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Localization of a Polynucleotide Binding Region in the HIV-1 Reverse Transcriptase: Implications for Primer Binding†

Robert W. Sobol,‡ Robert J. Suhadolnik,*,‡ Amalendra Kumar,§ Byeong Jae Lee,∥ Dolph L. Hatfield,∥ and Samuel H. Wilson§

Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140, and Laboratories of Biochemistry and of Experimental Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: Properties of primer recognition by purified human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) p66 homodimer have been investigated. Earlier studies had shown that RNA-directed DNA synthesis catalyzed by HIV-1 RT proceeds by an ordered mechanism in which template–primer combines with the free enzyme to form the first complex in the reaction scheme, and it was also shown that primer alone is a competitive inhibitor of template–primer. In this study, enzyme–primer binding has been further characterized utilizing $pd(T)_8$ and $pd(T)_{16}$ as model primers and UV cross-linking to covalently trap the enzyme–primer complexes. Competition experiments with several authentic primers, including $tRNA_3^{Lys}$, indicate that $pd(T)_n$ binds to the kinetically significant primer binding site of RT. Salt reversal experiments suggested that the free energy of $pd(T)_n$ binding to RT has a large nonelectrostatic component. Binding of $pd(T)_n$ to p66-RT is not affected by dNTPs and does not require the presence of template. The site of UV cross-linking of $pd(T)_{16}$ was localized to the NH_2-terminal half of p66 by use of V8 protease hydrolysis and microsequencing. Our results indicate that a polynucleotide binding site is in close proximity to residues in the peptide comprising amino acids 195~300. This region could be either a single-stranded template or single-stranded primer binding site; however, we have documented the specificity of binding with oligonucleotides that act as primer in the in vitro DNA synthesis reaction. Therefore, this $d(T)_{16}$ binding site may be part of a primer-binding groove within the HIV-1 reverse transcriptase.

Human immunodeficiency virus type 1 (HIV-1)[1] is a retrovirus widely considered to be the etiologic agent of acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983; Popovic et al., 1984). As with all retroviruses, an essential feature of HIV-1 replication is reverse transcription of the plus-strand RNA genome into DNA, a process requiring the RNA-dependent DNA polymerase, known as reverse transcriptase (E.C. 2.7.7.7) (Temin & Mizutani, 1970; Baltimore, 1970). Retroviral reverse transcriptases initiate DNA synthesis in vivo from the 3′-hydroxyl of a host cell tRNA annealed to its complementary region, or primer binding site, around 650 residues from the 5′-end of the viral genome (Panet et al., 1975). Previous work by Litvak and co-workers (Litvak & Araya, 1982; Sarih et al., 1988) and Haseltine et al. (1977) demonstrated specific binding of primer $tRNA^{Trp}$ by avian myeloblastosis virus (AMV) reverse transcriptase (RT); the AMV RT holoenzyme also is able to promote positioning of $tRNA^{Trp}$ at the primer binding site of the retroviral RNA genome (Litvak & Araya, 1982; Sarih et al., 1988). A recombinant HIV-1 RT expressed in yeast is able to form a specific complex with bovine $tRNA^{Lys}$, and this was with a higher binding affinity than for the control species $tRNA^{Trp}$ (Sallafranque-Andreola et al., 1989). Additionally, a recombinant HIV-1 RT expressed in *Escherichia coli* specifically binds $tRNA_3^{Lys}$, as demonstrated in Northwestern binding assays and gel mobility shift assays (Barat et al., 1989).

Kinetic studies of HIV-1 RT have indicated that the reaction pathway for DNA synthesis is ordered, with template–primer and free enzyme combining to form the first complex in the reaction sequence (Majumdar et al., 1988; Huber et al., 1989). Through the use of substrate analogues for dNTP and primer, further evidence was obtained for the formation of this template–primer/enzyme complex in the pathway, and it was found that the free enzyme can bind primer in the absence

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* To whom correspondence should be addressed.
‡ Temple University School of Medicine.
§ Laboratory of Biochemistry, NCI, NIH.
∥ Laboratory of Experimental Carcinogenesis, NCI, NIH.

[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; AMV, avian myeloblastosis virus; SSB, ssDNA binding protein from *E. coli*; DTT, dithiothreitol; $Sd(C)_{28}$, 28-residue oligodeoxycytidylate with sulfur substituted at a nonbridge oxygen of each phosphate atom; $d(C)_{19-24}$, mixture of 19–24-residue-long normal oligodeoxycytidylate; $d(T)_8$ and $d(T)_{16}$, oligomers of deoxythymidylate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NP40, Nonidet P-40; hnRNP, heterogenous nuclear ribonucleoprotein; dNTP, deoxynucleoside triphosphate; TNP-ATP, 2′,3′-di-*O*-(2,4,6-trinitrophenyl)adenosine triphosphate; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; MuLV, murine leukemia virus; PVDF, poly(vinylidene difluoride).

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of template (Majumdar et al., 1989).

Recently, the construction of a recombinant *E. coli* expression vector, pRC-RT, containing the precise HIV-1 RT coding region from HXB2 proviral DNA, as well as a method for the overexpression and purification of the HIV-1 RT, was obtained (Becerra et al., 1991). The RT polypeptide of 64 484 Da (p66) is purified as a homodimer and exhibits characteristics similar to that of virion-derived HIV RT. In the present study, utilizing this recombinant HXB2-derived RT (p66), we observed that enzyme–primer complex formation can be examined using UV cross-linking assays. Konigsberg, Williams, Sperling, and their associates (Merrill et al., 1984, 1988; Havron & Sperling, 1977) and Hockensmith et al. (1991, for review) have utilized ^{32}P-labeled oligothymidylic acid probes [i.e., $d(T)_8$] in the analysis of DNA–protein complexes by UV cross-linking. Their studies have shown that covalent, UV-induced cross-linking between $d(T)_n$ and either the *E. coli* SSB protein or A1 hnRNP, for example, is specific and that amino acids at the interface of the $d(T)_n$/protein complex have been identified (Merrill et al., 1984, 1988).

In the present study, complexes between the p66 homodimer form of RT and the primer analogues $d(T)_8$ and $d(T)_{16}$ are cross-linked following irradiation with UV light either by a 5-ns laser pulse or bench-top exposure. UV-induced cross-linking appeared to be a reasonable way of measuring the relative amount of enzyme–primer complex formed under equilibrium conditions. We used this approach to determine the ionic strength sensitivity and selected thermodynamic properties of primer binding. Further, using competition assays with labeled $d(T)_8$ or $d(T)_{10}$ as probe, binding constants for natural primer tRNAs have been determined. These studies suggest that the enzyme binds to its natural primer, $tRNA_3^{Lys}$, but binding is not tighter than to several other nonprimer natural tRNAs. This primer binding assay also has been extended to elucidate a region in the primary structure of HIV-1 RT that is in close proximity to the bound primer analogue [^{32}P]$pd(T)_{16}$ and is cross-linked to the primer by UV irradiation.

Materials and Methods

Materials. 5′OH $d(T)_8$, 5′OH $d(T)_{16}$, ribonucleotides, and deoxyribonucleotides were from Pharmacia; [γ-^{32}P]ATP was from ICN (>7000 Ci/mmol) or Amersham (>5000 Ci/mmol); T4 polynucleotide kinase was from USB; recombinant HIV-1 RT p66 homodimer was purified from *E. coli* as described (Becerra et al., 1991); 200 μg of rabbit liver $tRNA_3^{Lys}$ and a mixture of rabbit liver $tRNA_{1,2}^{Lys}$ were purified as described (Raba et al., 1979); *E. coli* $tRNA_1^{Val}$, $tRNA^{Phe}$, and $tRNA_f^{Met}$ were obtained from Boehringer Mannheim and then purified by gel electrophoresis; TNP-ATP was from Molecular Probes, Inc.; $Sd(C)_{28}$ and $d(C)_{19-24}$ were generous gifts from Drs. Jack S. Cohen and William Egan, respectively.

5′-Phosphorylation of $d(T)_n$. $d(T)_8$ and $d(T)_{16}$ were phosphorylated at the 5′-terminus with T4 polynucleotide kinase (E.C. 2.7.1.78) essentially as described by Maniatis et al. (1982) in a reaction mixture (total volume = 50 μL) containing the following: [γ-^{32}P]ATP (0.5 mCi), $d(T)_8$ or $d(T)_{16}$ (110 μM), and T4 polynucleotide kinase (10 units) in 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, and 5 mM DTT. This reaction mixture was incubated at 37 °C for 1 h, followed by the addition of ATP (final concentration = 0.16 mM) and T4 polynucleotide kinase (10 units) and a second incubation at 37 °C, 30 min. [^{32}P]-5′-pd$(T)_8$ or [^{32}P]-5′-pd$(T)_{16}$ were separated from unreacted ATP by passing the mixture over a Du Pont NENsorb 20 column, following the manufacturer's suggested protocol.

Photochemical Cross-Linking. Typically, 1 μg of p66 reverse transcriptase homodimer (M_r = 129 000) (in 2 μL of 50 mM Tris-HCl, pH 7.0, 75 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% NP40, and 10% glycerol) was mixed with [^{32}P]-5′-pd$(T)_8$ or [^{32}P]-5′-pd$(T)_{16}$ in a buffer containing 20 mM Tris-HCl, pH 8.3, 6 mM $MgCl_2$, and 10 mM DTT, with or without competitor as indicated in the figure legends. The final binding mixture in a volume of 15 μL contained 20 mM Tris-HCl, pH 8.2, 6 mM $MgCl_2$, 10 mM NaCl, 0.2 mM EDTA, 10 mM DTT, 2% glycerol, 0.02% NP-40, 1.25 μM [^{32}P]-5′-pd$(T)_8$ or [^{32}P]-5′-pd$(T)_{16}$, and RT homodimer as indicated. Following a 10-min incubation in 1.5-mL centrifuge tubes at 25 °C, the reaction mixture was spotted on Parafilm and immediately irradiated with 3.75×10^4 ergs/mm^2 in a Stratagene UV Stratalinker (254 nm). The photochemically cross-linked primer/protein complexes were analyzed by 12.5% SDS–PAGE (Laemmli, 1970). The dried gels were subject to autoradiography at −70 °C with X-Omat film (Kodak). Incorporation of [^{32}P]-5′-pd$(T)_8$ or [^{32}P]-5′-pd$(T)_{16}$ into p66-RT was determined by excising the radiolabeled complex from the gel followed by scintillation counting. Data for competition with unlabeled 5′-$d(T)_8$ (or other polynucleotides) were fitted to a theoretical curve for 5′-$d(T)_8$ binding, assuming 1.25 μM to be one-half $K_D^{d(T)_8}$, as follows: no competitor, 100% labeled probe bound; 1.25 μM competitor, 75.8%; 3.75 μM competitor, 50%; 6.25 μM competitor, 37.5%; 11.25 μM competitor, 24.8%. The theoretical curve was based on the assumptions of hyperbolic binding isotherms and the principle of isotope dilution. Results with polynucleotide inhibitors of [^{32}P]-5′-pd$(T)_8$ binding other than $d(T)_8$ could be analyzed by similar curve-fitting (termed reiterative) to obtain binding affinities relative to the affinity of $pd(T)_8$ (2.5 μM). The binding parameter of K_D^I (dissociation constant of inhibitor) also was calculated from the 50% competition values assuming the relationship

$$\begin{array}{ccc} RT + d(T)_8 & \overset{K_D^{d(T)_8}}{\rightleftharpoons} & RT\text{–}d(T)_8 \\ + & & \\ I & & \\ \Big\downarrow\Big\uparrow K_D^I & & \\ RT\text{–}I & & \end{array}$$

and $RT_{total} = RT_{free} + RT\text{-}d(T)_8 + RT\text{-}I$.

At half-maximal saturation observed with probe $d(T)_8$

$$[I]_{0.5} = K_i(1 + d(T)_8/K_d)$$

where $[I]_{0.5}$ is the inhibitor concentration resulting in 50% competition. As described under Results, the concentration of RT_{total} was taken as the concentration of homodimer in the binding mixture (~52 μM); this was calculated from the K_A value for p66 homodimerization of 5.1×10^4 M^{-1}. Although only ~5% of the enzyme would be in the homodimer form under our binding mixture conditions, enzyme/template–primer trapping experiments have shown that all of the RT is active, i.e., capable of template–primer binding and polymerase activity.[2]

Laser Cross-Linking. One microgram or 0.1 μg of p66-RT (as indicated in the figure legend) was mixed with [^{32}P]$pd(T)_{16}$ in a final volume of 15 μL as described above. Following a 10-min incubation in 1.5-mL centrifuge tubes at 25 °C, the reaction mixture was irradiated with a neodymium–yttrium–aluminum–garnet (Nd/YAG) laser (Model DCR-3G; Spec-

[2] W. Beard, and S. H. Wilson, manuscript in preparation, 1991.

tra-Physics, Inc., Mountain View, CA) directly in the centrifuge tube. The Nd/YAG laser used for the studies presented here produces pulsed radiation (5 ns in duration) at 1064 nm, which is converted to 266-nm photons using a combination of nonlinear optical "doubling" crystals, essentially as described elsewhere (Hockensmith et al., 1991). The laser-cross-linked primer–protein complex was analyzed by 12.5% SDS–PAGE (Laemmli, 1970). The dried gels were subject to autoradiography and quantified as described above.

Western Blot Analyses. Photo-cross-linked p66 and subsequent proteolysis products were resolved by SDS–PAGE as described above. The $[^{32}P]pd(T)_{16}$/protein complexes were transferred electrophoretically to nitrocellulose at 30 V (16 h, 4 °C) in a buffer containing 25 mM Tris-HCl (pH 8.5), 20 mM glycine, and 20% methanol by the method of Towbin et al. (1979); the blot was treated with goat whole serum in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) and reacted with monoclonal antibody mAB 19 at a 1:100 dilution for 2 h. Monoclonal antibody mAB 19 is specific for the first 13 amino acids of the NH_2 terminus of HIV-1 RT (Ferris et al., 1990). The nitrocellulose paper was incubated with biotinylated anti-mouse IgG (Cappel Laboratories) at a 1:500 dilution for 90 min and then with an avidin–biotinylated horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories) for 1 h. Color was generated by reaction with 0.3% Bio-Rad color development reagent and H_2O_2.

Hydrolysis of $[^{32}P]pd(T)_{16}$/p66-RT Complex with V8 Protease. $[^{32}P]pd(T)_{16}$ was cross-linked to p66-RT under reaction conditions identical with those described above, utilizing 3×10^4 ergs/mm^2, to generate the $[^{32}P]pd(T)_{16}$/p66-RT complex. Reaction mixtures containing 1 μg of p66-RT (15 μL) were incubated at 0 or 25 °C in the presence of 0.1% SDS and 1 μg of V8 protease in a final volume of 17 μL. Following the indicated time of hydrolysis (Figure 5), the reaction was terminated by the addition of 1 volume of 30% ice-cold TCA. Protein was precipitated by incubation on ice for 60–90 min and isolated by centrifugation at 14000*g* (10 min). The pellet was washed with ice-cold acetone, dried in vacuo (spin-vac), and dissolved in 20 μL of SDS–PAGE sample buffer. Proteins were analyzed by 15% SDS–PAGE. The radiolabeled proteolysis products were identified by autoradiography and quantified as described above.

Protein Microsequencing of $d(T)_{16}$ Cross-Linked Reverse Transcriptase and Related Proteolysis Products. Cross-linked RT was hydrolyzed with V8 protease by incubation in 0.1% SDS as described above and the proteins were separated by prolonged SDS–PAGE: 14 × 32 cm gel, 15% acrylamide; 10 mA, 40 h, 4 °C. The peptides were then electrotransferred to a 0.45-μm PVDF membrane (Immobilon P; Millipore Inc.) in 10 mM CAPS, pH 11.0/10% MeOH. $d(T)_{16}$ cross-linked peptides were identified by autoradiography and Coomassie brilliant blue staining. The stained, ^{32}P-labeled peptides were then subjected to microsequence analysis, kindly provided by Dr. Claude Klee, Laboratory of Biochemistry, NCI, NIH.

Radioactive Measurements. A Beckman LS-100C or Beckman LS-9000 liquid scintillation spectrometer was used for all radioactive measurements (counting efficiency, 99% for ^{32}P).

Results

UV Cross-Linking of p66-Reverse Transcriptase to $[^{32}P]$-5′-pd$(T)_8$ and $[^{32}P]$-5′-pd$(T)_{16}$. Earlier kinetic studies on the DNA polymerase reaction pathway for HIV-1 RT had suggested that there can be complex formation between the free enzyme and the primer (Majumdar et al., 1988, 1989; Huber et al., 1989). In this study, we examined complexes between the purified p66 homodimer of HIV-1 RT (Becerra et al., 1991) and the primer analogues $d(T)_8$ and $d(T)_{16}$ using UV cross-linking of preformed complexes. Cross-linked material was resolved in SDS–polyacrylamide gels; radioactive primer molecules covalently attached to RT were quantified by excising complexes from the gel followed by scintillation counting.

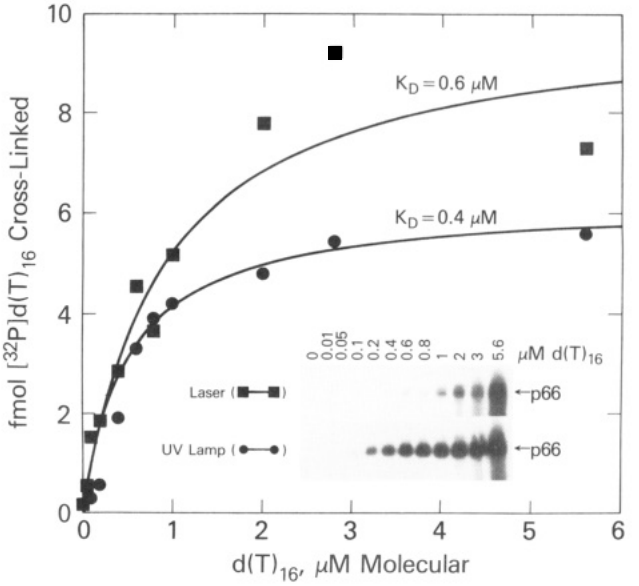

FIGURE 1: Saturation photochemical cross-linking of p66-RT with $[^{32}P]$-5′-pd$(T)_{16}$. The p66-RT reaction mixture, with increasing concentrations of $[^{32}P]$-5′-pd$(T)_{16}$ (3.2 mCi/μmol), was incubated, UV-irradiated by either (A) ~5-ns laser pulse or (B) bench-top exposure, and analyzed by 12.5% SDS–PAGE as described under Materials and Methods. Autoradiograms of the SDS–polyacrylamide gels are shown in the inset, along with the concentration of $[^{32}P]$-5′-pd$(T)_{16}$ in the binding mixtures. The upper autoradiogram was obtained with laser pulse and exposure for 1 h. The lower autoradiogram was obtained with the bench-top UV lamp and exposure for 3 h. Incorporation of $[^{32}P]$-5′-pd$(T)_{16}$ into p66-RT was determined by excising the radiolabeled p66-RT/$d(T)_{16}$ complex from the gel followed by scintillation counting.

As one of the aims in this study was to measure binding between the RT and the model primer $d(T)_n$, we first evaluated the question of whether the binding equilibrium

$$\text{enzyme} + d(T)_n \rightleftarrows \text{enzyme–}d(T)_n$$

is disturbed by the cross-linking procedure itself. One approach was to compare results obtained with a 5-ns pulse of laser-delivered UV light with those obtained with a 10-s pulse on the laboratory bench in a UV Stratalinker. The 5-ns pulse was considered to be fast enough to trap the complex without pulling the above-noted reaction to the right. The results, shown in Figure 1, are similar for the two methods; slightly more cross-linked complex was obtained with the laser-delivered pulse than with the bench-top pulse, however, the saturation curves obtained and the SDS–PAGE patterns of cross-linked material were almost identical and showed that labeled p66 was the predominant product. On the basis of these results, we chose to use a bench-top-delivered pulse for subsequent experiments. We found also that preexposure of the enzyme to a UV light pulse, by either method shown in Figure 1, had no effect on the subsequent binding activity of the enzyme. Therefore, the UV pulse under these conditions did not alter the binding activity of the enzyme.

To examine RT binding to other polynucleotides by a competition method, we chose to use $d(T)_8$ as the labeled probe. Cross-linking of the enzyme–$d(T)_8$ complex is illus-

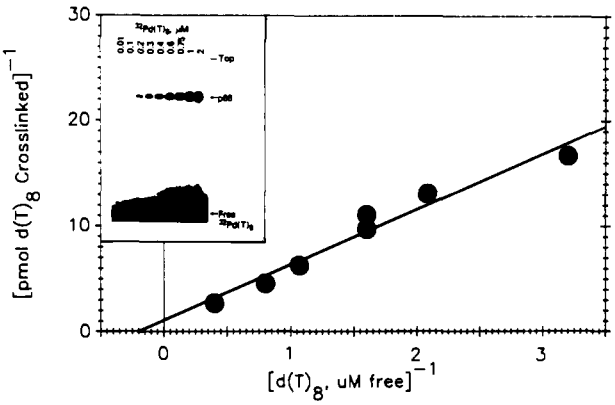

FIGURE 2: Saturation photochemical cross-linking of p66-RT with [^{32}P]-5′-pd(T)$_8$. The p66-RT reaction mixture, with increasing concentrations of [^{32}]-5′-pd(T)$_8$ (2.3 mCi/μmol), was incubated, UV-irradiated, and analyzed by 12.5% SDS–PAGE. A double-reciprocal plot is shown. An autoradiogram of the SDS–polyacrylamide gel is shown in the insert. Samples were electrophoresed on 12.5% SDS–polyacrylamide gels, the gels were dried, and the labeled p66 was visualized by autoradiography. Incorporation of [^{32}P]-5′-pd(T)$_8$ into p66-RT was quantified as described for Figure 1.

trated in the experiment shown in Figure 2. Binding and cross-linking resulted in a ^{32}P-labeled ~66 000 M_r species, but very little higher M_r or lower M_r material was observed. The amount of cross-linked 66 000 M_r material increased with concentration of d(T)$_8$ in a fashion similar to that seen for d(T)$_{16}$, except that the apparent K_D was ~2.5 μM instead of ~0.5 μM. Maximal cross-linking, obtained from extrapolation of a linear double-reciprocal plot of the data, correspond to ~5% of the 66 000 M_r polypeptide in the binding mixture.

Some properties and requirements for d(T)$_8$–RT complex formation were examined. No binding was observed with substitution of RT with BSA. The binding reactions in this study contain 6 mM $MgCl_2$, as is used for enzymatic activity measurements; however, primer–RT complex formation did not require Mg^{2+}, consistent with previous results on RT–primer–template complex formation (Huber et al., 1989). d(T)$_8$–RT complex formation was not altered by added nucleotides (ATP) or deoxynucleotides (dATP, dGTP, dCTP, dTTP) at concentrations as high as 100 μM (data not shown), suggesting that the d(T)$_8$ is probably not binding at the dNTP binding site. TNP-ATP, a fluorescent ATP derivative, which has been found to bind to the primer binding site of HIV-1 RT (LeGrice, 1990), inhibited complex formation (data not shown), and this was consistent with the interaction of d(T)$_n$ at the primer binding site of the RT. d(T)$_{16}$ was able to fully compete labeled d(T)$_8$.

Inhibition of p66-RT/Primer Complex Formation. As expected, d(T)$_8$, containing a 5′-phosphate group, was an effective inhibitor of complex formation by ^{32}P-labeled d(T)$_8$, whereas 5′-hydroxyl d(T)$_8$ was not as effective (Table I). Stronger binding for a 5′-phosphorylated d(T)$_8$ is not surprising with such a short oligonucleotide and is reminiscent of results with nucleic acid binding proteins, gene 5 protein of fd phage, and T4 gene 32 protein (O'Conner & Coleman, 1983; Karpel, 1990). The excess of unlabeled 5′-phosphorylated d(T)$_8$ required to reduce complex formation with labeled d(T)$_8$ probe was in good agreement with predictions from a theoretical second-order binding curve. Finally, the presence of poly[r(A)] in the binding mixture did not stimulate or grossly reduce binding, although in this experiment we may not have been able to detect tighter enzyme binding to this template–primer complex if the affinity of binding had been much higher than to primer alone. The specificity of d(T)$_n$ binding to RT was

Table I: Requirements for d(T)$_n$–RT Complex Formation

condition	pmol of d(T)$_n$ cross-linked[a]
complete	0.25 (100%)[c]
+ 1.25 μM 5′-pd(T)$_8$ (75.8%)[b]	0.2 (80%)[c]
+ 6.25 μM 5′-pd(T)$_8$ (37.5%)[b]	0.11 (44%)[c]
+ 12.5 μM 5′-pd(T)$_8$ (23.5%)[b]	0.06 (24%)[c]
+ 1.25 μM 5′-OH-d(T)$_8$	0.25
+ 6.25 μM 5′-OH-d(T)$_8$	0.25
+ 12.5 μM 5′-OH-d(T)$_8$	0.2
+ r(A)$_n$ (100 nm)	0.2
+ r(A)$_n$ (10 μM)	0.1

[a] With 1 μg (15 pmol) of p66-RT and 1.25 μM [^{32}P]d(T)$_8$. [b] Theoretical values for ^{32}P-labeled probe bound, assuming probe is at a concentration equal to one-half K_D. [c] Percent of complete cross-linking (0.25 pmol).

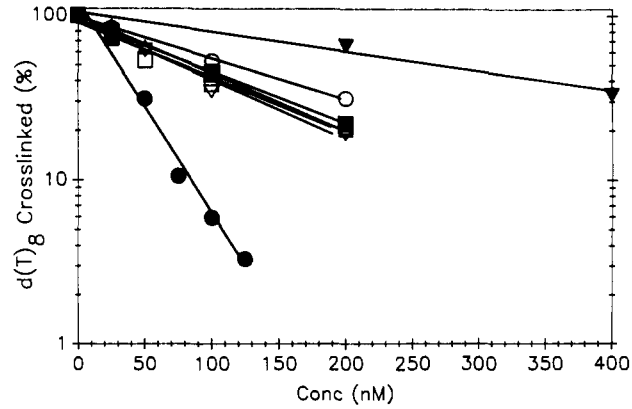

FIGURE 3: Oligonucleotide competition for the primer binding site of p66-RT. The affinity for binding to the primer binding site on p66-RT was determined in photochemical cross-linking competition assays under equilibrium binding conditions. Reaction mixtures contained p66-RT and [^{32}P]-5′-pd(T)$_8$ (as described under Materials and Methods) with Sd(C)$_{28}$ (●), d(C)$_{19-24}$ (▼), tRNA$_3^{Lys}$ (■), tRNA$_{1,2}^{Lys}$ (○), tRNA$_1^{Val}$ (□), tRNA^{Phe} (△), or tRNA$_f^{Met}$ (▽). d(T)$_8$/p66-RT complex formation was quantified as described for Figure 1.

further approached through use of two oligodeoxycytidylates that had been characterized earlier. These molecules, d(C)$_{19-24}$ and Sd(C)$_{28}$, are competitive inhibitors of polymerase activity vs the template–primer poly[r(A)]–poly[d(T)] and they also are functional primer substrates with poly[r(I)] as template. Thus, these molecules bind to the kinetically significant primer binding site on the enzyme. We evaluated the question of whether d(C)$_{19-24}$ or Sd(C)$_{28}$ could act as competitive inhibitors of d(T)$_8$ cross-linking, as expected if d(T)$_8$ binds at the primer site of the enzyme. The results, shown in Figure 3, indicate that both d(C)$_{19-24}$ and Sd(C)$_{28}$ block d(T)$_8$ cross-linking and this inhibition occurs in a competitive fashion as shown by the linear or near-linear semilog plot in Figure 3.

The reduction of labeled probe binding by unlabeled probe provides a baseline for comparative studies of binding by other polynucleotides using the competition approach. In finding a mathematical model (described under Materials and Methods) to analyze the competition results, we first considered the question of the actual concentration of primer binding activity in the binding mixture. The concentration of total p66 in the mixture was 1040 nM (monomer). Yet, from the 50% competition value observed with the very tight-binding oligonucleotide Sd(C)$_{28}$ ([I]$_{0.5}$ = 26 nM), it was clear that half of the binding activity could be sequestered by roughly 30 nM oligonucleotide. Hence, the binding activity clearly was in the range of 60 nM, rather than the 1040 nm p66 added to the mixture. We, therefore, considered that the active binding species was the homodimer form of p66 and used the known K_A for p66 dimerization, 5.1 × 10^4 M^{-1} (Becerra et al., 1991),

Table II: Summary of HIV-1 RT Binding Revealed By Competition Experiments

competitor	concn[a] required for 50% competition (M)	K_D[b] (M)
5′-pd(T)$_8$		2.5×10^{-6}
5′-pd(T)$_{16}$		0.5×10^{-6}
5′-OH-d(C)$_{19-24}$	200×10^{-9}	98×10^{-9}
tRNA$_3^{Lys}$	80×10^{-9}	30×10^{-9}
tRNA$_{1,2}^{Lys}$	120×10^{-9}	52×10^{-9}
tRNA$_1^{Val}$	80×10^{-9}	30×10^{-9}
tRNA^{Phe}	80×10^{-9}	30×10^{-9}
tRNA$_f^{Met}$	80×10^{-9}	30×10^{-9}

[a] [^{32}P]d(T)$_8$ probe is at 1.25 μM equal to approximately one-half K_D. [b] Values for 5′-pd(T)$_8$ and 5′-pd(T)$_{16}$ were measured directly, as in Figures 1 and 2. Other values were calculated by the competition equation described under Materials and Methods.

to calculate the amount of dimer present in the binding mixture. Thus, 1040 nM total p66 corresponded to a concentration of ~52 nM p66 homodimer, and the K_D values for the various tRNAs and oligonucleotides shown in Figure 3 were calculated on the basis of this 52 nM value (Table II). The K_D values obtained by this method were similar to estimates obtained by an empirical curve-fitting method (described under Materials and Methods).

Specificity of primer binding was further evaluated with purified rabbit liver tRNA$_3^{Lys}$, as well as reference species: rabbit liver tRNA$_{1,2}^{Lys}$ and *E. coli* tRNA$_1^{Val}$, tRNA^{Phe}, and tRNA$_f^{Met}$. Eighteen residues of tRNA$_3^{Lys}$ are complementary to the primer binding site in the HIV-1 5′-LTR, and tRNA$_3^{Lys}$ is thought to be the natural primer for HIV RT (Wain-Hobsin et al., 1985). Our results (Figure 3) indicate that all types of natural tRNA tested here were equally potent inhibitors of d(T)$_8$ probe binding; the tRNAs exhibited K_D values in the range of 30–52 nM. These results confirm that RT has the capacity to bind natural tRNAs. However, this enzyme did not appear to have the capacity to distinguish tRNA$_3^{Lys}$ from the reference tRNAs used here on the basis of primer binding alone.

Reverse Transcriptase—Ionic and Thermodynamic Properties of Primer Binding. The ionic strength requirement for binding between d(T)$_8$ and RT was evaluated (Figure 4). Binding was sensitive to the presence of NaCl in the binding mixture. Inhibition of complex formation follows a simple exponential pattern when plotted against ionic strength (Figure 4, inset A). From the slope of this plot, it is deduced that only one or two charge–charge interactions are involved in d(T)$_8$ complex formation with RT. Analysis of these data by the log K_A vs log [NaCl] plot method described by Record et al. (1976) revealed that binding is substantial (6×10^4 M^{-1}) when the salt concentration is extrapolated to the reference state of 1 M NaCl (Figure 4, inset B) and that the slope of the plot (−0.8) is consistent with the formation of only one ion pair. Therefore it appears from these experiments that a large portion (80%) of the free energy of complex formation between d(T)$_8$ and RT under our usual binding conditions is supplied by nonelectrostatic interactions. d(T)$_{16}$–RT complex formation exhibited similar NaCl sensitivity plots (data not shown).

Localization of the Primer Binding Site. Conditions were obtained for controlled proteolytic degradation of the native and/or d(T)$_{16}$ cross-linked RT. An identical proteolysis pattern was obtained with or without d(T)$_{16}$ cross-linking or UV irradiation (not shown). V8 protease at 1:1 weight ratio with total RT gives well-controlled degradation to domain peptides, leading first to a ≈40-kDa labeled peptide (^{32}P-p40) and then to a ≈16-kDa labeled peptide (^{32}P-p16); degradation was controlled by temperature and time of the V8 protease

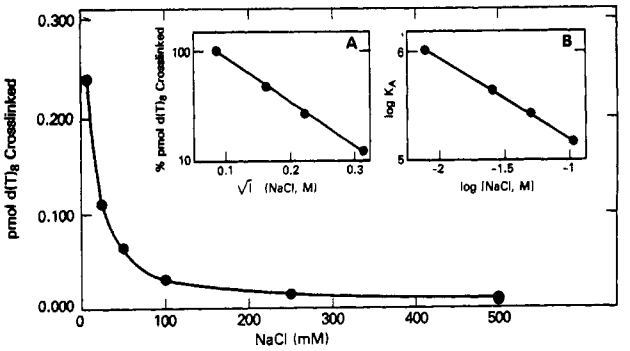

FIGURE 4: Ionic strength requirement for d(T)$_8$/p66-RT complex formation. For the characterization of the ionic strength requirement for complex formation, p66/d(T)$_8$ reaction mixtures were incubated with increasing concentrations of NaCl prior to photochemical cross-linking. Inset A: Replot of the picomoles of d(T)$_8$ cross-linked as a function of the square root of the ionic strength of the reaction buffer. The activity coefficient for NaCl concentration–ionic strength was taken as 1:1 in the concentration range shown. Inset B: Log K_A vs log [NaCl] analysis, according to Record et al. (1976). The ratio of bound and free d(T)$_8$ was used to calculate K_A at each salt concentration; stoichiometry between homodimer RT and d(T)$_8$ was taken as 1:1. Values plotted represent the average of three determinations. Quantification of cross-linked p66-RT/d(T)$_8$ complexes was conducted as described for Figure 1.

digestion (Figure 5). Coomassie blue staining of the corresponding gels (not shown) revealed a small amount of cross-linked peptide coincident with the p40 and p16 label and a much larger amount of peptide that was smaller by about 4 kDa, i.e., 36000 and 12000 Da, respectively. This suggested that the presence of the labeled d(T)$_{16}$ conferred slower migration of the cross-linked peptide as expected. For the p40 or p16 labeled peptides, the recovery of radioactive material originally present in p66 was ≥80% (Figure 5).

Localization of the labeled p40 and p16 peptides within the 560 amino acid sequence of p66 was investigated by direct protein microsequencing and by use of an NH$_2$-terminal region specific monoclonal antibody, mAB 19 (Ferris et al., 1990). Western blot analysis of cross-linked and un-cross-linked samples of RT was conducted after V8 protease digestion to produce p40 or p16 (Figure 6). Radiolabel in p40 precisely comigrated with a relatively minor dye-stained peptide that appeared just above the major dye-stained peptide of M_r = 36000. These two peptides, which differ by the mobility change expected for a d(T)$_{16}$ adduct, are designated 1 and 2, respectively, in Figure 6. They probably correspond to the same peptide sequence, as peptide 1 was not observed in the un-cross-linked samples. Peptides 1 and 2 contain the NH$_2$-terminal region of p66, since they are positive with monoclonal antibody mAB 19 and direct sequencing after transfer to PVDF membrane indicated that peptide 1 starts with proline 1 (Figure 6). The ratio of dye-stained material for peptides 1 and 2 is consistent with the extent of cross-linking expected for this experiment (10%).

Analysis of products from the ^{32}P-p16 production sample is shown in Figure 6, lanes 5 and 6. The radiolabeled material did not correspond to a mAB 19 positive band, indicating that ^{32}P-p16 is not from the extreme NH$_2$-terminal region of p66. Direct sequencing of the ^{32}P-p16 peptide is summarized in Figure 6. The membrane slice contained two sequences in roughly equal amounts, one beginning with proline 1 and the other with isoleucine 195. A major Coomassie blue stained peptide migrating at ~12000 M_r (p12) also started with isoleucine 195. We were able to resolve the two peptides comigrating at ~16000 M_r by prolonged SDS–PAGE as described under Materials and Methods: R_f values for ^{32}P-p16,

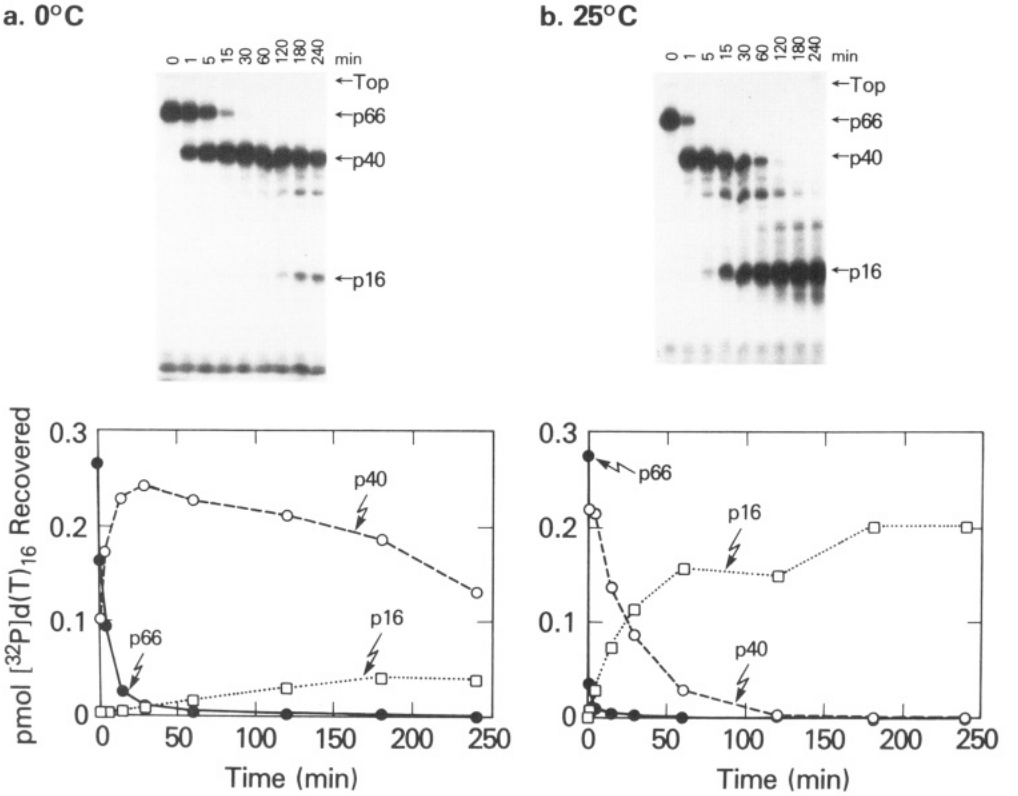

FIGURE 5: V8 protease hydrolysis of [^{32}P]-5′-pd(T)$_{16}$ cross-linked p66-RT. The d(T)$_{16}$ cross-linked RT was hydrolyzed with V8 protease (1:1 w/w) at 0 °C (A) or 25 °C (B) for 0 to 240 min, as indicated. Hydrolysis products were analyzed on 15% SDS–PAGE as described under Materials and Methods. Autoradiograms of SDS–polyacrylamide gels are shown. The picomoles of [^{32}P]-5′-pd(T)$_{16}$ cross-linked to intact p66 (0 min) and recovered following V8 proteolysis (as indicated) was determined by excising the radiolabeled complexes from the gel followed by scintillation counting. Quantification of the recovered [^{32}P]d(T)$_{16}$ is indicated in the plots below the corresponding autoradiograms.

p15, and p12 were 0.56, 0.62, and 0.94, respectively. The labeled peptide started with I 195, whereas a peptide migrating slightly faster, which was devoid of label, started with P 1. We conclude from these results that the peptide containing cross-linked d(T)$_n$ begins with I 195 and extends approximately 100 residues to approximately amino acid 300.

Discussion

Previous studies have shown that HIV-1 RT catalyzes RNA-directed DNA synthesis via an ordered mechanism in which template–primer binds to the enzyme prior to the first deoxynucleotide triphosphate. It was also found that free primer such as oligo[d(C)] could bind to the enzyme in the absence of template. In the present study, we have utilized d(T)$_8$ and d(T)$_{16}$ as model primers to characterize enzyme–primer complex formation and to study the specificity of primer recognition. d(T)$_8$ binds to p66-RT with a $K_D \approx 2.5$ μM in 10 mM NaCl, which corresponds to a binding free energy of about −8 kcal/mol. d(T)$_8$–RT complex formation is not interrupted by ribo- or deoxyribonucleotides (not shown) and does not require Mg^{2+} (Table I). Complex formation is sensitive to NaCl (Figure 4), yet our data indicate that the majority (≈80%) of the binding free energy is contributed by nonelectrostatic forces. In competition assays, the previously characterized primer analogues d(T)$_{16}$, Sd(C)$_{28}$, and d(C)$_{19-24}$ blocked d(T)$_8$–RT complex formation, indicating that d(T)$_8$ specifically binds to the kinetically significant primer binding site of the RT. Using this same approach, we determined that the binding constant, K_D^I, for the natural primer tRNA$_3^{Lys}$ is approximately 30 nM. This value is in line with the inhibition obtained kinetically with poly[r(A)]–d(T)$_{15}$ as template–primer. Yet, binding to several reference natural tRNAs was with a similar K_D^I as binding to tRNA$_3^{Lys}$.

Earlier studies had shown that photochemical cross-linking can be highly specific, such that only those residues in the vicinity of the oligonucleotide binding pocket are covalently linked (Merrill et al., 1984; Havron & Sperling, 1977). In this study, we have utilized the specificity of photochemical cross-linking of oligonucleotide–protein complexes in an analysis of HIV-1 RT p66 homodimer–d(T)$_8$ complex formation. In the analysis of the enzyme–primer complex, an overall second-order binding relationship was observed that can be formulated as follows:

$$\mathrm{RT} + \mathrm{d(T)_8} \overset{K}{\rightleftharpoons} [\mathrm{RT\text{–}d(T)_8}] \overset{k}{\longrightarrow} \mathrm{RT\text{–}d(T)_8}$$

where K is the dissociation constant for the complex, k is the UV cross-linking constant, and RT–d(T)$_8$ is the amount of cross-linked complex. Where Max is the RT–d(T)$_8$ formed when RT is completely complexed, the following equation can be derived:

$$\frac{1}{\mathrm{RT\text{–}d(T)_8}} = \frac{1}{\mathrm{Max}} + \frac{k}{\mathrm{Max}}\frac{1}{\mathrm{d(T)_8}}$$

This equation is applicable when the complex formation step is allowed to come to equilibrium before UV cross-linking is conducted and the equilibrium is not substantially disturbed due to cross-linking. This supposition is substantiated by a comparison of UV cross-linking via laser-delivered UV pulse (~5 ns) and bench-top exposure to a UV lamp (10 s), in which similar K_D values for d(T)$_{16}$ were obtained. Equilibrium binding conditions were evaluated to obtain photochemical cross-linking between d(T)$_8$ and p66 in a buffer system that would support RNA-directed DNA synthesis upon addition of template and dNTPs (Majumdar et al., 1989). Under these conditions, maximal photochemical cross-linking, in the absence of any noticeable photoinduced proteolysis, was achieved

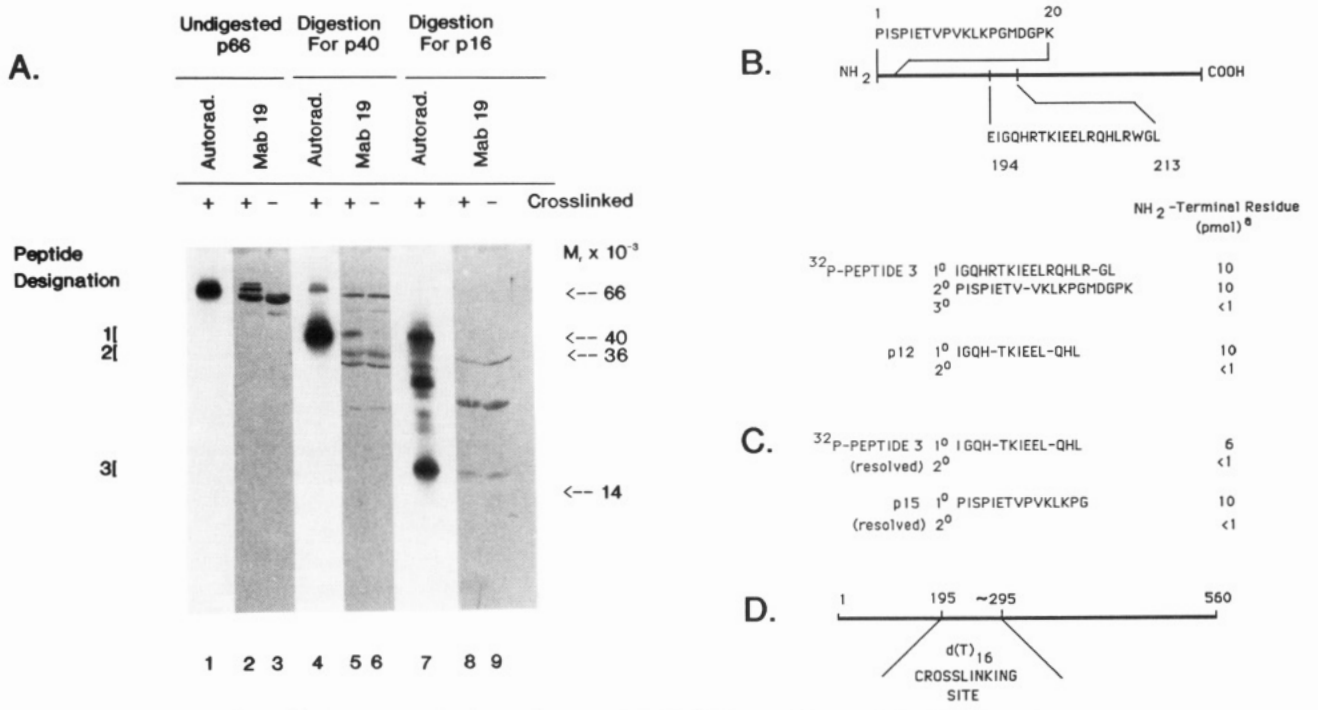

FIGURE 6: Localization of the $[^{32}P]pd(T)_{16}$ binding site of HIV-1 RT. (A) Western blot analysis of native and $[^{32}P]$-5′-$pd(T)_{16}$ cross-linked p66-RT. The native or $d(T)_{16}$ cross-linked RT was hydrolyzed with V8 protease (1:1 w/w) for 0 min (lanes 1–3), 5 min (lanes 4–6), or 120 min (lanes 7–9), resolved on 15% SDS–PAGE, and transferred electrophoretically to nitrocellulose as described under Materials and Methods. Autoradiograms (lanes 1, 4, and 7) and photographs of the immunoblots (lanes, 2, 3, 5, 6, 8, and 9) are shown. Identification of the NH_2-terminal region of p66-RT was determined by Western blot analysis with a 1:100 dilution of mAB 19, a monoclonal antibody specific for the first 13 amino acids of RT (Ferris et al., 1990). (B) Amino-terminal sequence analysis of the $[^{32}P]pd(T)_{16}$ conjugated peptide. The diagram shown at the top is a schematic representation of the HIV-1 RT primary structure, indicating the amino acid sequence beginning at residues 1 and 194. $[^{32}P]pd(T)_{16}$ cross-linked RT was hydrolyzed with V8 protease and separated by SDS–PAGE as described under Materials and Methods. The amino-terminal sequence of the ^{32}P-labeled peptide 3 (and the contaminating peptide, p15) and peptide p12 are shown. [Peptides 3 and p12 were isolated and sequenced in separate experiments.] [a]The picomoles of the NH_2-terminal residues obtained from automated Edman degradation refers to the amount of signal detected in the first cycle of sequence analysis; the last cycle corresponds to ≤1 pmol. Cycles that produced no known amino acid signal or no signal increase over the previous cycle are designated as –. (C) Summary of amino-terminal sequence analysis of the resolved $[^{32}P]pd(T)_{16}$-labeled peptide 3 and peptide p15. $[^{32}P]pd(T)_{16}$ cross-linked RT was hydrolyzed with V8 protease and separated by SDS–PAGE as described under Materials and Methods. The $[^{32}P]pd(T)_{16}$-labeled peptide 3 and contaminating peptide p15 were resolved by prolonged SDS–PAGE as described under Materials and Methods. The amino acid sequence analysis was as described above. (D) Schematic representation of HIV-1 RT primary structure. The indicated segment, spanning amino acids 195 to ~295–300, represents the region of the primary structure of HIV-1 RT that is cross-linked to $[^{32}P]pd(T)_{16}$ by UV irradiation.

at 3.75×10^4 ergs/mm^2. Further irradiation resulted in a net decrease in covalently bound RT–$d(T)_8$ formation, primarily as a result of photoinduced proteolysis (data not shown). As mentioned above and shown in Figure 2, $d(T)_8$ binding to p66-RT is saturable, with a K_D of ≈2.5 μM, whereas $d(T)_{16}$ exhibited a K_D value of ≈0.5 μM (Figure 1). As observed by Merrill et al. (1984) for another protein system, an increase in length of the oligonucleotide probe results in an increase in the percentage of protein molecules cross-linked.

Our results suggest that $d(T)_8$ binding is specific for the primer binding site. Competition experiments clearly indicate that $d(T)_8$–RT complex formation is not affected by added dNTPs at 100 μM. However, the modified nucleotide TNP-ATP does inhibit $d(T)_8$ p66-RT complex formation (not shown). This was expected since recent evidence indicates that TNP-ATP binds to the primer binding site of HIV-1 RT.[3] In addition, the primer analogues $Sd(C)_{28}$ and $d(C)_{19-24}$ exhibit inhibition constants similar to those determined kinetically (Majumdar et al., 1989).

The results showing binding between the enzyme and primer [$tRNA_3^{Lys}$, $Sd(C)_{28}$, and $d(C)_{19-24}$] are in line with a minimal reaction pathway for DNA synthesis proposed earlier from kinetic studies, in that free enzyme is able to bind primer in the absence of dNTP and template (Majumdar et al., 1989). The K_D values for primer binding observed here are in the range of those obtained by the kinetic approach. It is interesting to note the free energy of $tRNA_3^{Lys}$ binding in 10 mM NaCl and 6 mM $MgCl_2$ at pH 8.2 is equal to about −11.1 kcal/mol. This value is somewhat higher than the free energy of binding usually observed with sequence-nonspecific nucleic acid binding proteins, which is generally on the order of −9.5 kcal/mol (Williams & Chase, 1990). An improved understanding of this relatively high affinity protein–nucleic acid interaction between RT and $tRNA_3^{Lys}$ may lead to an approach for identifying a class of specific inhibitors of the enzyme. LeGrice and Darlix and their co-workers have implicated the anticodon loop of $tRNA_3^{Lys}$ in the interaction (Barat et al., 1989; LeGrice, 1990).

The ionic requirement for the interaction between $d(T)_8$ and RT was examined by conducting the binding reaction in the presence of increasing concentrations of NaCl. Although binding is inhibited by NaCl, a plot of the data according to Record et al. (1976) revealed a modest effect of increasing ionic strength on K_A. Thus, the relationship between log K_A and log [NaCl] was described by a linear plot with slope equal to about −1. This value is equal to $-m'\Psi$, where m' is the number of ion pairs formed between the protein and $d(T)_8$ and Ψ is the number of counterions bound per $d(T)_8$ phosphate (i.e., 0.88 in our calculations). Therefore, the protein forms only one ion pair with $d(T)_8$ binding, which is the overall binding free energy of −8.2 kcal/mol. The nonelectrostatic

[3] M. Delahunty, R. L. Karpel, and S. H. Wilson, manuscript in preparation, 1991.

contribution to this binding free energy was estimated by extrapolating the NaCl titration data to the reference state of 1 M NaCl. The nonelectrostatic contribution appears to be approximately 80% or −6.5 kcal/mol, consistent with several high-energy contacts between the primer and RT on the order of 1–2 kcal/mol each. Similar conclusions on the role of nonelectrostatic interactions have been obtained by Painter and co-workers (Painter & Furman, 1990), who used an intrinsic protein fluorescence based assay to measure binding.

Recent kinetic studies investigating primer recognition of HIV-1 RT have indicated that this enzyme is processive and proceeds via a stepwise, ordered mechanism (Majumdar et al., 1988, 1989). Utilizing the high-affinity primer analogue $Sd(C)_{28}$, it has been shown that the free enzyme recognizes both template–primer and primer alone (Majumdar et al., 1989). The natural primer for RNA-directed DNA synthesis is a specific tRNA which hybridizes to the primer binding site within the viral genome (Taylor, 1977). As such, a mechanistic model for reverse transcription has been developed (Gilboa et al., 1979). Subsequent evidence has indicated that some but not all of these RNA-dependent DNA polymerases specifically recognize their cognate tRNA primers. Avian myeloblastosis virus RT is found to be associated with its cognate primer $tRNA^{Trp}$ in virions and in vitro; the holoenzyme specifically binds $tRNA^{Trp}$ in the absence of template AMV RNA (Hazeltine et al., 1977; Panet & Berliner, 1978). However, MuLV RT does not specifically recognize its cognate primer $tRNA^{Pro}$, in that a specific MuLV-RT/$tRNA^{Pro}$ complex could not be identified (Hazeltine et al., 1977; Panet & Berliner, 1978). More recently, Litvak and co-workers and Darlix and co-workers have investigated tRNA recognition by recombinant HIV-1 RT expressed in yeast and in *E. coli*, respectively (Sallafranque-Andreola et al., 1989; Barat et al., 1989). Using glycerol gradient centrifugation to identify enzyme–primer complex formation, Sallafranque-Andreola et al. (1989) have shown that HIV-1 RT forms a complex with $tRNA^{Lys}$ (a mixture of Lys subspecies 1, 2, and 3) but not $tRNA^{Trp}$. However, complex formation with other tRNAs was not investigated. Subsequently, Barat et al. (1989) have investigated HIV-1 RT primer recognition utilizing a modified Northwestern-type blotting technique. Using this nonequilibrium binding method, their results suggest that HIV-1 RT only recognizes $tRNA_3^{Lys}$ but not $tRNA^{Trp}$ or $tRNA^{Pro}$. These two reports on specific recognition of free $tRNA_3^{Lys}$ are in contrast to the findings reported here but involved different reference tRNAs and different enzyme preparations. We are continuing these studies to examine possibilities of tRNA specific binding by RT.

The high efficiency of cross-linking of $d(T)_{16}$ at saturation (≈20%) and the specificity of $d(T)_n$ for binding to the kinetically significant primer binding site on HIV-RT prompted an extension of these studies to the localization of this polynucleotide binding site within the primary sequence of p66-RT. Controlled proteolysis of $d(T)_{16}$ cross-linked p66 with V8 protease resulted in the initial formation of a p40 complex, followed by a highly stable p16 complex, as determined by SDS–PAGE. The stability of RT to further proteolysis is not surprising. Larder and co-workers have reported that a p30 tryptic fragment of RT is stable to hydrolysis for several hours (Lowe et al., 1988). The localization of the bound $d(T)_{16}$ or polynucleotide binding site of p66 was determined by a combination of Western blot and protein microsequencing analyses. Western blot analysis clearly indicates that the p40 complex contains the amino-terminal region of RT. The p40 complex comprises both DNA [^{32}P-pd$(T)_{16}$; ≈4 kDa] and peptide (≈36 000 Da). Further, microsequence analysis of p40 (peptide 1) and a major Coomassie blue stained band of ≈36 000 Da (peptide 2) both had an N-terminal amino acid sequence of PISPIET, identical to that of the parent p66-RT (Figure 6). These data therefore, initially limit the primer binding site to amino acids 1∼312. This initial localization of the primer binding site to the N-terminal region of p66 is consistent with that reported by several groups, in which the DNA polymerase function of HIV-RT was demonstrated to be confined to the N-terminal region (amino acids 1–440) while the RNase H domain resides in the C-terminal region (Johnson et al., 1986; Larder et al., 1987; Lowe et al., 1988; Hizi et al., 1988; Prasad & Goff, 1989).

Continued V8 proteolysis of p40 results in a stable p16 complex. The generation of p16 is proportional to the hydrolysis of p40, suggestive of a precursor (p40)–product (p16) relationship. However, further analysis indicated that ^{32}P-p16 (peptide 3, Figure 6) is not recognized by mAb 19, suggesting that the p16 complex no longer contains the amino-terminal region. The results presented herein establish that the p16 fragment corresponds to amino acids 195∼300 of the primary sequence of HIV-1 RT, as determined by NH_2-terminal amino acid sequence of the p16 complex. These findings on localization of the primer binding site region are supported by several observations from other laboratories. Mutations within the NH_2-terminal region of RT, generated by the insertion of 5–28 random amino acids, eliminated polymerase activity (Prasad & Goff, 1989). Active-site analysis with the substrate dNTP-binding-site-directed reagent PLP has implicated Lys-263 in the dNTP binding domain (Basu et al., 1989). Whereas HIV-RT polymerase activity is sensitive to PLP, inhibition of PLP binding requires both template–primer and dNTP, suggesting a close relationship between the dNTP and template–primer binding site domains (Basu et al., 1989). Functional analysis of sequence-specific mutations and computer analysis of the HIV-1 RT amino acid sequence has led to the identification of a conserved Asp-Asp motif, common to many RNA polymerases (Kamer & Argos, 1984; Johnson et al., 1986; Larder et al., 1987). This Asp-Asp motif has been suggested to be involved in template binding (Kamer & Argos, 1984). However, Larder and co-workers have recently found that mutations within this Asp-Asp motif do not affect template–primer binding (Lowe et al., 1991), which supports the findings reported here since the Asp-Asp motif is outside the $d(T)_{16}$ cross-linking site or polynucleotide binding region of amino acids 195∼300 (Figure 6).

In summary, $d(T)_n$ specifically binds to HIV-1 RT p66 homodimer at the kinetically significant primer binding site and was used for the investigation of enzyme–primer complex formation and to characterize the specificity of primer recognition under equilibrium conditions. Primer binding involves as little as one charge–charge interaction, with the majority of the binding energy contributed by nonelectrostatic forces. As reported for the MuLV enzyme (Haseltine et al., 1977), the HIV-1 RT, under equilibrium binding conditions, does not specifically recognize $tRNA_3^{Lys}$. Finally, the [^{32}P]-5′-pd$(T)_{16}$ cross-linked polynucleotide binding site was localized to a protease domain fragment from the center of the RT primary structure, comprising an intact subdomain of ≈100 amino acids beginning at isoleucine 195. Strictly speaking, our results indicate that a polynucleotide binding site is in close proximity to residues in the peptide comprising amino acids 195∼300. This region could be either a single-stranded template or a single-stranded primer binding site. However, we have documented the specificity of binding with oligonucleotides that

act as primer in the in vitro DNA synthesis reaction, implicating this $d(T)_{16}$ binding site as part of a primer-binding groove within the HIV-1 reverse transcriptase.

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