the recombinant heterodimer HIV-1 RT (p66/p51) (see Materials and Methods), this enzyme is most likely directly responsible for the binding of the duplex DNA. The ready detection of the HIV-1 RT–DNA complex even without chemical cross-linking indicates that, unlike other DNA polymerases that have to be cross-linked to the DNA in order to remain detectable as complexes, HIV-1 RT forms a stable complex with the DNA. It should be noted that this interaction occurs independently of DNA synthesis since no dNTPs were present. The heterodimeric form p66/p51 of RT is the result of carboxyl-terminal proteolytic cleavage of one subunit of the homodimer (p66/p66) (DiMarzo Veronese et al., 1986). It was of interest to determine whether these two forms of HIV-1 RT differ in their nucleic acid binding capacity. It is notable that both the heterodimeric (p66/p51) and the homodimeric (p66/p66) isoform of HIV-1 RT form detectable complexes with the ds DNA with differences in their electrophoretic mobilities due to the disparity in their molecular weights (Figure 1). Thus, the structural differences between the homodimeric and heterodimeric forms of RT do not seem to affect significantly the DNA binding capacity. Consequently, each isoform can be considered suitable for studying the interactions between HIV-1 RT and oligonucleotide DNA. In light of this, we

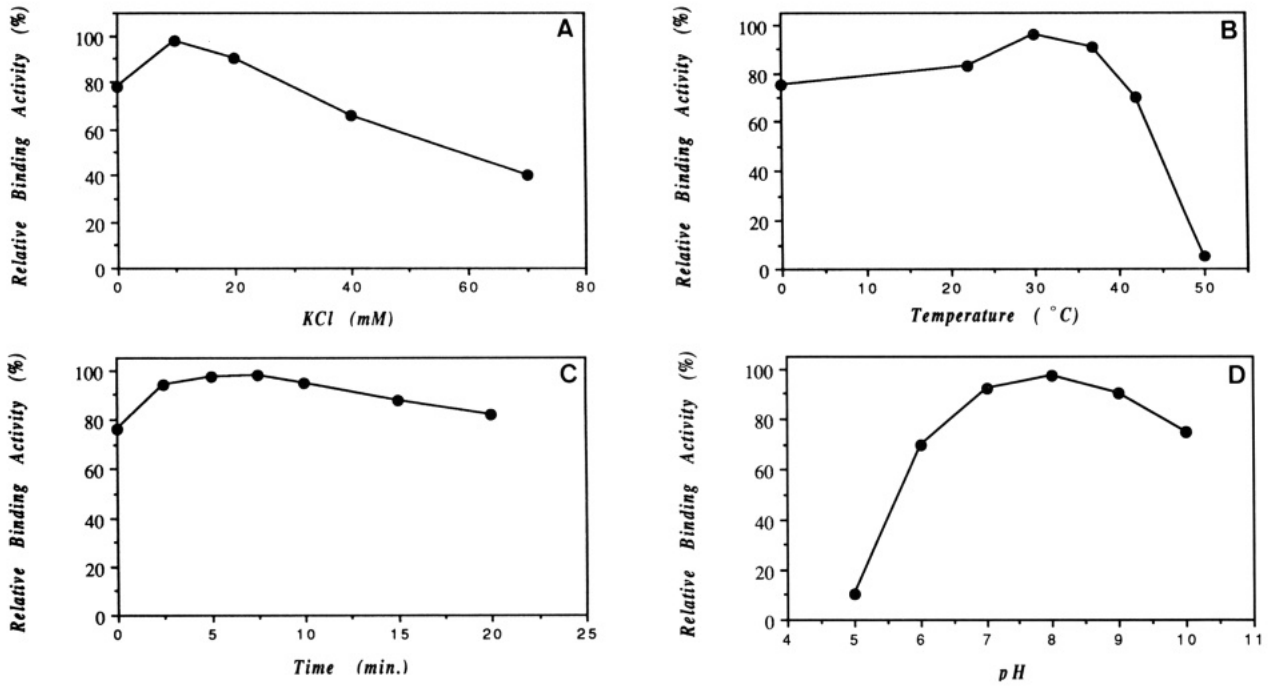

FIGURE 2: Conditions for the binding of HIV-1 RT to oligonucleotide DNA substrate. PAGE band mobility shift assays and quantification of binding products were as described under Materials and Methods. The standard binding conditions (unless otherwise stated) were as follows: 10 mM HEPES, pH 8.0; 50 mM ammonium sulfate; 0.25 mM DTT; 100 μg/mL BSA; and 10 mM KCl. (A) Effect of modifying concentrations of KCl on the binding of HIV-1 RT to oligonucleotide DNA. (B) Effect of temperature of the binding reaction. (C) Effect of the length of the incubation. (D) Effect of varying the pH on the binding of HIV-1 RT to DNA oligomer. The buffers used to cover the pH 5–10 range were sodium phosphate, HEPES, and glycine.

have designed further experiments to study the RT–nucleic acid interaction with only heterodimeric (p66/p51) RT.

Conditions for the Binding of HIV-1 RT to DNA. We have examined the effect of several variables on the extent of binding of the enzyme to DNA. First, the effect of KCl on DNA binding by RT was measured. Results shown in Figure 2A indicate that the maximum amount of HIV-1 RT–DNA complex is obtained in the presence of 10–20 mM KCl, whereas at higher salt concentrations the amount of complex is progressively diminished. Variations in temperature had a small effect on binding up to a temperature of 40 °C, but binding was completely abolished at temperatures exceeding 50 °C (Figure 2B). Measurements of incubation kinetics indicate that the complex is formed almost immediately after the reagents are mixed and remains stable for at least 20 min of incubation at 32 °C (Figure 2C). Maximum binding activity is observed within a pH range of 7–9 (Figure 2D). Finally, DNA binding activity did not change significantly in several tested buffer systems (Tris·HCl, HEPES–KOH, sodium phosphate buffer; data not shown).

A divalent metal cation activator, usually Mg^{2+}, is a universally required cofactor for DNA polymerases. One essential role of the metal is in the binding of incoming dNTPs. Furthermore, Mg^{2+} was detected as a potent effector of the binding interactions of different DNA polymerases, e.g., human DNA polymerase α with both templates and primers (Fisher & Korn, 1981). By contrast, the binding of DNA polymerase δ together with PCNA to the primer is independent of Mg^{2+}(Ng et al., 1993). We have used a PAGE band mobility shift assay to evaluate the role of Mg^{2+} in the formation of HIV-1 RT–DNA complex. As seen in Figure 3, Mg^{2+} is not required for the formation of a stable complex to be detected by the PAGE mobility band shift assay. This observation is consistent with the previous conclusions drawn from kinetic studies (De Stefano et al., 1993). The results indicated that RT binding to DNA–DNA duplexes does not require Mg^{2+}. However, this divalent cation is essential for the elongation of the primer DNA, and no DNA synthesis takes place without it (Hizi et al., 1991).

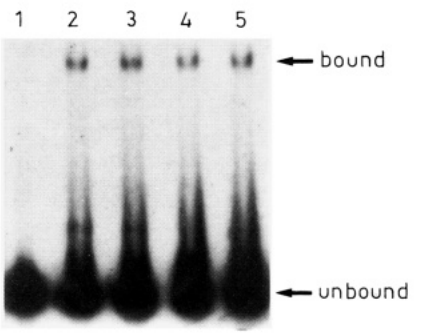

FIGURE 3: Effect of Mg^{2+} on complex formation. PAGE band mobility shift assays were performed under the standard binding conditions as described in Materials and Methods. Mixtures contained 5′-end-labeled 54-bp oligonucleotide DNA (oligo 1/oligo 2) with no enzyme (lane 1) or with HIV-1 RT (p66/p51) and either no added $MgCl_2$ (lane 2), 2.5 mM $MgCl_2$ (lane 3), 5 mM $MgCl_2$ (lane 4), or 15 mM $MgCl_2$ (lane 5). Arrows to the right of the figure indicate the migration positions of the enzyme–oligonucleotide complex (bound) and the oligonucleotide substrate alone (unbound).

Binding of HIV-1 RT to Different DNA Oligomers. So far we have analyzed the binding of HIV-1 RT to a ds oligonucleotide DNA with 2-nt overhangs. In order to determine whether the ds or ss DNA regions of the oligonucleotides are required for enzyme binding, we evaluated HIV-1 RT binding to either a ss 54-nt oligonucleotide (oligo 1) or a 56-bp blunt-ended duplex DNA (oligo 3/oligo 4) (which is identical to the 3′-recessed double-stranded DNA after filling) (see Chart 1). The ds DNA was designed to determine whether the presence of a recessed 3′ terminus in the DNA can affect the RT binding capacity. It is obvious that the extent of oligonucleotide binding by RT was indistinguishable for ss and ds DNAs (either blunt-ended or recessed) (Figure

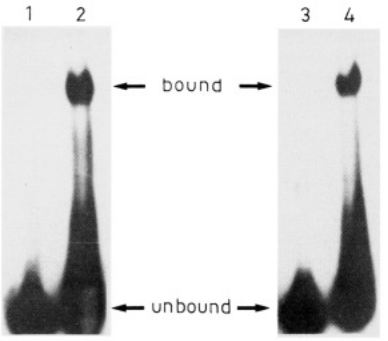

FIGURE 4: Complex formation between HIV-1 RT and various oligonucleotide DNA structures. PAGE band mobility shift assays were performed as described in Materials and Methods. Incubations contained either 5′-end-labeled 54-nt ss oligomer DNA (oligo 1) (lanes 1 and 2) or 56-bp blunt-ended ds oligomer DNA (oligo 3/oligo 4) (lanes 3 and 4), either with no RT (lanes 1 and 3) or with heterodimeric HIV-1 RT (lanes 2 and 4). Arrows in the middle of the figure indicate the migration positions of the enzyme–oligonucleotide complex (bound) and the oligonucleotide substrate alone (unbound).

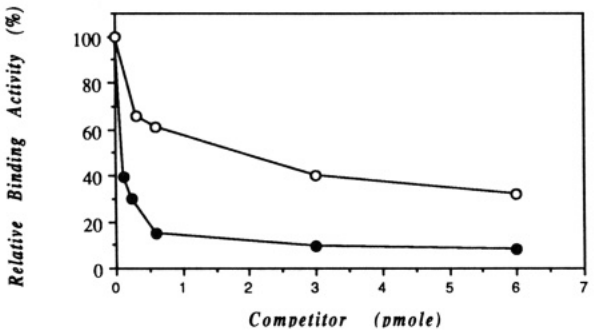

FIGURE 5: Effect of competition by single- or double-stranded DNA oligonucleotides on the binding of HIV-1 RT to 0.12 pmol of ^{32}P-labeled double-stranded DNA (oligo 1/oligo 2). PAGE band mobility shift assays and quantification of bound complex products were as described in Materials and Methods. Competitor DNA species were ss oligomer DNA (oligo 1) (- O -) and ds oligomer DNA (oligo 1/oligo 2) (- ● -).

4). To further support the ss DNA binding capacity of HIV-1 RT, we have also analyzed the direct binding of the enzyme to two other ss oligomer DNAs (72 and 34 bases long) with completely unrelated sequences (different also from oligo 1). It was found that HIV-1 RT is capable of forming stable complexes with both oligomer DNAs (data not shown). This property sets HIV-1 RT apart from other DNA polymerases, which were found to bind specifically either ds DNA (e.g., HSV-1 DNA polymerase) (Strick & Knopf, 1992) or ss DNA (e.g., DNA polymerase α) (Wong et al., 1986).

To further evaluate the specificity of complex formation between RT and various oligonucleotides, we performed competition experiments by incubating RT with a constant amount of the end-labeled 54-bp (oligo 1/oligo 2) ds DNA, in the presence of increasing amounts of nonradioactive competitors [either ss 54-nt oligonucleotide (oligo 1) or duplex 54-bp (oligo 1/oligo 2) DNA, with identical sequences]. Results presented in Figure 5 indicate that the binding of RT to the labeled template is diminished in the presence of either one of the unlabeled competitors. However, quantification of the extent of competition by each nonradioactive oligomer demonstrates that duplex and ss DNA compete at different efficiencies with the labeled ds oligonucleotide (Figure 5). Thus, while an equimolar amount of unlabeled duplex was sufficient to reduce the binding of the labeled ds DNA by approximately 50%, an excess of ss DNA as high as 14-fold was required to compete the same percent of the labeled

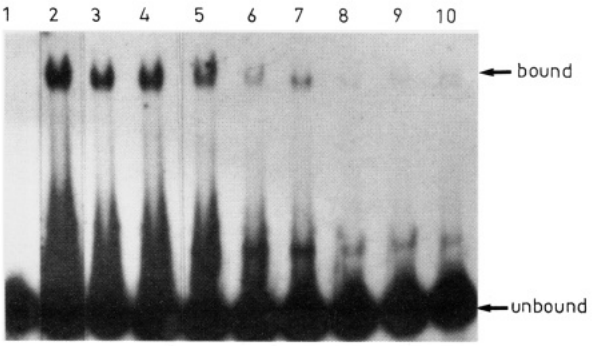

FIGURE 6: Effect of competition by different oligonucleotides on the binding of HIV-1 RT to 0.12 pmol ^{32}P-labeled DNA (oligo 1/oligo 2). PAGE band mobility shift assays were performed as described in Materials and Methods. Incubation mixtures contained 5′-end-labeled 54-bp ds DNA oligomer (lane 1). The binding activity of HIV-1 RT was analyzed in the absence (lane 2) or presence of two competitors: 21-nt oligonucleotide (oligo 6) (lanes 3–6) or 72-nt oligonucleotide (oligo 5) (lanes 7–10). The molar excesses of the competitors were 10-fold (lanes 3 and 7), 50-fold (lanes 4 and 8), 100-fold (lanes 5 and 9), and 500-fold (lanes 6 and 10). Arrows indicate the migration positions of the oligonucleotide substrate alone (unbound) and the enzyme–oligonucleotide complex (bound).

substrate. These data suggest, therefore, that the binding affinity of HIV-1 RT is higher for ds DNA than for ss DNA.

Next, we have compared the relative preference of HIV-1 RT for different nonradioactive competitors with unrelated sequences and various lengths. As shown in Figure 6, binding of labeled oligo 1/oligo 2 was efficiently diminished by a 10-fold molar excess of a 72-nt oligonucleotide (oligo 5). Conversely, the short 21-nt oligonucleotide (oligo 6) competed at a significantly lower efficiency, and no competition was observed even at a 100-fold molar excess. Recently, there have been reports of DNA binding proteins that bind preferentially pyrimidine-rich sequences (Kim et al., 1992; Meyer & Laine, 1991). Though the shorter 21-nt oligonucleotide has a higher pyrimidine content (62%) than the longer 72-nt oligonucleotide (52.7%), the longer oligonucleotide was an effective competititor. Thus, it seems that the binding activity of HIV-1 RT depends on the length of the oligonucleotides rather that on their sequence. The results also suggest that RT belongs to the class of non-sequence-specific DNA binding proteins.

Effect of dNTPs on the Formation of RT–Oligonucleotide Substrate Complex. We have used the gel retardation assay to examine the role of template complementary or non-complementary dNTPs in the formation of the RT–oligo DNA complexes. Each of the four dNTPs was added to the binding reactions with labeled 54-bp ds oligonucleotides (oligo 1/oligo 2) under conditions allowing DNA synthesis. Results presented in Figure 7A demonstrate that the addition of either the template-complementary dTTP or the noncomplementary nucleotides (dATP, dCTP, or dGTP) had no apparent effect on the extent of RT–oligonucleotide complex formation. Furthermore, denaturing gel analysis of aliquots of the binding reaction mixtures revealed the extension of the 54-base primer to 55 or 56 bases following the incorporation of either complementary dTTP or noncomplementary dATP, dCTP, or dGTP nucleotides (Figure 7B). This may indicate that neither DNA polymerization nor binding of the RT to the dNTP affects the stability of the complex. The complex was also unaffected by the presence of chain terminator ddTTP (data not shown).

Effect of Inhibitors of HIV-1 RT on Its Interaction with DNA Oligonucleotide. HIV-1 RT constitutes a potential specific target for antiviral chemotherapy (De Clercq, 1993).

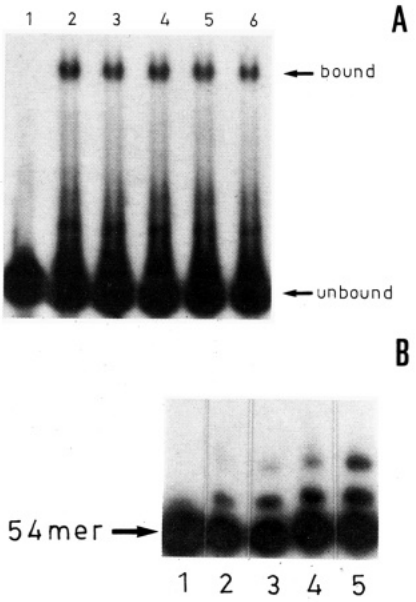

FIGURE 7: Effect of template-complementary and -noncomplementary dNTPs on complex formation between HIV-1 RT and DNA oligonucleotide. (A) PAGE band mobility shift assays were performed as described in Materials and Methods. Incubation mixtures contained 5′-end-labeled 54-bp dsDNA oligomer (oligo 1/oligo 2) (lane 1). The binding activity of HIV-1 RT was analyzed with no added dNTPs (lane 2) or with 50 μM dGTP (lane 3), 50 μM dATP (lane 4), 50 μM dCTP (lane 5), or 50 μM dTTP (lane 6). Marker arrows are as described in caption to Figure 3. (B) Denaturing gel analysis of aliquots of samples analyzed in panel A; the lanes do not correspond to the lanes in panel A. Samples were denatured and subjected to urea–PAGE analysis as described previously (Bakhanashvili & Hizi, 1991). The position of the 54mer DNA alone is indicated by an arrow. PAGE band mobility shift incubations were performed with no added dNTP (lane 1) or with 50 μM dGTP (lane 2), 50 μM dATP (lane 3), 50 μM dCTP (lane 4), or 50 μM dTTP (lane 5).

During DNA synthesis, the binding of HIV-1 RT to the template–primer precedes its binding to nucleotide triphosphate substrates (Reardon et al., 1991). Hence, the interaction of RT with template–primer might constitute a discrete step which may be a target for interference by anti-HIV-1 RT drugs. The binding assay developed was utilized to study the effects of various inhibitors of HIV-1 RT on the binding of the enzyme to template–primer. A number of structurally diverse compounds have been shown to be potent inhibitors of the DNA polymerase activity of HIV-1 RT. Nucleoside analogs, such as 3′-azidothymidine triphosphate and dideoxyinosine triphosphate, inhibit RTs by serving as DNA chain terminators. Several nonnucleoside inhibitors, e.g., TIBO compounds and nevirapine, were characterized as extremely effective and selective inhibitors of HIV-1 RT with no effect on HIV-2 RT (De Clercq, 1993). In addition, natural compounds isolated by us from Red Sea marine organisms were also reported to inhibit effectively either HIV-1 RT or both HIV RTs (Loya & Hizi, 1990; Loya et al., 1992). Recently, a novel marine natural inhibitor identified as 3,5,8-trihydroxy-4-quinolone was found by us to be a potent inhibitor of both HIV-1 and HIV-2 RTs (Loya et al., 1994).

We have investigated the interaction of HIV-1 RT with ds DNA oligomer in the presence of AZT-TP, TIBO, and the trihydroxyquinolone to characterize their mode of inhibition. As can be seen from the results of the experiment depicted in Figure 8, there is a complete destabilization of the RT–oligonucleotide complex after incubation with 50 μM trihydroxyquinolone, which was shown previously to possess an IC_{50} value for DNA-dependent DNA synthesis of about 43 μM (Loya et al., 1994). In contrast, addition of 20 nM AZT-TP, which has an IC_{50} value of 9 nM (Hizi et al., 1991), or of 40 μM TIBO, with an IC_{50} value of 2 μM (De Clercq, 1993), had no apparent effect on the stability of the RT–oligonucleotide complex. These results strongly demonstrate the differences in the modes of action of these compounds, especially those of the non-nucleoside compounds, TIBO and trihydroxyquinolone.

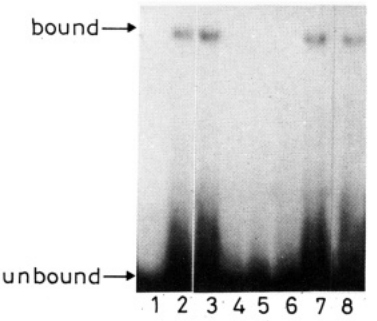

FIGURE 8: Effect of inhibitors of HIV-1 RT on the interaction with DNA. PAGE band mobility shift assays were performed as described under Materials and Methods. Incubation mixtures contained 5′-end-labeled 54-bp ds DNA oligomer (oligo 1/oligo 2) (lane 1). The binding activity of HIV-1 RT was analyzed with no inhibitor (lane 2) or with increasing concentrations of trihydroxyquinolone: lane 3, 10 μM; lane 4, 50 μM; lane 5, 100 μM; and lane 6, 200 μM. The binding activity was also analyzed in the presence of either 20 nM AZT-TP (lane 7) or 40 μM TIBO (lane 8). Marker arrows are as described in the caption to Figure 3.

DISCUSSION

In the present study, the physical binding of HIV-1 RT to various DNA structures was investigated. We have developed a PAGE band mobility shift assay to characterize the interactions between HIV-1 RT and labeled oligonucleotide substrates. A comparison of the interactions of the homodimeric (p66/p66) and the heterodimeric (p66/51) isoforms of HIV-1 RT with the template–primers demonstrates that both isoforms interact with the oligonucleotide substrates similarily (Figure 1). This is in line with recent kinetic studies demonstrating that both isoforms of HIV-1 RT interact with poly(rA)$_n$·(dT)$_{16}$ in a similar fashion with the same interaction sites (Beard & Wilson, 1993). It should be emphasized that, similar to DNA polymerase α (Wong et al., 1986) and unlike DNA polymerase δ (Ng et al., 1993), HIV-1 RT binds DNA without the involvement of any other auxiliary proteins.

Analysis of the interaction of herpes simplex virus type 1 (HSV-1) DNA polymerase with DNA oligomer by gel retardation assay revealed two bands (Strick et al., 1992). It was suggested that the two bands represent DNA molecules with one or two ends bound by the HSV polymerase. The single retarded band obtained with both isoforms of HIV-1 RT indicates that only one molecule of enzyme binds to the DNA oligomer.

It was of interest to examine the effect of composition (ss DNA *versus* ds DNA), sequence length, and type of overhang of the oligonucleotide duplexes on their interaction with RT. Our results demonstrate that HIV-1 RT supports complex formation with (a) ss DNA template, (b) ds DNA with a recessed 3′ terminus, and (c) blunt-ended duplex DNA (Figures 1 and 4). The binding to all three of these DNA substrates is a distinguishable feature of HIV-1 RT. Thus, HSV-1 DNA polymerase binds DNA with recessed 3′ ends significantly more efficiently than it binds either ss DNA or blunt-ended ds DNA. Calf thymus DNA polymerase δ complexed to PCNA binds only ds DNA with recessed ends, and the binding is primer dependent. Data presented in Figure

4 strongly indicate that a stable RT–oligonucleotide complex is formed independently of the presence of primer. The binding of HIV-1 RT to blunt ends may reflect the need of the enzyme to remain bound to the template–primer after completion of synthesis until strand transfer occurs and that the enzyme must recognize blunt-ended structures as potential substrates for dNTP addition sites. Recent studies have suggested that HIV-1 RT can add additional non-template-directed nucleotides onto the DNA strand of a blunt-end heteroduplex (Peliska & Benkovic, 1992). Consequently, it is conceivable that the DNA polymerase active site of HIV-1 RT is involved in the interaction with blunt-end structures.

The length of the template strand probably influences the stability of the RT complexes with DNA oligomer. Furthermore, HIV-1 RT binds to various oligonucleotide substrates with no known nucleotide sequence dependence. Therefore, this enzyme can be grouped with a class of non-sequence-specific DNA binding proteins.

Recent studies have shown that RT binds with higher affinity and greater stability to DNA oligonucleotide-primed RNA *versus* DNA oligonucleotide-primed DNA template (De Stefano et al., 1993). It was also shown that the HIV-1 RT binds specifically and efficiently $tRNA^{Lys3}$ that serves as a primer for DNA synthesis. Our preliminary data indicate that ribosomal RNA can compete efficiently with DNA oligomer (data not shown). This indicates that both RNA and DNA templates may bind to similar sites on the RT. We have shown that the complex remains stable following the addition of either the complementary nucleotide (dTTP) or non-complementary nucleotides (dATP, dCTP, or dGTP) (Figure 7). In contrast, it was reported that the stability of the complex formed among DNA polymerase δ, PCNA, and oligo DNA was influenced by the presence of individual dNTPs (Ng et al., 1993). These results were explained by assuming that complex reduction was due to the catalytic destabilization resulting from nucleotide misincorporation.

In the continuing search for anti-HIV agents, the disruption of the interaction between RT and DNA substrates represents a new alternative target for therapeutic intervention. We have utilized this assay to evaluate the effect of several inhibitors of HIV-1 RT on the interaction with template–primer. The chain terminator AZT-TP has no apparent influence on the interaction of the enzyme with oligonucleotide substrates (Figure 8). Similarily, the complex was unaffected by the presence of ddTTP (data not shown), suggesting that nucleoside analogs generally do not influence the binding of the RT to template–primer. Other unrelated non-nucleoside inhibitors, TIBO and the newly identified 3,5,8-trihydroxy-4quinolone, were studied also for their effect on HIV-1 RT, thus complementing the results obtained in kinetic studies. Trihydroxyquinolone, in contrast to TIBO, strongly affects the formation of RT–oligonucleotide DNA complexes, suggesting a difference in the mode of action of these two compounds. Recently it was shown that TIBO does not affect the kinetics of binding and dissociation of primer–template with HIV-1 RT (Divita et al., 1993). Interestingly, while TIBO is a selective inhibitor of only HIV-1 RT, trihydroxyquinolone was found to be an effective inhibitor of both HIV RTs.

Two X-ray crystallographic studies with nevirapine (Kohlstaedt et al., 1992) or ds DNA template–primer (Jacobo-Molina et al., 1993) have resulted in the three-dimensional structure of the p66/p51 heterodimer of HIV-1 RT. The DNA polymerase domain of the p66 subunit is composed of four subdomains and has a large cleft which accommodates the template–primer binding sites. Nevirapine is almost completely buried in a hydrophobic pocket in the p66 cleft near but not overlapping the DNA polymerase active site. This is supported by a biochemical analysis that shows that nevirapine, TIBO, and other nonnucleoside analogs specific to HIV-1 RT interact mainly with tyrosines 181 and 188, which are located adjacent to the highly conserved putative DNA polymerase active site. Our present study with TIBO (Figure 8) indeed supports the notion that, despite the binding to the residues in close proximity to the DNA polymerase active site, the compound does not affect the binding of the template–primer DNA *per se*. The three-dimensional studies elucidate that the residues shown previously by mutational analysis to interact with deoxynucleotide analogs are located more distal to the DNA polymerase active site. This is in line with our results with AZT-TP (Figure 8) and dTTP (not shown), which do not affect the binding to DNA. The results with the trihydroxyquinolone indicate that this inhibitor has a mechanism of inhibition not shared by the other two groups of inhibitors. It would be interesting, therefore, to localize, both by biochemical and X-ray crystallographic studies, the interaction sites of the compound with HIV-1 RT.

The localization of nucleic acid binding sites in RT is an important step toward the design of novel inhibitors of RT function. Recent studies have indicated that two regions of nucleic acid binding capacity are found within the N-terminal half of p66 (residues 1–230 and residues 273–302) (Kumar et al., 1993). Further evaluation of nucleic acid binding by mutant RTs may help to identify the amino acids involved in RT–nucleic acid interactions and the mechanism by which these amino acids recognize the template–primer.

ACKNOWLEDGMENT

We deeply thank Dr. M. Fry for thorough reading of the manuscript and for many helpful suggestions, Dr. S. Loya for reading the manuscript, and Dr. S. Hughes for purified HIV-1 RT.

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