Affinity Labeling and Functional Analysis of the Primer Binding Domain of HIV-1 Reverse Transcriptase†

M.-L. Andreola,‡ L. Tarrago-Litvak,‡ A. S. Levina,§ T. I. Kolocheva,§ R. El Dirani-Diab,‡ V. I. Jamkovoy,⊥ N. L. Khalimskaya,⊥ P. J. Barr,∥ S. Litvak,*,‡ and G. A. Nevinsky*,§

Institut de Biochimie Cellulaire du CNRS, 1 rue Camille Saint Saëns, 33077 Bordeaux Cedex, France, Institute of Bioorganic Chemistry, Siberian Division of the Russia Academy of Sciences, 630090 Novosibirsk, Russian Federation, Novosibirsk State University, 630090 Novosibirsk, Russian Federation, and Chiron Corporation, 4560 Horton Street, Emeryville, California 94608

Received August 12, 1992; Revised Manuscript Received December 10, 1992

ABSTRACT: Six affinity reagents containing chemically reactive groups, either on the phosphate residue at the 5′-end or on the 5′- or 3′-end internucleoside phosphate linkages of the oligothymidylate primers, were used to covalently modify the human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT). After covalent binding of these modified primer analogs to the enzyme, the addition of [α-^{32}P]dTTP, in the presence of a complementary template, led to elongation of the primer. This reaction was catalyzed by the active site of the enzyme carrying the covalently bound primer. The relative efficiency of labeling of the p66/p51 heterodimer compared to the p66/p66 and p51/p51 homodimers of HIV-1 RT was in agreement with the previously determined affinity of the various enzyme forms toward different primers. The analogues preferentially modified the p66 subunit of the HIV-1 RT heterodimer. The labeling of all RT forms by synthetic primer analogues showed significant and specific competition by the natural primer of HIV-1 RT, tRNA^{Lys}. In addition, the kinetics of inactivation of RT by primer analogues was studied. The affinity of the enzyme to those derivatives in the presence of poly(A) template was about 5–10 times higher than in the absence of template. Moreover, the maximal rates of HIV-1 RT inactivation by analogues in the absence of template were 3–4 times higher. Our results suggest that the mechanism of oligonucleotide primer binding to HIV-1 RT is different in the presence or absence of template. However, in both cases the nucleotide at the 3′ position of the primer appears to interact with the same specific domain of the enzyme active site.

Human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) catalyzes the synthesis of double-stranded proviral DNA from a single-stranded retroviral RNA genome [for recent reviews, see Jacobo-Molina and Arnolds (1991) and Barber et al. (1990)]. At least three enzymatic activities are associated with this protein: an RNA-dependent DNA polymerase activity involved in the synthesis of the (−) strand of DNA; a DNA-dependent DNA polymerase activity which synthesizes the second, (+) strand of the proviral genome; and a ribonuclease H activity (RNase H) located in the C-terminal domain of the protein (Prasad & Goff, 1989).

Functional RT purified from virions (Di Marzo Veronese et al., 1986) or infected cells (Lightfoote et al., 1986) consists of two polypeptides of 66 and 51 kDa. Both DNA polymerase and RNase H activities reside within the p66 subunit. In the dimeric forms of HIV-1 RT (p66/p51, p66/p66, or p51/p51), there are two potential binding sites for the template, primer, and dNTP precursors (Hansen et al., 1987).

The sequence of the HIV-1 primer binding site indicates that HIV-1 RT uses tRNA^{Lys,3} for the initiation of cDNA synthesis (Wain-Hobson et al., 1985). A specific interaction between RT p66/p51 and the primer tRNA^{Lys,3} anticodon loop region has been demonstrated by cross-linking analysis using a platinum derivative (Barat et al., 1991). By the nuclease footprinting technique, the anticodon and diHU region of the tRNA were shown to interact with the enzyme (Sarih-Cottin et al., 1992). In addition, the binding of tRNA^{Lys} induces significant structural changes in HIV-1 RT (Robert et al., 1990).

A better understanding of the mechanism of action of HIV-1 RT will be important for finding specific inhibitors of the infection and of acquired immunodeficiency syndrome (AIDS). Specific labeling of RT by substrate analogues could be used for the selective inhibition of the enzyme. Cheng et al. carried out photoaffinity labeling experiments with the heterodimeric and homodimeric forms of HIV-1 RT using unmodified $(pA)_{12-18}$·$d(pT)_{10}$ template·primer and [α-^{32}P]dTTP. In the case of the p66/p51 heterodimer (the enzymatic form believed to be involved in viral replication), cross-linking occurred exclusively to the p66 subunit (Cheng et al., 1991).

In the present study, we have used the highly selective method of affinity labeling based on the "catalytic competence" of a covalently bound protein and its initiating substrate. This approach has been successfully applied to studies of the active sites of DNA-dependent RNA polymerases (Grachev et al., 1987), *Escherichia coli* DNA polymerase I (Mitina et al., 1990), and DNA primase (Foiani et al., 1989). Affinity labeling of HIV-1 RT was accomplished by using oligothymidylate derivatives containing chemically reactive groups in the thiophosphate residue at the 5′-end, as well as in the internucleotide thiophosphate group of the 5′- or 3′-end, of the oligonucleotide. The results of the labeling of the heterodimer RT p66/p51 were compared to those obtained using the homodimers p66/p66 and p51/p51 and to data obtained, in parallel, from enzymatic kinetic studies.

† This work was supported by the Agence Nationale de Recherches contre le SIDA (ANRS), the Association des Recherches pour le Cancer (ARC), the Conseil Régional d'Aquitaine, the Centre National de la Recherche Scientifique (CNRS), and the University of Bordeaux II. M.-L.A. has a postdoctoral fellowship from the ANRS.

* Corresponding authors.
‡ Institut de Biochimie Cellulaire du CNRS.
§ Institute of Bioorganic Chemistry.
⊥ Novosibirsk State University.
∥ Chiron Corp.

0006-2960/93/0432-3629$04.00/0

EXPERIMENTAL PROCEDURES

Materials

Unlabeled nucleotides, polynucleotides, and $d(pT)_{12-18}$ were obtained from Sigma or Pharmacia. Radioisotopes were purchased from Commissariat à l'Energie Atomique-Saclay (CEA-Saclay, France). Bovine pancreatic DNase I was from Amersham. T4 polynucleotide kinase was from BRL. Adenosine 5′-*O*-(3-thiotriphosphate) was purchased from Serva. *N*,*N*,*N*′-Tri(2-chloroethyl)-*N*′-(*p*-formylphenyl)propylenediamine-1,3 (RCl_3) is a product from the Novosibirsk Institute of Bioorganic Chemistry.

Methods

Purification of HIV-1 Reverse Transcriptase. The p66/p51 form of RT was obtained as described previously (Sallafranque-Andreola et al., 1989). The p66/p66 and p51/p51 RT forms were purified as described for the p66/p51 form, but a protease-deficient yeast strain was used [JSC 302, derivative of AB 116: Mat a, leu 2, trp 1, ura 3-52, prB1-1122, pep 4-3, prCl-407 [cir°]] transformed with either the expression vector pBS24RT5 or pBS24RT6, respectively (Bathurst et al., 1990).

Reverse Transcriptase Assays. The kinetic parameters of DNA synthesis for all template-primer complexes used were determined at 30 °C. The reaction mixture (50 μL) contained the following standard components: 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 80 mM KCl, 10 μM dTTP, and 1 μCi [^{3}H]dTTP (56 Ci/mmol). In all cases, saturating concentrations of poly(A) (1.8 A_{260} units/mL) were used and did not inhibit the polymerization reaction. The optimal concentration of template was found by using high concentrations of primers: $5–10K_m$, $K_m = 1.6 \pm 0.3$ μM (Nevinsky et al., 1992). Otherwise, primer concentrations varied between 0.3 and 15 times the K_m values. The polymerization reaction was initiated by adding the enzyme (5–20 nM).

For DNA synthesis primed with oligothymidylates or their derivatives, the radioactivity of samples was measured as described previously (Nevinsky et al., 1990), using 5% trichloroacetic acid containing 50 mM sodium pyrophosphate during the precipitation and washing of samples.

Determination of Kinetic Parameters. To measure the initial rates of the polymerization reaction, samples of the reaction mixture were drawn at different times and the initial rates were determined from the tangents of the data curves at zero time. Apparent Michaelis–Menten parameters, K_m and V_{max}, were determined according to Cornish-Bowden direct linear plots (Cornish-Bowden, 1976). Errors of K_m and V_{max} were within 10–40%.

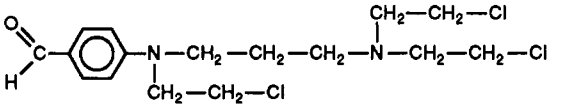
Synthesis of Oligonucleotides. The synthesis of $d(pT)_{10}$ and $d(pT)_8$ was described earlier (Levina et al., 1985; Veniaminova et al., 1987). Oligonucleotides $(pA)_n$ were obtained by hydrolysis of poly(A) using snake venom endonuclease from *Naja naja oxiana* (Mudrakovskaya et al., 1990). Oligonucleotides were purified as described by Levina et al. (1985). The following millimolar absorption coefficients were used to determine the oligonucleotide concentrations: (a) $d(pT)_8$, 69.0; $d(pT)_{10}$, 87.0, determined at 267 nm (Levina et al., 1985; Bukhrashvili et al., 1989); (b) $(pA)_7$, 75.4; $(pA)_{10}$, 103.5; $(pA)_{14}$, 141, determined at 260 nm (Veniaminova et al., 1990).

Synthesis of Oligothymidylate Derivatives. Step 1: Synthesis of Oligothymidylates with a Phosphorothioate Group. (a) The synthesis of a decanucleotide containing an internucleoside thiophosphate group on the 3′-end, $d[(Tp)_8Tp(S)T]$, was carried out according to Veniaminova et al. (1990): dithymidylylmethyl phosphonate was synthesized on a solid support containing dimethoxytriethylthymidine. The sulfurization of the internucleoside H-phosphonate linkage was carried out as described previously (Stec & Zon, 1984). The solid support was transferred to an automatic synthesizer and all the following steps of the synthesis were performed as described (Veniaminova et al., 1990). For the oxidation of H-phosphonate linkages, 25 mM iodine was used to decrease the oxidation of internucleoside thiophosphate to phosphate groups. After cleavage from the solid support, the product was purified by ion-exchange and reverse-phase chromatography (Levina et al., 1985). The ratio of $d[(Tp)_8Tp(S)T]$ to $d[(Tp)_9T]$ was estimated to be about 1:1 according to the elution profile.

(b) The synthesis of the decathymidylate containing an internucleoside thiophosphate group on the 5′-end, $d[Tp(S)T(pT)_8]$, was carried out by the phosphonate method (Veniaminova et al., 1990). Oxidation of trialkylphosphonate during all steps of the synthesis (excluding the last) was carried out by using a 0.2 M iodine solution in a mixture of pyridine/acetic acid (9:1), and the subsequent steps were as above. The ratio of thiophosphate- to phosphate-containing oligonucleotides was approximately 3:1.

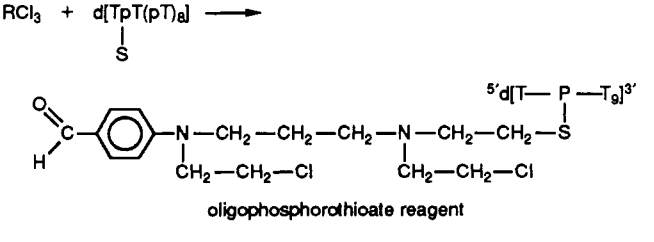
(c) A 5′-thiophosphorylated octanucleotide, $d[p(S)T(pT)_7]$, was synthesized from $d[pT(pT)_7]$ as described by Oshevski (Oshevski, 1982). Purification of the thiophosphorylated compound was performed by microcolumn chromatography.

Step 2: Synthesis of Chemically Reactive Derivatives of Oligophosphorothioates: RA and RCl Reagents. The thiophosphorylated oligonucleotides were alkylated with the trifunctional reagent RCl_3 (Oshevski, 1982):



Oligophosphorothioates ($2 A_{268}$ units) in 50 mM Hepes buffer, pH 7.0, were mixed with an RCl_3 solution (0.22 mg/30 μL DMSO). After incubation for 2 h at 20 °C, the samples were precipitated with cold acetone. The yield of oligothymidylate derivatives was about 70–90%. The products were homogeneous after purification and analysis by microcolumn ion-exchange and reverse-phase chromatography (Grachev et al., 1980; Levina et al., 1985). The product of alkylation was measured using the maximal absorption of RCl_3 at 350 nm. In all cases, the ratio A_{350}/A_{268} indicated that one formylphenyl residue/mol oligonucleotide was present.

An example of the formation of an oligophosphorothioate derivative is given below:



The oligophosphorothioate reactive derivatives can interact through either the aldehyde group (RA reagents) or the chlorine from the 2-chloroethyl group (RCl reagents). Analysis of the reactive chlorine was carried out after reduction

Table I: Oligothymidylate Derivatives

$d(pT)_n \rightarrow$	Oligophosphorothioate →	RA reagents →	RCl reagents
$d(pT)_{10}$	$d[Tp(S)T(pT)_8]$	(I) 5′-RA-$d(pT)_{10}$	(IV) 5′-RCl-$d(pT)_{10}$
$d(pT)_{10}$	$d[(Tp)_8Tp(S)T]$	(II) 3′-RA-$d(pT)_{10}$	(V) 3′-RCl-$d(pT)_{10}$
$d(pT)_8$	$d[p(S)T(pT)_7]$	(III) RA-$d(pT)_8$	(VI) RCl-$d(pT)_8$

of the aldehyde group (RA reagents) to alcohol with 0.1 M $NaBH_4$ (10 min, 20 °C).

The reduced oligothymidylate derivatives (RCl reagents) were incubated for 24 h at 40 °C in the presence of 0.3 M $Na_2S_2O_3$. RCl–$d(pT)_n$ derivatives contained 80–90% reactive chlorine.

The six different oligothymidylate derivatives (3 RA reagents and 3 RCl reagents) obtained are shown in Table I.

Modification of the HIV-1 RT by the Oligothymidylate Derivatives. (1) Determination of K_d Values for the Binding of the Oligothymidylate Derivatives to RT. Modification of HIV-1 by RA reagents (I), (II), and (III) was used for the quantitative analysis of the interaction between the enzyme and the primer. The reaction mixture (50–100 μL) contained the standard components: 50 mM Tris-HCl, pH 8.0, 5 mM $Mg(CH_3COO)_2$, and 0.5 mM DTT. The poly(A) template, when used, was present at a concentration of 1.8 A_{260} units/mL. RA derivatives were added to different concentrations. The modification reaction (30 °C) was started by the addition of RT (50–100 nM). Aliquots were taken at different times, and the DNA polymerase activity was determined as described under Reverse Transcriptase Assays (that is, standard components plus 0.5 A_{260} units/mL of poly(A)·$d(pT)_{12-18}$ template·primer).

The dissociation constants, K_d, of the complexes formed between RT and the RA derivatives were evaluated using the Kitz and Wilson equation (Kitz & Wilson, 1962) based on the dependence of the apparent rate constant (k_{app}) of enzyme inactivation on the reagent concentration:

$$1/k_{app} = 1/k + K_d/kx_0$$

where x_0 is the initial concentration of the affinity reagent and k is the rate constant of RT inactivation. The apparent rate constant of inactivation, k_{app}, was determined as the slope of the semilogarithmic plot of the inactivation kinetics.

After incubation with RA reagents, RT activity decreased due to the formation of a Schiff base between the protein and the reagent. If the modified enzyme assay was carried out for less than 15 min, $NaBH_4$ addition did not affect the efficiency of inactivation. Thus, the activity of modified RT was measured before the Schiff base was reduced with $NaBH_4$.

(2) Analysis of the Modification Products. The alkylating derivatives (IV), (V), and (VI) were obtained from RA derivatives (I), (II), and (III), respectively, by reduction with $NaBH_4$ immediately before the modification experiments with the enzyme. Freshly prepared $NaBH_4$ (5 mM final concentration) was added to a solution of 0.05 mM RA reagent. After 30 min of incubation at 0 °C, the mixture was kept for 20 min at 20 °C. Then aliquots were used for RT modification.

Modification Step. A mixture containing 12 mM Tris-HCl, pH 8.0, 1 μM RT, 1 μM $(pA)_{14}$ as template, and one of the oligothymidylate RA or RCl derivatives as primer (concentrations indicated in the figure captions) was incubated for 2 h at 30 °C. The modification reaction was stopped by cooling the mixture to 0 °C and adding 1 μL of 50 mM DTT for the RCl reagents or 1 μL of 50 mM DTT and 1 μL of 0.1 M $NaBH_4$ for the RA reagents. The mixture was kept for 30 min at 0 °C and then for 30 min at 30 °C.

Elongation Step. The elongation of the reagents bound during the modification step was initiated by the addition of 10 μCi [α-^{32}P]dTTP (3000 Ci/mmol) and 5 mM $Mg(CH_3COO)_2$. After 90 min at 30 °C, samples were subjected to electrophoresis on a polyacrylamide gel (12% acrylamide) in the presence of sodium dodecyl sulfate (SDS) as described in Laemmli (1970). The gel was stained with Coomassie brilliant blue, dried, and subjected to autoradiography on 3MR films.

RESULTS

Synthesis of Reactive Derivatives of Oligothymidylates. As was shown earlier by Oshevski (Oshevski, 1982), the T4 phage polynucleotide kinase transfers the thiophosphate residue of ATP-γ-S onto the 5′-hydroxyl of deoxyoligonucleotides. This enzymatic approach was used to obtain 5′-thiophosphorylated octathymidylate. In addition, chemical synthesis was used to obtain two decathymidylates containing an internucleoside thiophosphate group near the 5′- or the 3′-end.

The reactive derivatives of the oligothymidylates were prepared by alkylation with the trifunctional agent RCl_3. Treatment of oligophosphorothioates by RCl_3 under mild conditions resulted in the complete alkylation of the thiophosphate group. The oligonucleotide derivatives (I), (II), and (III) were thus obtained. The 2-chloroethyl group at the nitrogen atom having the *p*-formylphenyl residue is inactive due to the electron-acceptor effect of the formyl group. It may be activated by reduction of the formyl residue with sodium borohydride under mild conditions. After reduction, as described in the Methods section, the reagents (IV), (V), and (VI) were obtained.

The oligonucleotide analogues may act either as aliphatic amino group modifying reagents or as alkylating reagents. (a) In the first case, the aldehyde group of reagents (I), (II), and (III) can react with primary amines, yielding Schiff bases with the protein. Reduction with $NaBH_4$ increases the stability of the linkage. In the case of RA reagents (I), (II), and (III), the strong electron-acceptor effect of the aldehyde group inhibits the alkylating activity of the (2-chloroethyl)amino group. Thus, under physiological conditions, these compounds are not reactive for the alkylating function (Grachev et al., 1987; Oshevski, 1982). (b) In the second case, the limiting step of the reaction of RCl reagents (IV), (V), and (VI) with the nucleophilic amino groups of the protein is the formation of a highly reactive aziridinium ion (Gall et al., 1979). The RCl reagents obtained after reduction with $NaBH_4$ presented a 1000-fold increase in the alkylating activity of the (2-chloroethyl)amino group. The half-life of this reaction was about 50 min at 37 °C.

In our studies of HIV-1 RT, both functions of the RA and the RCl reagents were used separately.

Interaction of Oligothymidylate Derivatives with RT. It is usually difficult to achieve a highly selective modification of polyfunctional enzymes by using ligands or analogues which have low affinities for the active site. For this reason, the apparent Michaelis–Menten parameters (K_m and V_{max}) of RT p66/p51 for the primers $d(pT)_{10}$ and $d(pT)_8$ and their derivatives were determined to ascertain if the oligo derivatives

Table II: Determination of Apparent Michaelis–Menten Constants of $d(pT)_{10}$, $d(pT)_8$, and RA Derivatives to RT p66/p51

primer	K_m (μM)	V_m (%)
$d(pT)_{10}$	1.2 ± 0.3	100
5′-RA-$d(pT)_{10}$	0.35 ± 0.1	85
3′-RA-$d(pT)_{10}$	1.4 ± 0.5	27
$d(pT)_8$	6.0 ± 2.0	95
RA-$d(pT)_8$	2.0 ± 1.0	30

Table III: Dissociation Constants and Rate of Inactivation of RT and RA Reagent Complexes in the Presence or Absence of Poly(A) Template

	+poly(A)		−poly(A)	
primer	K_d (μM)	$k \times 10^{-3}$ (min^{-1})	K_d (μM)	$k \times 10^{-3}$ (min^{-1})
5′-RA-$d(pT)_{10}$	0.4 ± 0.2	5.7 ± 1.7	20 ± 7	20 ● 5
3′-RA-$d(pT)_{10}$	1.5 ± 0.5	5.4 ± 1.0	11 ± 5	14 ± 3
RA-$d(pT)_8$	3.0 ± 1.2	6.3 ● 2	30 ± 10	15 ± 3

were likely to be of use for modification. The rate of elongation of analogues with [^{3}H]dTTP was measured as a function of the primer concentration, in the presence of saturating concentrations of poly(A). The apparent K_m and V_{max} values for $d(pT)_{10}$, $d(pT)_8$, RA-$d(pT)_8$, 5′-RA-$d(pT)_{10}$, and 3′-RA-$d(pT)_{10}$ are given in Table II. The apparent K_m values for RA-$d(pT)_8$ and 5′-RA-$d(pT)_{10}$ were 3 times lower than for $d(pT)_8$ and $d(pT)_{10}$, respectively. On the other hand, the introduction of the RA residue onto the sulfur atom of d[$(Tp)_8$Tp(S)T] (to give the analogue II, 3′-RA-$d(pT)_{10}$) did not appreciably alter the apparent K_m value of the decathmidylate primer compared to $d(pT)_{10}$. In all cases the values of V_{max} for the RA derivatives were lower than those of the unmodified primers. Such phenomena may be due to the formation of additional contacts between RT and the RA residues.

Affinity of RA Derivatives to RT. The dissociation constants (K_d) of the complexes formed between RT and RA reagents (I), (II), and (III) were determined according to Kitz and Wilson (Kitz & Wilson, 1962) from the following affinity modification scheme:

$$E + X \overset{K_d}{\rightleftharpoons} E{\cdot}X \overset{k}{\rightarrow} EZ$$

where E·X is the enzyme-reagent complex, EZ is the product of the modification, and k is the rate constant of E to EZ transformation.

The incubation of RT with the RA reagents, in the presence or absence of a template, resulted in the inactivation of the enzyme. The K_d values of complexes formed between RT and RA reagents were determined from the plot of k_{app} versus the analogue concentration. A typical experiment, with analogue RA-$d(pT)_8$ in the presence or the absence of poly(A), is shown in Figure 1. The K_d values of such complexes are presented in Table III. In the absence of poly(A), the affinity of RT to RA derivatives (I), (II), and (III) was about at least 10 times lower than in the presence of the template. In the presence of poly(A), the apparent K_m (Table II) and K_d (Table III) values for RA derivatives (I), (II), and (III) were virtually identical. From a comparison of the apparent K_m and K_d values, it appears that the two approaches, affinity modification in the presence of poly(A) template and the polymerization reaction, resulted in the same affinity parameters for the active site.

The maximal rates of enzyme inactivation (k) by the RA reagents are shown in Table III. In the presence of poly(A), the values corresponding to different analogues were very similar. On the other hand, when the enzyme was incubated with the analogues in the absence of template, the rate of enzyme inactivation increased in all cases, by a factor of 2–4. These data show that complex formation between the enzyme and the RA–primer derivatives was different in the presence or absence of a complementary template.

Affinity Labeling of RT p66/p51 by Oligothymidylate Derivatives. The information obtained by using ligand affinity modification depends on the specificity of interaction with the ligand binding site. The modification of the enzyme by the primer derivatives in the presence of a template is due to the "catalytic competence" of the enzyme; the subsequent addition of [α-^{32}P]dTTP results in the elongation of the covalently bound primer by the radioactive nucleotide due to the catalytic action of the active site. As described above, the modification of the enzyme by primer analogues led to the inactivation of RT. The decrease of RT activity likely reflects the specific binding of oligothymidylate derivatives to the template and/or the primer binding site. However, part of the enzyme inactivation could be the consequence of reactions elsewhere in the enzyme molecule between essential amino acids and derivatives. One advantage of our approach is that

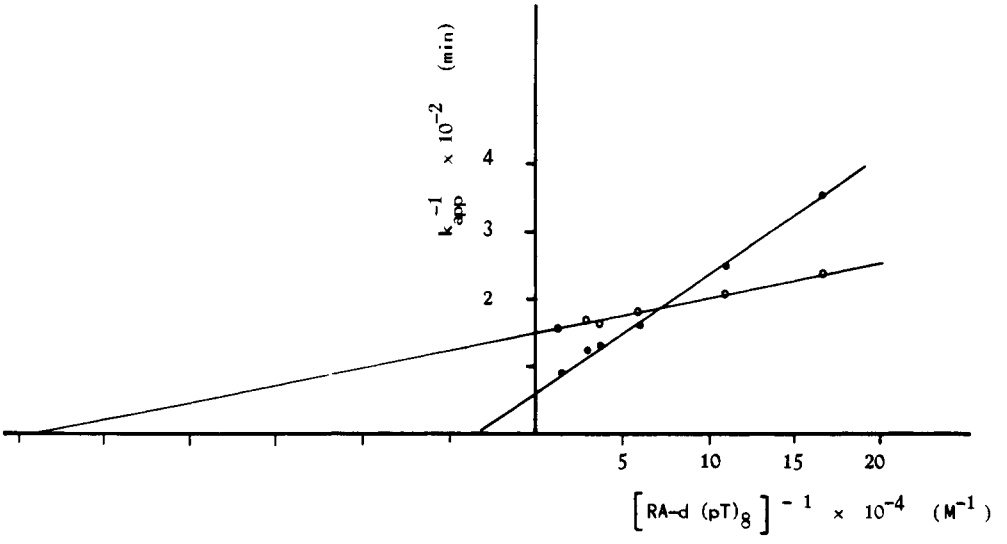


FIGURE 1: Reciprocal plot of the dependence of the k_{app} of RT inactivation upon the concentration of primer derivative. RT p66/p51 was incubated in the presence (○) or absence (●) of poly(A) template and the primer derivative RA-$d(pT)_8$, as described in Methods.

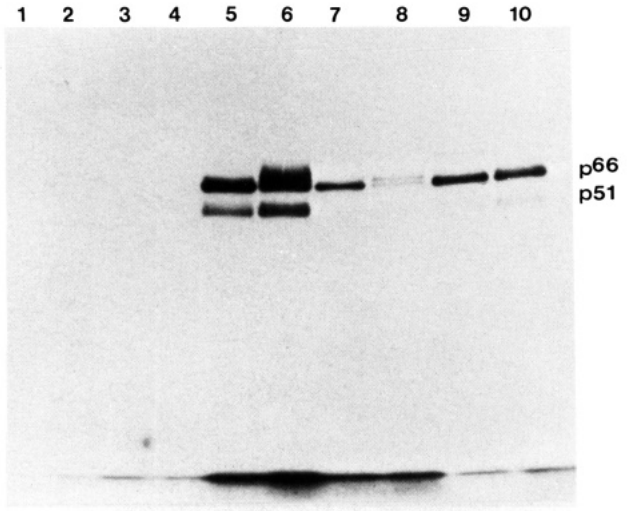


FIGURE 2: Affinity labeling of RT p66/p51. RT p66/p51 (1 μM) was incubated with the template $(pA)_{14}$ (1 μM) and the corresponding oligothymidylate derivative (1 μM). After reduction with $NaBH_4$ and DTT, [α-^{32}P]dTTP was added for 1.5 h at 30 °C. Samples were analyzed by electrophoresis on 12% polyacrylamide gel in the presence of SDS. Controls are shown in lanes 1–4: (1) RT plus [α-^{32}P]dTTP; (2) RT plus $(pA)_{14}$ plus [α-^{32}P]dTTP; (3) RT plus 5′-RA-$d(pT)_{10}$; (4) RT plus 3′-RA-$d(pT)_{10}$. Lanes 5–10 show the complete reaction mixture plus (5) 5′-RA-$d(pT)_{10}$, (6) 5′-RCl-$d(pT)_{10}$, (7) 3′-RA-$d(pT)_{10}$, (8) 3′-RCl-$d(pT)_{10}$, (9) RA-$d(pT)_8$, or (10) RCl-$d(pT)_8$.

the analogues which are bound outside the active center will not be elongated by the radioactive precursor and, thus, will not be visualized during subsequent analysis.

The affinity labeling was performed with the homo- and heterodimeric recombinant forms of HIV-1 RT (Bordier et al., 1990). The first experiments were carried out with RT p66/p51 in the presence of a template. Because long templates such as poly(A) are unable to enter polyacrylamide gel, shorter templates containing 10–14 nucleotides were used, and this led to a reduction in the maximal rate of polymerization (V_{max}) by a factor of 4–6. The decrease is compensated by the $p(A)_{14}$ template being short enough to migrate well into the gel, but long enough to permit a convenient polymerization rate.

HIV-1 RT was incubated in the presence of the template and oligothymidylate analogues. The enzyme modification reaction was terminated by the addition of $NaBH_4$ and DTT in the case of RA derivatives or DTT in the case of RCl derivatives. The elongation step was initiated by the addition of [α-^{32}P]dTTP. Results of the affinity labeling are shown in the autoradiogram of Figure 2.

The most extensive labeling of RT was found with analogues IV and I (Figure 2, lanes 5 and 6). With both reagents, there was an effective labeling of the p66 subunit in the heterodimer. Although the p51 subunit was also modified, its labeling, as determined by densitometry, represented only 20 and 45%, respectively, of that of the p66 subunit. Under the same experimental conditions, analogs II, III, V, and VI labeled the p66 subunit of the heterodimer to a much greater extent that the p51 subunit (lanes 7–10). However, when autoradiograms were exposed for longer times, a labeled band could be seen at the position of the p51 subunit. In all cases, for analogs II, III, V, and VI, the p51 labeling represented only 12–15% of the p66 band. Controls were carried out in the presence of [α-^{32}P]dTTP alone, in the presence of the radioactive substrate together with $(pA)_{14}$, or with either 5′-RA-$d(pT)_{10}$ or 3′-RA-$d(pT)_{10}$ in the absence of template. As shown in Figure 2 (lanes 1–4), no labeling of the enzyme was observed.

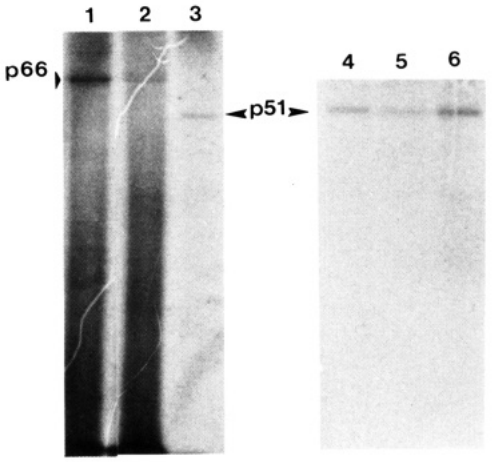


FIGURE 3: Affinity labeling of the three forms of RT, p66/p51, p66/p66, and p51/p51. RT (1 μM) was incubated with $(pA)_{14}$ (1 μM) and oligothymidylate (1 μM). Modification was performed as described in Methods. RT p66/p51 (lane 1), p66/p66 (lane 2), and p51/p51 (lane 3) were modified with 3′-RA-$d(pT)_{10}$. RT p51/p51 was also modified with RA-$d(pT)_8$ (lane 4), 5′-RA-$d(pT)_{10}$ (lane 5), and 3′-RA-$d(pT)_{10}$ (lane 6).

In some cases, additional radioactive products different from those migrating at the position of the p66 or p51 subunits were observed. It is possible that, after the modification step, a population of RT molecules and noncovalently bound template·primer remains. Probably the free RT can elongate the free template·primer, giving products of low molecular weight. Moreover, some bands of molecular weight greater than the p66 subunit (lanes 6, 8, 9, and 10) can be observed. We suspect that some noncovalently bound derivatives, after elongation by RT, may react with the surface of the enzyme, giving nonspecifically labeled bands.

Parameters which control the RT labeling were studied. The extent of labeling depended on the preincubation time of RT and analogue before addition of [α-^{32}P]dTTP. The maximal level of modification of RT was observed after 2 h of incubation. Increasing the incubation time further led to the appearance of additional radioactive bands of lower molecular weight than RT (data not shown). RT labeling also depended on the concentration of the analogues. The maximum was reached when the reagent concentration was equal to that of the template. At higher concentrations, there was a decrease in the level of RT modification (data not shown).

The influence of Mg^{2+} on the complex formation between the template·primer analogue and RT was studied at the modification and elongation steps of the procedure. With either homo- or heterodimeric RT forms, no label was observed when Mg^{2+} was omitted from the incubation mixture. If Mg^{2+} was absent from the modification step but added during the elongation step, high incorporation of [α-^{32}P]dTMP by RT was seen (not shown). While the polymerase activity of RT was absolutely Mg^{2+} dependent, no Mg^{2+} was necessary for the modification of RT by the analogue. As shown by others, Mg^{2+} is not required for the UV-dependent cross-linking of [^{32}P]$(pA)_{12-18}$·dT_{10} to the different HIV-1 RT forms (Cheng et al., 1991).

Comparison of the Modification of Different Forms of RT: Role of the Analogue Structure in the Efficiency of Labeling. The labeling of the three forms of RT, namely, p66/p51, p66/p66, and p51/p51, after modification by RA derivatives I, II, and III is shown in Figure 3. In all experiments, the same concentrations of protein and derivatives were used. As evaluated by densitometry, the labeling of p66/p66 (lane 2) and p51/p51 (lane 3) was 10 and 17%,

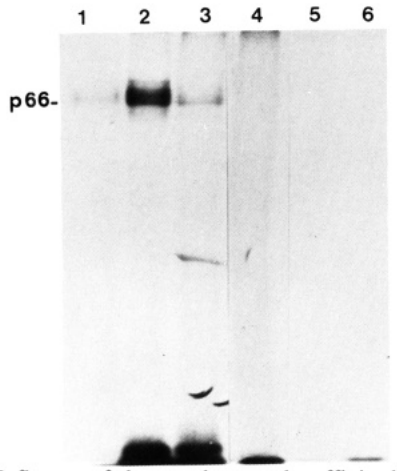


FIGURE 4: Influence of the template on the affinity labeling. RT p66/p51 (1 μM) was modified with the 5′-RA-d(pT)$_{10}$ derivative as described in Methods. The templates, used at 1 μM concentrations, were d(pA)$_{14}$ (lane 1), (pA)$_{14}$ (lane 2), (pA)$_{10}$ (lane 3), (pA)$_7$ (lane 4), d(G)$_{12-18}$ (lane 5), and d(T)$_{12-18}$ (lane 6).

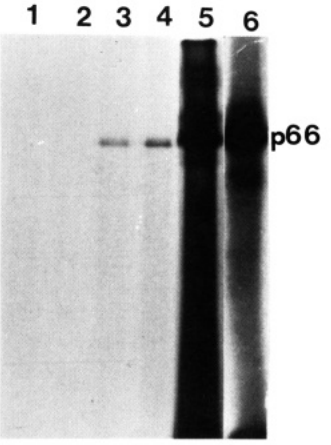


FIGURE 5: Modification of RT p66/p51 in the presence or absence of the template. Lane 1: RT p66/p51 (1 μM) was incubated with RA-d(pT)$_8$ for 2 h as described in Methods. Reduction was performed by the addition of $NaBH_4$ and DTT. Then 1 μM d(G)$_{12-18}$ was added, together with [α-^{32}P]dTTP and Mg^{2+}, to perform the elongation step. Lane 2: Same as lane 1, but d(G)$_{12-18}$ was present from the beginning of the modification step. Lane 3: The modification step was performed by incubating RT with (pA)$_{14}$ and the RA-d(pT)$_8$ analogue in the presence of d(G)$_{12-18}$, a noncomplementary template. After reduction with $NaBH_4$, [α-^{32}P]dTTP and Mg^{2+} were added to perform the elongation step. Lane 4: Modification was performed as described in Methods with 1 μM RT, 1 μM RA-d(pT)$_8$, and 1 μM (pA)$_{14}$. After reduction with $NaBH_4$, the elongation step was initiated by the addition of [α-^{32}P]dTTP and Mg^{2+}. Lane 5: Modification was performed with 1 μM RT and 1 μM RA-d(pT)$_8$ in the absence of the template. (pA)$_{14}$, 1 μM, was added, together with [α-^{32}P]dTTP and Mg^{2+}, after reduction with $NaBH_4$, to allow DNA synthesis. Lane 6: Same as lane 5, but, 10 units of *E. coli* DNase I were added, and incubation was carried out for 30 min at 30 °C after the elongation step.

respectively, of the p66/p51 label (lane 1). This result is in agreement with the relative specific activities and relative affinities of the homo- and heterodimeric forms of RT for d(pT)$_{10}$ primers. As was shown earlier, the specific activity of HIV-1 RT p66/p51 heterodimer was about 10 times higher than that of the homodimers (Bordier et al., 1990). Moreover, the p66/p66 and p51/p51 forms of RT have affinities for the primer d(pT)$_{10}$ 1 order of magnitude lower than that of the p66/p51 heterodimer (Nevinsky et al., 1992).

The best labeling of p66/p51 and p66/p66 (not shown for p66/p66) was obtained with the 5′-RA-d(pT)$_{10}$ analogue, while the opposite situation was observed for the p51/p51 homodimer. In the presence of p51/p51, the most intense modification was obtained with 3′-RA-d(pT)$_{10}$ (lane 6), followed by RA-d(pT)$_8$ (lane 4) and 5′-RA-d(pT)$_{10}$ (lane 5).

Influence of the Template on the Modification Reaction of RT p66/p51. Although there were some differences in the labeling of homo- and heterodimers by the RA reagents, it is important to note that, in all cases, enzyme labeling occurs only in the presence of the complementary template. When (pA)$_{14}$ was substituted for d(G)$_{12-18}$ or d(T)$_{12-18}$, no labeling of RT forms was observed (Figure 4, lanes 5 and 6). A different situation was found when a DNA template, rather than RNA, was used. In the presence of d(pA)$_{14}$, there was incorporation of [α-^{32}P]dTMP by RT (lane 1), but the label was less than 2% of (pA)$_{14}$ (lane 2). This result agrees with the relative maximal rates determined in the polymerization reaction, in that V_{max} when poly(dA) was used as template was only 0.5% of the V_{max} for poly(A) (Nevinsky et al., 1992). Changing the template from (pA)$_{14}$ (Figure 4, lane 2) to (pA)$_{10}$ decreased the labeling to only 4% (lane 3). No labeling was detected with (pA)$_7$ template (lane 4).

As shown above, labeling occurred only in the presence of the complementary template. Moreover, RT interacts differently with the oligothymidylate derivatives during the modification step, depending on whether or not the template is present (Table III). To confirm these observations, affinity labeling of RT was performed under different conditions.

As described in the Methods section, affinity labeling of RT is performed in two subsequent steps: the modification step and the elongation step. Under standard conditions, the modification step led to the covalent binding of the modified (dT)$_{10}$ primer to the enzyme in the presence of the (pA)$_{14}$ complementary template. Then, elongation of the primer due to the catalytic competence of the enzyme was performed after addition of [α-^{32}P]dTTP and Mg^{2+}. Under these conditions, labeling of the p66 subunit was observed, as shown in Figure 5, lane 4. On the contrary, no labeling was observed when the (pA)$_{14}$ template was replaced by a noncomplementary template, oligo(dG)$_{12-18}$, which was added either during the elongation step (lane 1) or during the modification step (lane 2). This could be due to the fact that although the primer was bound to RT, the elongation step and subsequent labeling were impossible because of the absence of complementary template. To test this hypothesis, the following experiment was carried out. The enzyme was incubated with the oligothymidylate derivative primer in the absence of (pA)$_{14}$ template. The modification step was terminated with an excess of $NaBH_4$ and DTT, and then (pA)$_{14}$ and [α-^{32}P]dTTP were added. This procedure led to an abundant incorporation of dTTP in the enzyme, as well as in noncovalently bound products, which appear as a smear in Figure 5, lane 5. A comparison of these results with those shown in lane 4, where the labeling was performed under standard conditions, indicates that the enzyme recognized the primer differently depending on whether or not the template was present during the modification step. As shown in Table III, the dissociation constant value in the presence of template was lower than that in the absence of template. This means that the enzyme binds better to the primer·template complex than to the primer in the absence of template. It may be that, during the modification step, few primers are bound to the RT because of this low dissociation constant. During the elongation step, free primers can anneal by complementary interaction to (pA)$_{14}$, and then the enzyme can polymerize on this template·primer complex. Under these conditions, "switching" of the template and primer is possible because there is no covalent binding of the primer to the enzyme and a strong RT

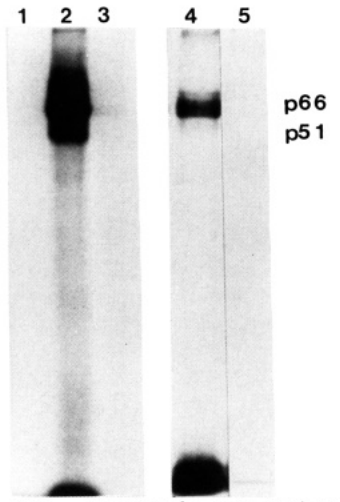


FIGURE 6: Competition with $tRNA^{Lys}$. RT p66/p51 (1 μM) (lanes 1, 2 and 3) or RT p66/p66 (1 μM) (lanes 4 and 5) was used. Lanes 2 and 4: RT was incubated with 1 μM RA-d(pT)$_8$ in the presence of 1 μM (pA)$_{14}$. Lanes 1 and 5: Same as lane 2, but 0.5 μM $tRNA^{Lys}$ was added during the modification step, before the reduction with $NaBH_4$. Lane 3: RT was incubated with the analogue and 0.5 μM of $tRNA^{Lys}$ in the absence of template. After the modification step and the reduction, (pA)$_{14}$ was added, together with [α-^{32}P]dTTP and Mg^{2+}.

activity is possible. As seen in lane 5, most of the products were not covalently bound to the enzyme and appeared as a smear in the gel (lane 5).

To determine whether some primer was bound to the enzyme in the absence of template, the effect of DNase I on the products of synthesis was followed. Enzyme was incubated with the primer in the absence of template, and then the elongation step was initiated by the addition of (pA)$_{14}$, Mg^{2+}, and [α-^{32}P]-dTTP; after 1.5 h, *E. coli* DNase I was added for 30 min. As shown in Figure 5, lane 6, the elongation product was protected from nuclease action by its interaction with RT. Therefore, the oligo(dT) derivative was covalently bound to the enzyme in the absence of template. In competition experiments, short oligomers, such as oligo(dG)$_{12-18}$, were not able to compete when present in the reaction with other reagents (Figure 5, lane 3), because RT probably has a higher affinity for (pA)$_{14}$ than for oligo(dG)$_{12-18}$.

The conclusion from the experiments shown in Figure 5 and the data in Table III is that the enzyme binds to the primer differently depending on the presence or absence of template. However, in either case, the 3′-end unit of the primer interacts with the same specific binding site on the active center of the enzyme.

Competition by $tRNA^{Lys}$ for the Primer·Template Binding Site. The labeling test based on the catalytic competence of the enzyme indicates that the 3′-end of the analogue is properly set at the active site of RT. According to our previous work (Bordier et al., 1990), $tRNA^{Lys}$ is a competitive inhibitor of the template·primer complex of RT p66/p51 (K_i = 80 nM). At concentrations of RT pp66/p51 and $tRNA^{Lys}$ of about 1 μM, practically all the molecules of the enzyme should be complexed with tRNA. As shown in Figure 6, $tRNA^{Lys}$ "protected" HIV-1 RT from covalent binding by oligothymidylate derivatives, and as a consequence, there is no elongation of the RA-d(pT)$_8$ analogue (Figure 6, lane 1). Due to the high affinity of $tRNA^{Lys}$ for RT, once the tRNA is bound to the enzyme, neither (pA)$_{14}$ nor RA-d(pT)$_8$ can displace the natural primer of the enzyme. The same results were obtained with the p66/p66 form of HIV-1 RT (Figure 6, lanes 4 and 5). This confirms our previous data and indicates a competition between $tRNA^{Lys}$ and the template·primer complex for the binding site of HIV-1 RT.

DISCUSSION

The highly selective method of affinity labeling by analogues of initiating substrates was first reported for *E. coli* RNA polymerase (Grachev et al., 1987). Using a similar approach, we describe here the interaction of HIV-1 RT with a series of modified oligonucleotides. These compounds contained reactive aromatic residues on the 5′-thiophosphate and internucleoside thiophosphate groups at the 5′- and 3′-ends of the oligothymidylates. After reacting the enzyme with the active site-directed affinity labeling reagent, the protein was still catalytically competent and could incorporate the labeled precursor, [α-^{32}P]dTTP, into the 3′-terminus of the primer. This reaction due to the "catalytic competence" of the enzyme showed that the 3′-end, which is crucial for the initiation of cDNA synthesis, was localized at or near the catalytic site.

The kinetic parameters of HIV-1 RT for the modified oligothymidylates (RA and RCl derivatives) were determined. The introduction of hydrophobic residues at the 5′-end of the primers led to an increase of their affinity for RT (Table II). For 5′-RA derivatives, the apparent K_m value is 3 times lower than the apparent K_m for the corresponding unmodified oligo-(dT). However, the increased affinity can not be attributed to the RA group alone, because 3′-RA-d(pT)$_{10}$ and d(pT)$_{10}$ have similar K_m values, whereas the K_m for RA-d(pT)$_8$ is 7-fold greater than that of 5′-RA-d(pT)$_{10}$. This increased stabilization has also been observed for other DNA polymerases and was attributed to the attachment of different hydrophobic residues, ethidium, phenazinium, or daunomycin, to the 5′-phosphate of the primer (Lokhova et al., 1991).

All RA and RCl derivatives, irrespective of the point of introduction of the active residue, could specifically be used as primers by HIV-1 RT in DNA synthesis. The specificity of the reaction was confirmed by the observation that $tRNA^{Lys}$, the natural primer of HIV-1 RT, strongly competed with the template·primer binding site on the enzyme. The best labeling of RT was obtained with the analogues 5′-RA-d(pT)$_{10}$ and 5′-RCl-d(pT)$_{10}$ (Figure 3). At equal concentrations of enzyme, template, and primer, a weaker signal was observed in the case of the other four analogues.

The amount of dTMP incorporated into the different primers could be due to the relative amount of primer covalently attached to the enzyme, but alternatively, the radioactivity may reflect the catalytic competence of the enzyme after modification by the different primer analogues. Indeed, the K_d for the 3′-RA-d(pT)$_{10}$ analogue is higher than the K_d for 5′-RA-d(pT)$_{10}$. This indicates that the affinity of RT for analogues modified at the 3′-end is lower than for 5′-end modified analogues. When RT labeling using the 5′- or 3′-modified analogues is compared, the best labeling was obtained with the 5′-RA-d(pT)$_{10}$. This could indicate a better modification of RT with 5′-RA-d(pT)$_{10}$ than with 3′-RA-d(pT)$_{10}$. But also, the modification with the 3′-RA and 3′-RCl reagents may place the modifying group too close to the active site and partially disrupt the ability of the enzyme to incorporate a dTMP residue.

Also, the difference in length between RA-d(pT)$_8$ or RCl-d(pT)$_8$ analogues compared to the 5′-modified d(pT)$_{10}$ analogues may account for the difference in labeling, because the affinity of RT for d(pT)$_8$ is lower than for d(pT)$_{10}$ (Nevinsky et al., 1992).

According to a general model of template·primer interaction with DNA polymerases, the 3′-end of the primer makes a

crucial contribution to the binding of DNA polymerases to primers (Majumdar et al., 1988, 1989; Nevinsky et al., 1990). For this reason, the 5′-end is a convenient point to introduce different groups, and then the modified primers can be used as tools to analyze the mechanism of DNA replication. The utilization of modified primers led to a strong inhibition of the DNA polymerase activity of HIV-1 RT. These modified primers may be used as potential specific inhibitors or inactivators of reverse transcriptases. In that sense, it has been shown elsewhere that unmodified oligonucleotides were able to inhibit HIV-1 RT activity (Majumdar et al., 1988). Moreover, a phosphorothioate oligonucleotide, $Sd(C)_{28}$, was able to bind the enzyme with an exceptionally high affinity and competitively inhibited the synthesis directed by a heterologous template·primer (Majumdar et al., 1989).

The inhibition kinetics of HIV-1 RT by oligothymidylate derivatives allowed us to calculate the rate of inactivation, k, and the K_d of the enzyme for the different reagents. As shown in Table III, RT interacted differently with oligothymidylates depending on the presence or absence of poly(A) template. Selective ^{32}P labeling of the enzyme demonstrated that, in all complexes formed between RT and the RA reagents, the 3′-end unit of the primer was localized on the enzyme at the catalytically correct position. It has been shown that the main template·primer recognition process by HIV-1 RT involves high-affinity binding to the primer portion of the template·primer complex (Majumdar et al., 1989).

We have shown that the shortest primers utilized by some DNA polymerases, as well as by AMV (avian myeloblastosis virus) and HIV-1 RT, were dNMP, dNDP, and dNTP (Knorre et al., 1988; Nevinsky et al., 1992). The 3′-end unit of a longer primer contributed significantly to the interaction between the primer and the DNA polymerases or reverse transcriptases. In the presence of a template, all the nucleotide units of the primer interacted with the template exclusively by Watson–Crick hydrogen bonds. All these results strongly suggest that, in the presence of the appropriate template, no significant interaction is observed between the 5′-end of the primer and the enzyme. Another possibility is that the 5′-end of the primer, in the absence of the template, moves more easily than in the presence of the complementary template and, therefore, can find more reactive groups on the protein.

In the case of pro- and eukaryotic DNA polymerases, reactive derivatives were used to specifically modify the template binding site (Knorre et al., 1988). No primer binding site labeling was detected in the absence of a template complementary to the primer analogue (Mitina et al., 1990; our unpublished data). In the case of *E. coli* RNA polymerase, labeling was only observed in the presence of a promoter-containing template complementary to the reagent (Grachev et al., 1987).

Our results strongly suggest that the "catalytically competitive" modification of the primer binding site by primer analogues, in the absence of template may occur only for the viral reverse transcriptase. According to Majumdar et al. (1988, 1989) and Kolocheva et al. (1989) and to our results, HIV-1 RT is able to bind the primer chain before the template. Our observations may thus be related to the utilization of a tRNA as a natural primer. In contrast with other DNA polymerases, reverse transcriptase must select its specific primer from the total population of host cellular tRNAs (Araya & Litvak, 1982). The use of tRNA analogues as primers for the DNA synthesis reaction catalyzed by HIV-1 RT could be an interesting tool to assess this hypothesis.

ACKNOWLEDGMENT

The authors are very grateful to Dr. Michel Fournier for his generous gift of bovine $tRNA^{Lys}$ and Dr. J. J. Toulmé for discussions and suggestions.

REFERENCES

Araya, A., & Litvak, S. (1982) *Trends Biochem. Sci. 7*, 361–364.

Barat, C., Le Grice, S. F., & Darlix, J.-L. (1991) *Nucleic Acids Res. 19*, 751–757.

Barber, A. M., Hizi, A., Maizel, J. V., Jr., & Hughes, S. H. (1990) *AIDS Res. Hum. Retroviruses 6*, 1061–1072.

Bathurst, I. C., Moen, L. K., Lujan, M. A., Gibson, H. L., Feucht, P. H., Pichuantes, S., Craik, C. S., Santi, D. V., & Barr, P. J. (1990) *Biochem. Biophys. Res. Commun. 171*, 589–595.

Bordier, B., Tarrago-Litvak, L., Sallafranque-Andreola, M.-L., Robert, D., Tharaud, D., Fournier, M., Barr, P. J., Litvak, S., & Sarih-Cottin, L. (1990) *Nucleic Acids Res. 18*, 429–436.

Bukhrashvili, I. S., Chinchaladze, D. Z., Lavrik, O. I., Levina, A. S., Nevinsky, G. A., & Prangishvili, D. A. (1989) *Biochim. Biophys. Acta 1008*, 102–107.

Cheng, N., Painter, G. R., & Furman, P. A. (1991) *Biochem. Biophys. Res. Commun. 174*, 785–789.

Cornish-Bowden, A. (1976) in *Principles of enzyme kinetics*, pp 27–28, 189–192, Butterworth, London and Boston.

Di Marzo Veronese, F., Copeland, T. D., De Vico, A. L., Rahman, R., Oroszlan, S., Gallo, R. C., & Sarngadharan, M. G. (1986) *Science 231*, 1289–1291.

Foiani, M., Lindner, A. J., Hartmann, G. R., Lucchini, G., & Plevani, P. (1989) *J. Biol. Chem. 264*, 2189–2194.

Gall, A. A., Karbatov, V. A., Mustaev, A. A., & Shishbin, G. V. (1979) *Izv. Sib. Otd. Akad. Nauk SSSR, Ser. Khim. Nauk 4*, 99–104.

Grachev, M. A., Mustaev, A. A., & Oshevski, S. J. (1980) *Nucleic Acids Res. 8*, 3413–3426.

Grachev, M. A., Kolocheva, T. I., Lukhtanov, E. A., & Mustaev, A. A. (1987) *Eur. J. Biochem. 163*, 113–121.

Hansen, J., Schulze, T., & Moelling, K. (1987) *J. Biol. Chem. 262*, 12393–12396.

Jacobo-Molina, A., & Arnold, E. (1991) *Biochemistry 30*, 6351–6361.

Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.

Knorre, D. G., Lavrik, O. I., & Nevinsky, G. A. (1988) *Biochimie 70*, 655–661.

Kolocheva, T. I., Nevinsky, G. A., Volchkova, V. A., Levina, A. S., Khomov, V. V., & Lavrik, O. I. (1989) *FEBS Lett. 248*, 97–100.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Levina, A. S., Nevinsky, G. A., & Lavrik, O. I. (1985) *Bioorg. Khim. 11*, 358–369.

Lightfoote, M. M., Coligan, J. E., Folks, T. M., Fauci, A. S., Martin, M. A., & Venkatesan, S. (1986) *J. Virol. 60*, 771–775.

Lokhova, I. A., Nevinsky, G. A., Godovikova, T. S., Ivanova, E. M., Koshkin, A. A., Sergeev, D. S., Frolova, E. I., Rudenko, N. K., Khonov, V. V., Kavsan, V. M., Zaryfova, U. F., & Lavrik, O. I. (1991) *FEBS Lett. 281*, 111–113.

Majumdar, C., Abbotts, J., Broder, S., & Wilson, S. M. (1988) *J. Biol. Chem. 263*, 15657–15665.

Majumdar, C., Stein, C. A., Cohen, J. S., Broder, S., & Wilson, S. M. (1989) *Biochemistry 28*, 1340–1346.

Mitina, R. L., Mustaev, A. A., Zaychikov, E. F., Khomov, V. V., & Lavrik, O. I. (1990) *FEBS Lett. 272*, 181–183.

Mudrakovskaya, A. V., & Jamkovoy, V. I. (1990) in *The enzymes of microorganisms and biopolymers degradation* (Debabov, V. G., & Gordon, I. O., Eds.) pp 199–206, NPO Medbioeconomic, Moscow.

Nevinsky, G. A., Veniaminova, A. G., Levina, A. S., Podust, V. N., Lavrik, O. I., & Holler, E. (1990) *Biochemistry 29*, 1200–1207.

Nevinsky, G., Andreola, M.-L., Jamkovoy, V. I., Levina, A. S., Barr, P. J., Tarrago-Litvak, L., Tharaud, D., & Litvak, S. (1992) *Eur. J. Biochem. 207*, 351–358.

Oshevski, S. I. (1982) *FEBS Lett. 143*, 119–123.

Prasad, V. R., & Goff, S. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3104–3108.

Robert, D., Sallafranque-Andreola, M.-L., Bordier, B., Sarih-Cottin, L., Tarrago-Litvak, L., Graves, P. V., Barr, P. J., Fournier, M., & Litvak, S. (1990) *FEBS Lett. 277*, 239–242.

Sallafranque-Andreola, M.-L., Robert, D., Barr, P. J., Fournier, M., Litvak, S., Sarih-Cottin, L., & Tarrago-Litvak, L. (1989) *Eur. J. Biochem. 184*, 367–374.

Sarih-Cottin, L., Bordier, B., Musier-Forsyth, K., Andreola, M.-L., Barr, P. J., & Litvak, S. (1992) *J. Mol. Biol. 226*, 1–6.

Stec, W., & Zon, G. (1984) *Tetrahedron Lett. 25*, 5275–5278.

Veniaminova, A. G., Levina, A. S., Nevinsky, G. A., & Podust, V. N. (1987) *Mol. Biol. (Moscow) 21*, 1378–1385.

Veniaminova, A. G., Gorn, V. V., Zenhova, N. A., Komarova, V. F., & Repkova, M. N. (1990) *Biorg. Khim. 16*, 941–950.

Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S., & Alizon, M. (1985) *Cell 40*, 9–17.