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Identification of the Primer Binding Domain in Human Immunodeficiency Virus Reverse Transcriptase†

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ABSTRACT: We have labeled the primer binding domain of HIV1-RT with 5'-^{32}P-labeled $(dT)_{15}$ primer using ultraviolet light energy. The specificity of the primer cross-linking to HIV1-RT was demonstrated by competition experiments. Both synthetic and natural primers, e.g., $p(dA)_{15}$, $p(dC)_{15}$, and $tRNA^{Lys}$, inhibit $p(dT)_{15}$ binding and cross-linking to the enzyme. The observed binding and cross-linking of the primer to the enzyme were further shown to be functionally significant by the observation that $tRNA^{Lys}$ inhibits the polymerase activity on poly(rA)·$(dT)_{15}$ template-primer as well as the cross-linking of $p(dT)_{15}$ to the enzyme to a similar extent. At an enzyme to $p(dT)_{15}$ ratio of 1:3, about 15% of the enzyme can be cross-linked to the primer. To identify the domain cross-linked to $(dT)_{15}$, tryptic peptides were generated and purified by a combination of HPLC on a C-18 reverse-phase column and DEAE-Sephadex chromatography. A single peptide cross-linked to $p(dT)_{15}$ was identified. This peptide corresponded to amino acid residues 288-307 in the primary sequence of HIV1-RT as judged by amino acid composition and sequence analyses. Further, Leu(289)-Thr(290) and Leu(295)-Thr(296) of HIV1-RT appear to be the probable sites of cross-linking to the primer $p(dT)_{15}$.

Reverse transcriptases have been reported to utilize specific host tRNA for priming the viral first-strand DNA synthesis from the RNA template in vivo (Harada et al., 1975, 1979; Litvak et al., 1982). Avian myeloblastosis virus reverse transcriptase (AMV-RT)[1] uses $tRNA^{Trp}$ and MuLV-RT uses $tRNA^{Pro}$ while HIV1-RT utilizes $tRNA^{Lys}$ as a primer (Araya et al., 1980; Garret et al., 1984). The p66/51 heterodimeric form binds $tRNA^{Lys}$ in vitro with high affinity (Bordier et al., 1990; Barat et al., 1989, 1991). In vitro kinetic studies with synthetic homopolymers as template-primer have demonstrated

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[1] Abbreviations: HIV1-RT, human immunodeficiency virus 1 reverse transcriptase; AMV-RT, avian myeloblastosis virus reverse transcriptase; MuLV-RT, murine leukemia virus reverse transcriptase; dNTP, deoxynucleoside 5'-triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; DTT, dithiothreitol; NP-40, Nonidet P-40; TCA, trichloroacetic acid; TEAA, triethylammonium acetate.

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that primer binding to the free enzyme initiates the template-primer recognition followed by the binding of deoxynucleotides in HIV1-RT (Majumdar et al., 1989). To identify the primer binding site of HIV1-RT, we have used a synthetic primer, $p(dT)_{15}$, and a recombinant p66 homodimeric form of HIV1-RT.[1]

Our laboratory had earlier shown that radiolabeled nucleotides and primers can be covalently cross-linked to the DNA polymerase class of enzymes using UV irradiation (Modak & Gillerman-Cox, 1982). This technique has also been used successfully to map the oligonucleotide binding site of a single-strand DNA binding Al hnRNP protein (Merrill et al., 1984, 1988). Using this technique, our laboratory has recently identified important domains and amino acid residues involved in the primer binding site of MuLV RT and the Klenow fragment of *Escherichia coli* DNA polymerase I (Tirumalai & Modak, 1991a,b). There are several advantages of UV-mediated cross-linking as opposed to cross-linking via chemically reactive groups. UV energy causes "zero length" cross-linking and is thus expected to cause minimal perturbation of the enzyme–substrate complex as compared to the addition of a bulky chemical group that may react with distant sites. Since many amino acids, including serine, cysteine, isoleucine, threonine, and tyrosine (Havron & Sperling, 1977; Maly et al., 1980; Paradiso et al., 1979; Paradiso & Konigsberg, 1982), have been found to be cross-linked to nucleotides, nucleic acids bound to any environment can theoretically cross-link to the enzyme protein. Most importantly, in UV-mediated cross-linking, the amino acids identified are indeed in close proximity to the nucleotide in the three-dimensional structure of the protein (Brayer & McPherson, 1983). On the basis of these considerations, we utilized this technique for labeling and identification of the primer binding domain of HIV1-RT. To achieve this goal, the protocol for photochemical cross-linking of primer to HIV1-RT was optimized, and the relevance of the cross-linking reaction to the functional binding of primer was established. Analysis of the amino acid sequence of the primer-cross-linked peptide was carried out to identify the likely amino acid residues that are in close proximity of the primer nucleotides.

Materials and Methods

The synthetic oligonucleotide $(dT)_{15}$ was synthesized using an Applied Biosystem (280A) synthesizer and purified on a NENsorb column following the protocol described by the manufacturer. The other synthetic oligonucleotides, template-primers, ssDNA–agarose, and nucleotide/deoxynucleoside triphosphates were obtained from Pharmacia. [γ-^{32}P]ATP was from New England Nuclear. Trypsin (TPCK-treated) was obtained from Worthington. HPLC-grade acetonitrile and water were obtained from Fisher Scientific Co.

Enzyme. HIV1-RT (p66 homodimer) was purified from an *E. coli* clone, pRC-RTi$_5$, which was a generous gift from Dr. Samuel H. Wilson of the National Cancer Institute. The purification procedure was a slight modification from that described earlier (Basu et al., 1989). The *E. coli* cells were sonicated, and the extracts were clarified by centrifugation at 40000*g* for 30 min. The enzyme was then purified by sequential chromatography on DEAE-cellulose, phosphocellulose, and ssDNA–agarose. At each step, the activity was monitored using poly(rC)·$(dG)_{15}$ as template-primer and [^{3}H]dGTP as a substrate (Basu et al., 1989). The fractions were analyzed on a 10% SDS–polyacrylamide gel, and protein bands were visualized by Coomassie brilliant blue staining. The crude extracts contained both the p66/66 homodimer and p66/51 heterodimers, and their differential chromatographic behavior on phosphocellulose and ssDNA columns permitted their isolation. The fractions enriched in p66 homodimers and p66/51 heterodimers were individually pooled and processed separately. The final purification of RT was achieved by FPLC on a Q-Sepharose (Pharmacia) column (20 × 1 cm) using the protocol described earlier by Muller et al. (1989). The final enzyme preparation was more than 95% pure as judged by 10% SDS–polyacrylamide gel electrophoresis and Coomassie brilliant blue staining.

5′-End-Labeling of $(dT)_{15}$. Oligonucleotide $(dT)_{15}$ was phosphorylated at the 5′-OH terminus with [γ-^{32}P]ATP and polynucleotide kinase as described by Maniatis et al. (1982). The reaction was carried out in a final volume of 100 μL containing 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.3 mM $(dT)_{15}$, 0.5 mCi of [γ-^{32}P]ATP (30 Ci/mmol final concentration), and 500 units of polynucleotide kinase. After 1 h at 37 °C, 10 μL of 2 mM ATP and 500 units of polynucleotide kinase were added, and incubation was continued for 30 min. Reaction was terminated by the addition of EDTA (10 mM), and labeled primer was separated from free ATP by HPLC on an Oligo column (DuPont) using a 0–1 M NaCl gradient in phosphate buffer, pH 6.8. The concentration of the labeled oligonucleotide was determined by measuring the absorbance at 260 nm, and specific radioactivity was quantitated using a Packard Scintillation counter.

Photochemical Cross-Linking of HIV1-RT to [5′-^{32}P]-$(dT)_{15}$. HIV1-RT (2 μg, about 15 pmol of p66 homodimer) was mixed with desired concentrations of [^{32}P]$(dT)_{15}$ in 30 μL of a reaction mixture containing 50 mM Hepes–KOH, pH 7.8, 10% glycerol, 6 mM $MgCl_2$, 2 mM DTT, and 0.1% NP-40. At the end of 30 min of incubation on ice, the samples were layered on parafilm wells and irradiated for 15 min with UV light (a mineral lamp Model R-52 with maximum intensity at 254 nm, manufactured by Ultra-violet Products Inc., with an output of 200 μW/cm^2 at a distance of 10 cm was used). The UV energy output was measured by a dosimeter (International Light Inc.). For determination of the extent of HIV-RT cross-linked to [^{32}P]$(dT)_{15}$, the cross-linked enzyme sample was subjected to 8% SDS–polyacrylamide gel electrophoresis and located by autoradiography. The extent of cross-linking was quantitated by excising the gel regions containing radioactive bands, drying under vacuum, and subjecting to liquid scintillation spectrometry. For the determination of the extent of cross-linking, the specific radioactivity was calculated from the decay table supplied by NEN. For competition studies, varying amounts of competitors, as indicated in the figure legends, were included in the incubation mixture.

CNBr Fragmentation of [^{32}P]$(dT)_{15}$*-Cross-Linked HIV1-RT and Peptide Separation.* HIV1-RT (20 μg) cross-linked to [^{32}P]$(dT)_{15}$ was precipitated by the addition of 10% TCA and washed extensively with 5% TCA and finally with ethanol. The dry protein pellet was dissolved in 50 μL of 70% formic acid containing 25 mg/mL CNBr and incubated at room temperature for 24 h in the dark. The solution was diluted 10-fold with water, dried under vacuum, dissolved in 50 μL of buffer containing 50 mM Tris-HCl, pH 6.7, 2% SDS, 5% 2-mercaptoethanol, and 20% glycerol, and resolved on a 12–20% gradient SDS–polyacrylamide gel. The $(dT)_{15}$-cross-linked peptides were located by autoradiography.

Preparative-Scale Cross-Linking of HIV1-RT to $(dT)_{15}$ *and Isolation of Cross-Linked Enzyme.* Ten nanomoles of HIV1-RT was mixed with 30 nmol of [^{32}P]$(dT)_{15}$ in a final volume of 2 mL containing 50 mM Hepes–KOH, pH 7.8, 10% glycerol, 10 mM NaCl, 2 mM DTT, 0.1% NP-40, and 6 mM

$MgCl_2$. The mixture was incubated on ice for 30 min and divided into 400-μL aliquots and placed in plastic wells (2.5 cm in diameter and 2 cm deep) surrounded by finely crushed ice. The solution was irradiated for 15 min at 10 cm from the source of light (approximately 3×10^4 erg/mm^2). Irradiated enzyme was then subjected to SDS–PAGE to separate $(dT)_{15}$-cross-linked protein from non-cross-linked protein on an 8% polyacrylamide gel, where $p(dT)_{15}$-cross-linked enzyme migrates much slower than the non-cross-linked enzyme. Cross-linked protein was located by autoradiography, the radioactive band was excised, and the $[^{32}P](dT)_{15}$-cross-linked protein was electroeluted and lyophilized. Labeled protein was repeatedly washed with 75% methanol to remove SDS and finally dried under vaccum. The recovery of the labeled protein by this procedure was greater than 80%.

Isolation of HIV1-RT Tryptic Peptides Cross-Linked to $[^{32}P](dT)_{15}$. The labeled protein pellet [$(dT)_{15}$-cross-linked HIV1-RT] isolated as described above was suspended in 50 μL of 8 M urea and then diluted to 400 μL with 0.1 M NH_4HCO_3, pH 8.0, to give a final urea concentration of 1 M. Trypsin (TPCK) was then added at a protein:trypsin ratio of 50:1. After 2 h of incubation at 37 °C, a second aliquot of trypsin (50:1 protein:trypsin ratio) was added, and the incubation was continued overnight. The digests were directly injected onto a Vydac C-18 reverse-phase column that was equilibrated with 10 mM sodium phosphate, pH 6.8 (solvent A). Peptides were eluted at a flow rate of 0.7 mL/min using a linear gradient of solvent B (70% acetonitrile in 10 mM sodium phosphate, pH 6.8) as follows: 0–100 min (0–40% B), 100–140 min (40–70% B). One-minute fractions were collected directly in 1.5-mL microfuge tubes, and the associated radioactivity was monitored by Cerenkov counting. Radioactive fractions from three such batches of 10-nmol scale were pooled and further purified on a DEAE-Sephadex column by the method of Pandey and Modak (1988) with the following modifications. Fractions were loaded on a 2-mL DEAE-Sephadex column preequilibrated with 50 mM NH_4HCO_3 (pH 8.0) and washed with 20 mL of the same buffer. The column was then washed with the above buffer containing 100 mM NaCl, and finally the $(dT)_{15}$-bound peptides were eluted with 50 mM bicarbonate buffer containing 400 mM NaCl. Samples (100 μL) were collected, and the radioactive fractions were further purified on a C-18 reverse-phase column using shallow gradients of acetonitrile as described in the legend to Figure 5. The radioactive peptides were desalted according to the procedure of Merrill et al. (1988) on a NENsorb column equilibrated with 20 mM TEAA buffer, pH 6.8. The column was washed with 10 mL of the same buffer, and the peptides were eluted with 50% methanol in water. Labeled peptides were subjected to amino acid composition and sequence analyses. The PTC-based amino acid composition analysis was carried out by HPLC on Waters NovaPak columns according to the method described by Stone and Williams (1986). The sequence analysis was carried out at the proten chemistry facility of Yale University on a gas-phase automated sequenator (Applied Biosystems 470A) under the supervision of Dr. Kenneth R. Williams.

Results

Binding of $p(dT)_{15}$ to p66 HIV1-RT was found to be very significant. We applied the nitrocellulose membrane filter binding technique to examine the binding of $(dT)_{15}$ to HIV1-RT, using the protocol described earlier for the determination of the K_D value for HIV1-RT–$Sd(C)_{28}$ interaction (Majumdar et al., 1989). Though this technique does not allow measurements under true equilibrium conditions, Majumdar et al. (1989) have found that K_D values for oligonucleotide binding measured by the filter binding assay are very close to the K_i value measured by kinetic experiments. The equilibrium dissociation constant (K_D) for the $(dT)_{15}$–HIV1-RT interaction, as measured by direct filter binding assay, was about 3×10^{-7} M.

Table I: Effect of Competitors and Ionic Strength on $[^{32}P](dT)_{15}$ Cross-Linking to HIV1-RT

addition	pmol of $(dT)_{15}$ cross-linked	% cross-linking
(1) Enz + $[^{32}P](dT)_{15}$[a]	2.2	100
(2) heat-inactivated enzyme	0	0
(3) +$p(dT)_{15}$ (3 μM)	1.1	50
(4) +$p(dT)_{15}$ (6 μM)	0.75	34
(5) +$p(dA)_{15}$ (6 μM)	0.66	30
(6) +$p(dG)_{15}$ (6 μM)	0.85	40
(7) +$p(dC)_{15}$ (6 μM)	0.98	45
(6) +TTP (100 μM)	2.2	100
(7) +dATP (100 μM)	2.15	98
(8) +ATP (100 μM)	2.1	95
(9) +poly(rA) (0.15 μM)	2.26	103
(10) +poly(rA) (1.5 μM)	1.15	53
(11) + NaCl (20 mM)	1.55	71
(12) +NaCl (50 mM)	1.0	45
(13) +NaCl (10 mM)	0.11	5
(14) +NaCl (200 mM)	0.02	1

[a] Primer cross-linked to 2 μg (15 pmol of p66 homodimer) of HIV1-RT in the presence of 45 pmol of $[^{32}P](dT)_{15}$, 6 mM $MgCl_2$, and 10 mM NaCl was taken as 100%.

Table II: Primer Cross-Linking to HIV1-RT in the Presence of Increasing Concentrations of $[^{32}P](dT)_{15}$[a]

addition	pmol of $(dT)_{15}$ cross-linked	% protein cross-linked
Enz + $[^{32}P](dT)_{15}$		
0.5 μM	1	6.6
1.0 μM	1.6	10.7
1.5 μM	2.2	14.7
2.0 μM	2.3	15.3
2.5 μM	2.3	15.3

[a] 2 μg of HIV1-RT was cross-linked with increasing concentrations of $[^{32}P](dT)_{15}$, and the extent of cross-linking was estimated as described under Materials and Methods.

UV-Mediated Cross-Linking of $[^{32}P](dT)_{15}$ *to HIV1-RT.* We observed that $(dT)_{15}$ bound to HIV1-RT can be covalently cross-linked by UV energy. Figure 1 shows the extent of cross-linking of $[5'\text{-}^{32}P](dT)_{15}$ to HIV1-RT p66 (homodimer) as a function of UV energy (UV exposure time). Maximum cross-linking was observed around 15 min of UV exposure, which corresponds to the UV dose of about 3×10^4 erg/mm^2 at a distance of 10 cm from the lamp. After 15 min, the extent of cross-linking appeared to level off. Under these conditions, no detectable UV-induced proteolysis was noticeable as observed by SDS–PAGE and autoradiography. Short preincubation of enzyme with primer was sufficient to reach equilibrium, since preincubation times ranging from 5 to 30 min showed a similar extent of cross-linking (data not shown). Therefore, incubation of enzyme with primer in further studies was restricted to 15 min. Detailed characterization of the cross-linking reaction is shown in Table I. Cross-linking was strictly dependent on the presence of the active enzyme molecule, since heat-inactivated enzyme did not show any cross-linking. Substitution of HIV1-RT with BSA also showed no cross-linking (data not shown). The extent of cross-linking increases with the increase in primer concentration, the optimum ratio being 3 mol of primer to 1 mol of enzyme (Table II). The efficiency of cross-linking of primer to enzyme under these conditions was measured by separating cross-linked

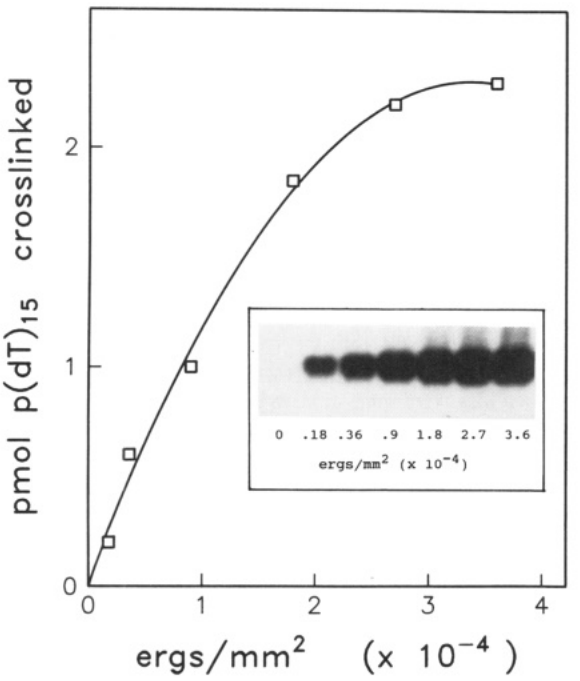

FIGURE 1: UV-mediated cross-linking of [5′-^{32}P](dT)$_{15}$ to HIV1-RT: HIV1-RT (15 pmol of p66 homodimer) was mixed with 45 pmol of [5′-^{32}P](dT)$_{15}$ in 30 μL of buffer containing 50 mM Hepes–KOH, pH 7.8, 10 mM NaCl, 2 mM DTT, 0.1% NP-40, and 10% glycerol and irradiated with increasing doses of UV light (254 nm). The extent of cross-linking was measured by separating cross-linked protein from (dT)$_{15}$ by 8% SDS–PAGE and the incorporated radioactivity measured as described under Materials and Methods. The inset shows an autoradiogram of the SDS–polyacrylamide gel.

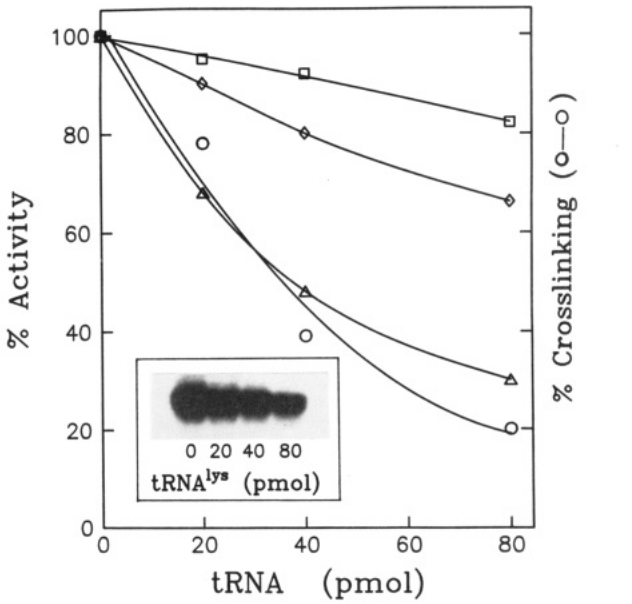

FIGURE 2: Effect of tRNA^{Lys} on DNA polymerase activity and [5′-^{32}P](dT)$_{15}$ cross-linking to HIV1-RT. DNA polymerase activity was measured on (rA)$_n$·(dT)$_{15}$ template-primer using 100 ng of HIV1-RT in the presence of varying tRNA concentrations as described under Materials and Methods. (□) tRNA^{fMet}; (◇) tRNA^{mix}; (△) tRNA^{Lys}. For cross-linking, HIV-RT (15 pmol) was preincubated with [5-^{32}P](dT)$_{15}$ (20 pmol) along with the indicated concentrations of tRNA^{Lys}. The extent of cross-linking was measured as described under Materials and Methods. The autoradiogram of the SDS–polyacrylamide gel is shown in the inset.

protein from free protein and primer by SDS–polyacrylamide gel electrophoresis followed by the quantitation of radioactivity in the appropriate bands. At an enzyme:primer ratio of 1:3, about 15% of the enzyme molecules are cross-linked to the primer. The binding mixture contained 6 mM $MgCl_2$, which is optimal for the enzyme activity (Cheng et al., 1987), although binding of primer to the enzyme does not appear to require the presence of divalent metal ions (Table I), which is consistent with the earlier observation of Huber et al. (1989). We chose to carry out cross-linking studies in the presence of 6 mM $MgCl_2$ since it represented conditions similar to those used for catalysis. Binding of primer by HIV1-RT (as judged by the extent of cross-linking) was found to be highly susceptible to the ionic strength of the incubation mixture; virtually no cross-linking is observed at 200 mM NaCl (Table I). These results indicate that ionic interactions may play a role in the binding of primer to HIV1-RT.

Specificity of p(dT)$_{15}$ Binding to HIV1-RT. To assess the specificity of p(dT)$_{15}$ binding to HIV1-RT, competition experiments with various mono, oligo-, and polynucleotides and with tRNAs were carried out. For these studies, the enzyme was incubated in the standard preincubation mixture (see Materials and Methods) along with varying concentrations of different competitors. The results are shown in Table I. The presence of ribo- or deoxyribonucleotides does not interfere with p(dT)$_{15}$–RT complex formation. Addition of unlabeled p(dT)$_{15}$ effectively inhibits the cross-linking of ^{32}P-labeled oligonucleotide. A 4-fold excess of unlabeled primer reduces the extent of cross-linking by about 65%. Other oligonucleotides such as p(dG)$_{15}$, p(dC)$_{15}$, and p(dA)$_{15}$ were also found to be effective inhibitors of complex formation between [^{32}P](dT)$_{15}$ and HIV1-RT. tRNA^{Lys} has been found to be present in the HIV virions, and HIV1-RT has been reported to utilize this tRNA as a primer in vitro for reverse transcription (Barat et al., 1991). We therefore examined the effect of tRNA^{Lys} on the catalytic activity of HIV1-RT on the poly(rA)·(dT)$_{15}$ reaction. Simultaneously, the effect of tRNA^{Lys} on the cross-linking of (dT)$_{15}$ was measured. The results shown in Figure 2 clearly show that tRNA^{Lys} is a strong inhibitor of DNA polymerase activity and the inhibitory activity correlates well with the observed inhibition of p(dT)$_{15}$ cross-linking to the enzyme. Barat et al. (1989) have shown that other tRNAs are not able to compete for binding of tRNA^{Lys} to HIV1-RT. Thus, to further assess the specificity of p(dT)$_{15}$ binding to the primer binding site, the effect of tRNA^{fMet} and a mixture of tRNA on HIV1-RT activity on (rA)$_n$·(dT)$_{15}$ was examined. The results (Figure 2) show that a strong inhibition is observed only with tRNA^{Lys} while only a marginal effect is observed with tRNA^{fMet}. These results strongly suggest that (dT)$_{15}$ binds to HIV1-RT at a functionally significant primer binding site. The presence of poly(rA), a complementary polynucleotide template for (dT)$_{15}$ primer, at low concentrations (0.1 mol of template to 1 mol of primer) does not significantly alter the binding of primer to the enzyme. However, at a concentration of 1:1 (molar ratio) of template to primer, a 50% reduction of the cross-linking was observed (Table I).

Localization of the (dT)$_{15}$ Cross-Linking Site. In order to determine the number of sites in HIV1-RT that contain cross-linked (dT)$_{15}$, we subjected the radiolabeled enzyme–primer adduct to cyanogen bromide fragmentation and separated the CNBr fragments on a 12–20% gradient SDS–polyacrylamide gel. Upon autoradiography, a major radioactive band corresponding to a molecular weight of about 19 000 was observed (Figure 3). Table III lists the CNBr fragments and their location in the primary amino acid sequence of HIV1-RT p66 polypeptide. The radioactive band corresponds to two CNBr peptides, CB3 and CB6. [Both of these peptides have molecular weights around 14 000 and upon cross-linking to p(dT)$_{15}$ would migrate slowly.] There are two

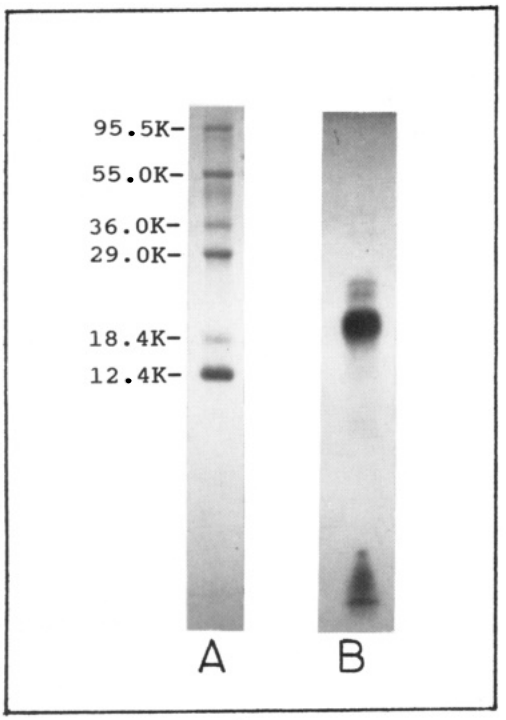

FIGURE 3: SDS–PAGE of [5′-^{32}P](dT)$_{15}$-labeled HIV1-RT CNBr peptides. HIV1-RT (20 μg) was cross-linked by UV energy to [5′-^{32}P](dT)$_{15}$ (45 nmol) and subjected to CNBr cleavage in 70% formic acid as described under Materials and Methods. The CNBr peptides were analyzed on a 12–20% gradient SDS–polyacrylamide gel followed by autoradiography. Lane A, molecular weight markers; lane B, autoradiogram of the gel showing labeled CNBr peptide.

Table III: CNBr Peptides of HIV1-RT p66 Subunit

peptide	start[a]	stop	fragment size	
			no. of AAs	mol wt
1	1	16	16	1420
2	17	41	25	2900
3	42	164	123	14100
4	165	184	20	2400
5	185	230	46	5020
6	231	357	127	14700
7	358	560	204	24000

[a] The numbers represent residues in the primary amino acid sequence of HIV1-RT.

minor radioactive bands (less than 5% of the major band) migrating around 23 000–24 000 daltons which do not correspond to any HIV1-RT CNBr peptide cross-linked to p(dT)$_{15}$. These bands may have appeared due to incomplete cleavage by CNBr. Thus, cross-linking of p(dT)$_{15}$ to HIV1-RT does not appear to be random and seems to be restricted to one or two CNBr peptides.

Identification of the Primer Binding Domain. To identify the domain(s)/peptide(s) of p66 cross-linked to (dT)$_{15}$, 10 nmol of p66 HIV1-RT was cross-linked after equilibrating with 30 nmol of [5′-^{32}P](dT)$_{15}$ in the buffer system described under Materials and Methods. Since approximately 15% of the protein cross-links to the primer, excess un-cross-linked enzyme protein and primer were removed by preparative SDS–PAGE. Comparison of the stained gel with the corresponding autoradiogram revealed that the cross-linked and the un-cross-linked protein separate by about 1 cm (data not shown). The radiolabeled band was excised and the protein recovered as described under Materials and Methods. The p(dT)$_{15}$-labeled tryptic peptides generated by digesting the primer-cross-linked HIV1-RT (see Materials and Methods) were purified by a combination of HPLC on reverse-phase C-18 columns under

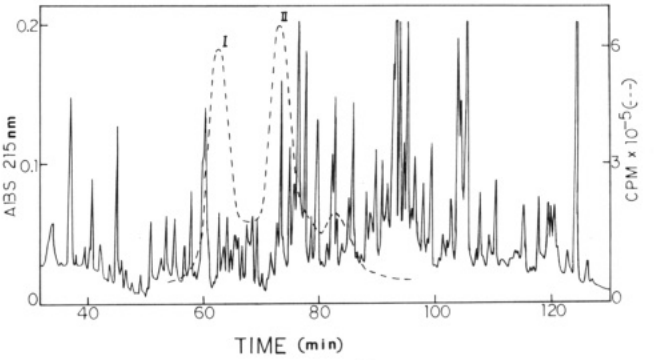

FIGURE 4: Tryptic peptide map of [5′-^{32}P](dT)$_{15}$-labeled HIV1-RT. Tryptic peptides were generated from [5′-^{32}P](dT)$_{15}$-cross-linked HIV1-RT as described under Materials and Methods and fractionated by HPLC on a C-18 reverse-phase column equilibrated with 10 mM sodium phosphate buffer, pH 6.8 (buffer A). Peptides were eluted with increasing concentrations of buffer B (70% acetonitrile in 10 mM sodium phosphate buffer, pH 6.8) as follows: 0–10 min (0% B), 10–110 min (0–40% B), 100–150 min (40–70% B). One-minute fractions were collected. The 215-nm absorbance profile (solid line) of the tryptic peptides and the associated radioactivity (dashed line) are depicted in the figure.

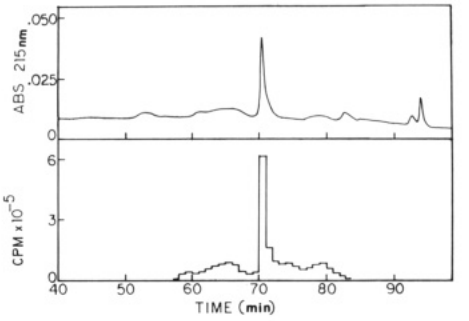

FIGURE 5: Purification of (dT)$_{15}$-cross-linked HIV1-RT tryptic peptide. Radioactive peak II (see Figure 4) was purified on a DEAE-Sephadex column as described under Materials and Methods, and the 400 mM NaCl wash was loaded on a C-18 reverse-phase column equilibrated as described in Figure 4. The elution profile was as follows: 0–10 min (0% B), 10–130 min (0–30% B).

neutral conditions and ion-exchange chromatography on DEAE-Sephadex. The tryptic peptides were first subjected to HPLC on a C-18 reverse-phase column equilibrated with phosphate buffer, pH 6.8 (Figure 4). Two major radioactive peaks eluting at about 65 and 72 min (labeled I and II) were observed. Each peak was separately processed on DEAE-Sephadex followed by C-18 reverse-phase HPLC. Peak I was found to be free p(dT)$_{15}$, as no amino acids were found associated with it as judged by amino acid composition analysis. Peptide(s) in peak II was (were) further purified by DEAE-Sephadex chromatography by batchwise elution with increasing NaCl concentration. The radioactive fractions were directly adsorbed onto a Vydac C-18 column which was further developed by shallow gradients of acetonitrile. A typical HPLC pattern is shown in Figure 5. By the radioactivity associated with the peak, the recovery of the cross-linked peptide at this stage was about 20%, which represents about 2% of the starting protein. Table IV shows the amino acid composition of the purified peptide II, which indicates that the peptide corresponds to amino acid residues 288–307 of the primary amino acid sequence of HIV1-RT (Ratner et al., 1985). This peptide was not merely a contaminant tryptic peptide which happens to coeluate with p(dT)$_{15}$ since free p(dT)$_{15}$ (peak I) elutes at a different time and moreover this peptide elutes along with the radioactivity even when chromatographed on a Vydac C-4 column (data not shown). All

Table IV: Amino Acid Composition of $(dT)_{15}$-Cross-Linked Peptide

amino acid	residues/mol of peptide: found	residues/mol of peptide: expected from sequence 288–307[a]
Cys		
Asp	0.9	1
Thr	2.2	2
Ser	0.3	
Glu	5.6	6
Pro	1.5	1
Gly	0.4	
Ala	2.5	3
Val	0.8	1
Met		
Ile	0.8	1
Leu	4.3	4
Tyr		
Phe		
His	0.3	
Lys		
Arg	1.1	1

[a] The numbers represent residues in the primary amino acid sequence of HIV1-RT.

Table V: Amino Acid Sequence Analysis of $(dT)_{15}$-Cross-Linked Peptide

cycle	amino acid identified[a]: amino acid[c]	amino acid identified[a]: amino acid	amino acid identified[a]: residue[b]
1	Ala	Thr	296
2	Leu	Glu	297
3	Val	Glu	298
4	Ile	Ala	299
5	Pro	Glu	300
6	Leu	Leu	301
7		Glu	302
8		Leu	303
9		Ala	304
10		Glu	305
11		Asn	306
12		Arg	307

[a] Each cycle yielded two distinct amino acid residues. [b] The numbers in this column represent the amino acid residues in the primary amino acid sequence of HIV1-RT. [c] The amino acid sequence in this column did not match with any HIV1-RT tryptic peptide; however, the appearance of these amino acids can be explained by splitting the sequence as follows: (1) Ala-Leu; (2) Thr-Glu-Val-Ile-Pro-Leu, which corresponds to residues 288–289 and 290–295.

the amino acids present in this tryptic peptide were found in the composition analysis, indicating that the amino acid–p-$(dT)_{15}$ adduct is not stable to acid hydrolysis. Instability of the amino acid–$p(dT)_{15}$ adduct to acid hydrolysis is not surprising as it has been observed before for the adduct formed between the tryptic peptide T-8 of *E. coli* SSB protein and $p(dT)_8$ (Merrill et al., 1984).

Approximately 0.4 nmol (as judged by the associated radioactivity) of this peptide was subjected to amino acid sequence analysis. The results of this analysis are shown in Table V. Two sequences based on amino acids found at each cycle could be inferred from these data as shown below:

(I) Ala-Leu-Val-Ile-Pro-Leu

(II) Thr-Glu-Glu-Ala-Glu-Leu-Glu-Leu-Ala-Glu-Asn

The overall composition of the above two sequences matches well with the amino acid composition analysis (Table IV). Sequence I apparently does not not match with any tryptic peptide of HIV1-RT while sequence II represents amino acid residues 296–307 in the primary amino acid sequence of HIV1-RT which is a fragment of a tryptic peptide. A careful examination of the above sequences together with the results obtained from amino acid composition analysis suggested that sequence I may actually represent two sequences which can be written as follows: IA, Ala-Leu; IB, Thr-Glu-Val-Ile-Pro-Leu (the first two residues of this peptide are the same as in sequence II shown above). Thus, by combining sequences IA, IB, and II, an authentic tryptic peptide corresponding to residues Ala(288)-Arg(307) can be generated. These results then suggest that there is a possible break between Leu-(289)-Thr(290) and Leu(295)-Thr(296) as a result of $p(dT)_{15}$ cross-linking.

The above results indicate that the tryptic peptide spanning residues Ala(288)-Asn(306) forms a part of the primer binding domain and that $p(dT)_{15}$ cross-links between Leu(289)-Thr-(290) and Leu(295)-Thr(296).

Discussion

Replicative DNA polymerases, including HIV1-RT, require both template and hydrogen-bonded primer to initiate DNA synthesis. Therefore, in the active-site pocket of HIV1-RT, sufficient space to accommodate both template and primer must exist. In order to identify the putative domain(s) within the enzyme protein that recognize(s) the primer nucleotides, we used $(dT)_{15}$ as a primer, since HIV1-RT utilizes this primer quite efficiently for the synthesis of DNA. Furthermore, in the reaction scheme of HIV1-RT, Wilson and colleagues have shown that primer recognition and binding precede the binding of template and substrate dNTPs (Majumdar et al., 1988). Binding of HIV1-RT and $p(dT)_{15}$ in vitro is quite strong with an apparent equilibrium dissociation constant of about 10^{-7} M, which is consistent with the dissociation constant for primer binding reported by Majumdar et al. (1988). In order to determine the primer binding domain of HIV1-RT, we resorted to photoaffinity labeling using ^{32}P-labeled $(dT)_{15}$ as a primer probe. Results presented in Figure 2 shows some characteristics of the photoaffinity labeling reaction. Under optimal conditions, nearly 15% of the protein can be covalently cross-linked to the primer. The specificity of primer binding (and therefore cross-linking) was demonstrated by the fact that (i) denatured protein does not cross-link to the primer, (ii) addition of other oligomeric primers as well as $tRNA^{Lys}$ but not dNTPs results in a competitive decrease in the cross-linking of $p(dT)_{15}$ to the enzyme. Most importantly, $tRNA^{Lys}$ inhibits the cross-linking of $p(dT)_{15}$ and DNA synthesis on poly-(rA)·$(dT)_{15}$ catalyzed by HIV1-RT, to a similar extent (Figure 2). These results establish that $p(dT)_{15}$ cross-linking to HIV1-RT represents a true primer binding reaction.

The initial efforts to identify the primer binding domain in HIV1-RT utilized CNBr fragmentation of the primer-cross-linked enzyme. Results indicated that only one or two CNBr peptides contained the radiolabeled primer. Tryptic peptide mapping of the primer-cross-linked enzyme revealed the identity of a smaller peptide domain that was present in the N-terminal region of HIV1-RT. Thus, the primer recognition appears to reside within amino acid residues 288–307 in the primary amino acid sequence of HIV1-RT. Amino acid sequence analysis of the primer-cross-linked peptide (see Results) suggested at least two locations in the primer cross-linking reaction, both involving Leu-Thr (residues 289–290 and 295–296). The results, however, do not indicate which of the nucleotides of $(dT)_{15}$ are involved in cross-linking to the protein. Cross-linking of the oligonucleotide to the peptide is most probably occurring within the peptide bond which results in weakening and subsequent cleavage of the involved peptide bonds. It is not clear whether the cleavage occurs before or during Edman degradation. The two cleavage sites would yield three fragments that represent a single tryptic

peptide, cross-linked to $p(dT)_{15}$. The chemistry of the adduct formed between $p(dT)_{15}$ and the peptide is not clear at present. The adduct between $(dT)_{15}$ and the peptide is apparently not stable to Edman degradation as inferred from the fact that (i) all the expected amino acids are obtained in good yields and (ii) more than 90% of the input radioactivity associated with $p(dT)_{15}$ remained bound to the PVDF membrane to which the peptide was bound for sequence analysis. Photo-cross-linking of nucleotides to peptide bonds has been observed before for N_3-dATP cross-linking to *E. coli* pol I (Rush & Konigsberg, 1990); however, in their analysis, such cross-linking appears to interfere with Edman chemistry which is not apparent in our studies.

Recent studies from our laboratory have identified at least three peptides that cross-link to primer $(dT)_8$ in MuLV-RT (Tirumalai & Modak, 1991) and span residues Leu(72)-Arg(80), Gly(602)-Lys(609), and Asp(615)-Lys(622). The first of these peptides lies in the putative DNA polymerase domain while the other two are contained in the RNase H domain of that enzyme (Johnson et al., 1986; Tanese & Goff, 1988; Kotewicz et al., 1988). None of these peptides are in the homologous region of the primer binding peptide of HIV1-RT that we have identified in the present studies. Recent studies on MuLV- and AMV-RTs (Luo & Taylor, 1990; Oyara et al., 1989) have indicated that there is a spatial relationship between the polymerase and the RNAse H active sites such that these domains are separated by one or two helical turns of the RNA/DNA hybrid molecule. It has also been demonstrated that MuLV-RT can be separated into independently active polymerase and RNase H domains which are contained in the N-terminal and C-terminal portions of the enzyme, respectively (Kotewicz et al., 1988; Tanese & Goff, 1988). In HIV1-RT, such distinction does not appear to exist. Removal of the C-terminus from HIV1-RT, which generates the p51 subunit of the enzyme (Starnes et al., 1988; Hizi et al., 1988; Tisdale et al., 1988), as well as insertional mutations in the C-terminus of HIV1-RT (Prasad & Goff, 1989; Hizi et al., 1989) results in loss of the DNA polymerase activity contained in the N-terminus polymerase domain. These reports and our present studies suggest that there are structural differences between the organization of the polymerase domain of HIV1-RT and other viral RTs such as MuLV-RT.

Recent studies by Sarngadharan and colleagues (DeVico et al., 1991) have shown that an antibody raised against a region within the C-terminal end of HIV1-RT blocks DNA polymerase activity and full protection from antibody-mediated inactivation was observed in the presence of template-primer. The authors have suggested that in HIV1-RT, the polymerase and the RNase H domains are folded to form a continuous pocket that serves as a template-primer binding cavity. If this is true, then the fact that the $(dT)_{15}$ primer cross-links only in the N-terminal (polymerase) domain of HIV1-RT and not to the sequences present in the C-terminus (RNase H) region would indicate that both C- and N-terminal regions may be required for the binding of the template strand but not for the primer strand. The exact functional role of the p66 C-terminus with respect to the polymerase activity is not yet clear.

In conclusion, we have shown that $p(dT)_{15}$ specifically binds and cross-links to the kinetically significant primer binding domain of HIV1-RT. The domain represented by the residues Ala(288)-Arg(307) is involved in the primer binding function. Furthermore, the Leu-Thr pair within this sequence appears to provide actual contact sites for the primer nucleotides. Future studies with primers labeled at defined sites (e.g., 3′-OH) would clarify the orientation of the primer and the interacting surface of the protein.

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Interactions of Saturated Diacylglycerols with Phosphatidylcholine Bilayers: A ^{2}H NMR Study†

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ABSTRACT: The interactions of a series of saturated diacylglycerols (DAGs) with fatty acid side chain lengths of 6–14 carbons with multilamellar phospholipid bilayers consisting either of dipalmitoylphosphatidylcholine (DPPC) or of a mixture of DPPC and bovine liver phosphatidylcholine (BL-PC) extracts were studied by ^{2}H NMR spectrometry. We found that the perturbation induced by the DAGs into the bilayer structure strongly depends on the length of the DAG fatty acid side chain. Shorter chain 1,2-*sn*-dihexanoylglycerol and, to a larger degree, 1,2-*sn*-dioctanoylglycerol (diC_8) induce transverse perturbation of the bilayer structure: the order parameters of the phospholipid side chains are increased by the intercalating DAG molecules in the region adjacent to the phospholipid headgroups and decreased toward the terminal methyls, corresponding to the bilayer interior. The longer chain DAGs ($C \geq 12$) studied in this and previous [De Boeck & Zidovetzki (1989) *Biochemistry 28*, 7439] work induce lateral phase separation of the lipids into DAG-enriched gellike domains and relatively DAG-free regions in the liquid-crystalline phase. Each of the DAGs studied induces a decrease in the area per phospholipid molecule, and a corresponding *increase* in the lateral surface pressure of the bilayers. Since numerous biochemical studies consistently report that diC_8 is the most effective of saturated DAGs in activating protein kinase C, we may conclude that the activation of this enzyme is associated with a transverse perturbation of the lipid bilayer structure and a decreased ordering in the interior of the bilayer membrane, and is less affected by the lateral phase separation of the lipids into regions of different fluidities, as induced by the longer chain DAGs. The DAG-induced lateral phase separation may, however, play a role in activating other enzymes, such as pig pancreatic phospholipase A_2.

Diacylglycerols (DAGs)[1] are endogenous second messengers, produced as a result of cell activation by a variety of stimuli [see Berridge (1987) for a review]. Several exogenously added DAGs will stimulate a variety of biological responses. Exogenous (mostly short-chained) DAGs stimulate protein kinase C (PK-C) in C62B glioma cells (Brooks et al., 1982), pituitary HL-60 cells (Ebeling et al., 1985), and human carcinoma A431 cells (Davis et al., 1985a,b). Tumor-promoting activity of 1,2-*sn*-dioctanoylglycerol (diC_8) and 1,2-*sn*-didecanoylglycerol (diC_{10}) on mouse skin was reported by Verma (1988), Smart et al. (1989), and Hansen et al. (1990). Recently, 1,2-*sn*-dihexanoylglycerol (diC_6) and diC_8 were shown to enhance light-induced stomatal opening in plants (Lee & Assmann, 1991). In the studies where the homologous series of saturated short-chained DAGs was employed, the emerging consensus is that the most potent PK-C activator is diC_8 with a somewhat smaller effect of diC_6 and diC_{10}, while shorter or longer chain DAGs are usually ineffective (Mori et al., 1982; Jetten et al., 1985; Ebeling et al., 1985; Cohn et al., 1985; Go et al., 1987; Sekiguchi et al., 1988). The biological effects of short-chain DAGs were reviewed by Abdel-Latif (1986).

A growing number of studies indicate that exogenously added DAGs can stimulate other intracellular enzymes. Goppelt-Strübe et al. (1987) reported that diC_8 inhibits membrane-bound lysophosphatide acyltransferase, probably

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‡ Deceased.

[1] Abbreviations: BL, bovine liver; DAG, diacylglycerol; diC_6, 1,2-*sn*-dihexanoylglycerol; diC_8, 1,2-*sn*-dioctanoylglycerol; diC_{10}, 1,2-*sn*-didecanoylglycerol; diC_{12}, 1,2-*sn*-didodecanoylglycerol; diC_{14}, 1,2-*sn*-ditetradecanoylglycerol; DPPC, dipalmitoylphosphatidylcholine; DPPC-d_{62}, diperdeuteriopalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; PC, phosphatidylcholine; PK-C, protein kinase C.

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