Articles

Identification of the Nucleotide Binding Site of HIV-1 Reverse Transcriptase Using dTTP as a Photoaffinity Label

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ABSTRACT: We have utilized UV-induced cross-linking of [*methyl*-^{3}H]dTTP to identify the nucleotide binding site on heterodimeric HIV-1 reverse transcriptase (RT). RT was derivatized by irradiating a solution containing [*methyl*-^{3}H]dTTP and purified recombinant RT for 10 min. The UV-induced cross-linking reaction between dTTP and RT is linear with time of UV exposure up to 10 min, and it has been determined previously that dTTP cross-linking is half-maximal at 90 μM [Cheng, N., Painter, G. R., & Furmann, P. A. (1991) *Biochem. Biophys. Res. Commun. 174*, 785–789]. Under these reaction conditions, only the 66-kDa subunit of the 66-kDa/51-kDa RT heterodimer was labeled with dTTP. The [*methyl*-^{3}H]dTTP-labeled RT was fragmented with trypsin and endoproteinase Asp-N, and peptides were purified on reversed phase HPLC. The peptide covalently linked to [*methyl*-^{3}H]dTTP was subjected to amino acid sequence analysis. The sequencing data localized the nucleotide binding site of RT to Lys-73 in the vicinity of several mutation sites linked to antiviral drug resistance. Since most effective anti-AIDS compounds are inhibitors of RT, information about its dNTP binding site may make it possible to understand the basis for the antiviral activity of nucleoside analogs such as AZT, ddI, and ddC. This information may also be useful for a more rationally based design of anti-HIV agents.

Reverse transcriptase (RT) (E.C. 2.7.7.49) is an enzyme of central importance in the replication of human immunodeficiency virus type 1 (HIV-1),[1] converting the viral RNA genome to double-standed DNA (Varmus & Swanstrom, 1985). Because HIV-1 is the etiological agent of AIDS, RT has been the focus of intense investigation.

HIV-1 RT is a bifunctional enzyme; it has a DNA polymerase activity that utilizes either RNA or DNA as the template and a RNase H activity (Varmus, 1988). The active enzyme purified from the virions consists of two asymmetric subunits—one of 66 kDa (p66) and one of 51 kDa (p51) (Hoffman et al., 1985; DiMarzo et al., 1988). The p66 subunit contains both the polymerase and the RNase H activities. The p51 subunit, derived from a carboxyl terminal truncation of the p66 subunit, contains the polymerase domain, but lacks the RNase H domain (Hansen et al., 1987; Hizi et al., 1988; LeGrice et al., 1991; Lowe et al., 1988). A bireactant–biproduct mechanism has been proposed for DNA synthesis catalyzed by RT (Majundarm et al., 1988). In this mechanism binding of the reactants is ordered, with template–primer binding first followed by the binding of the 2′-deoxynucleoside 5′-triphosphate having Watson–Crick complementarity to the next appropriate template residue. The resulting ternary complex undergoes a conformational change to form the catalytically competent complex. The 5′-monophosphate of the bound nucleotide is then incorporated into the nascent chain by an S_N2-type reaction (Hopkins et al., 1989).

To date, the most effective anti-HIV compounds are inhibitors of RT. These drugs, such as AZT, ddI, and ddC, are nucleoside analogs that function as chain terminators when incorporated by the RT into the nascent viral DNA (Mitsuya et al., 1990; St. Calir et al., 1987). Therefore, an understanding of the topography of the RT nucleotide binding site is relevant to the design of new drugs that might help to control AIDS. The crystal structure of RT has been reported at 7.0- and 3.5-Å resolution (Arnold et al., 1992; Kohlstaedt et al., 1992); however, electron density maps are not yet detailed enough to give a precise location of the dNTP binding pocket of RT. Attempts to identify the binding site have employed chemical modification techniques. Treatment of RT with pyridoxal phosphate followed by reduction with sodium borohydride results in the derivatization of Lys-263 of RT and a drastic decrease in polymerase activity. It has also been observed that pretreatment of the enzyme–template–primer complex with dNTP blocks the incorporation of pyridoxal phosphate. These observations suggest that Lys-263 is directly involved with dNTP binding (Basu et al., 1989). However site-specific mutagenesis experiments that alter Lys-263 to either Ile or Ser do not affect either the K_m value for dTTP or the K_i value for AZTTP (Martin et al., 1993).

Another approach for investigating the dNTP binding domain is photochemical cross-linking. Upon UV irradiation, many amino acids have been shown to form covalent adducts with nucleic acids (Williams & Konigsberg, 1991) and nucleotides (Modak & Gillerman-Cox, 1982). Consequently, site-specific photochemical cross-linking to proteins has been used to identify segments of primary sequence involved in nucleotide binding (Williams & Konigsberg, 1991). Here

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography.

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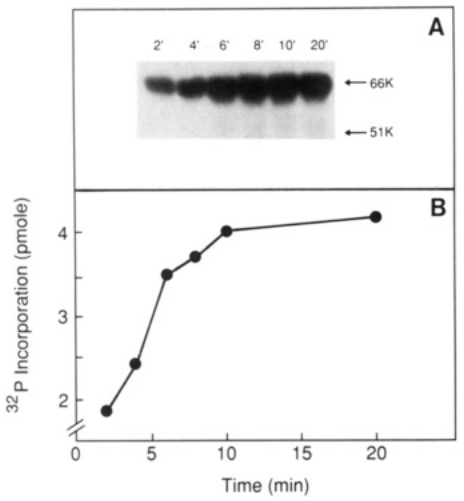

FIGURE 1: Incorporation of [α-^{32}P]dTTP into RT as a function of UV irradiation time. (A) Samples of RT were irradiated for up to 20 min. The RT concentration was 50 μg/mL, and that of [α-^{32}P]-dTTP was 100 μM. Cross-linking reactions were performed as described in the Materials and Methods. Following the reaction, the samples were analyzed by SDS–PAGE and the bands corresponding to the enzyme were cut out and ^{32}P quantitated by Cerenkov counting. (B) The picomoles of ^{32}P bound to the enzyme was plotted vs the time of UV irradiation.

we report the use of [*methyl*-^{3}H]dTTP as a photoaffinity label for cross-linking to the dNTP binding domain of HIV-1 RT. Following the proteolysis of the labeled RT, the peptide containing the dTTP was isolated and sequenced. The result indicates Lys-73 as the residue that is cross-linked to dTTP. This information in conjunction with the resistance profile of anti-HIV nucleoside analogs identifies a portion of the primary sequence involved in substrate recognition and binding.

MATERIALS AND METHODS

Materials. Trypsin was purchased from Worthington (Freehold, NJ), endoproteinase Asp-N was from Boehringer Mannheim (Indianapolis, IN), and dTTP was purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). [*methyl*-^{3}H]dTTP (81 Ci/mmol) and [α-^{32}P]dTTP (3000 Ci/mmol) were obtained from Du Pont-New England Nuclear (Wilmington, DE). All other reagents were anlytical grade or better. Highly purified HIV-1 RT from an overproducing *Escherichia coli* clone was a generous gift from Dr. Paul Ray of the Division of Molecular Genetics and Microbiology, Burroughs Wellcome Co.

SDS–PAGE Analysis of [a-^{32}P]dTTP-Cross-Linked RT. A standard 100-μL reaction mixture containing 5 μg of purified RT, 5 mM $MgCl_2$, 1 mM DTT, 200 μM dTTP, 5 μCi of [α-^{32}P]dTTP, and 50 mM HEPES (pH 7.0) was placed on the cover of a 96-well microtiter plate. A 254-nm UV lamp (model UVGL-35, UVP) was used to irradiate the preparation for various amounts of time, as indicated in Figure 1. The reaction mixtures were kept on ice, 8 cm from the UV lamp. Following irradiation, 50 μL of the samples was analyzed on 7.5% SDS–PAGE (Laemmli, 1970). The extent of [α-^{32}P]-dTTP incorporation was measured by Cerenkov counting of RT bands cut out of the gel.

Preparative-Scale [methyl-^{3}H]dTTP Cross-Linking to RT. A 2-mL cross-linking reaction containing 50 mM HEPES (pH 7.0), 0.9 mg of purified RT, 200 μM dTTP, 150 μCi of [*methyl*-^{3}H]dTTP, 5 mM $MgCl_2$, and 1 mM DTT was cross-linked as described above. After the reaction, the sample was desalted on a NAP-5 column (Pharmacia) and lyophilized. The lyophilized, cross-linked protein was incubated in 0.2 mL of solubilization buffer (0.4 M ammonium bicarbonate, 8 M urea, and 4.5 mM DTT) at 55 °C for 15 min. Free cysteines in the protein were alkylated by incubating with 10 mM iodoacetamide at room temperature for 20 min. The sample was diluted with 0.8 mL of water and digested for 24 h at 37 °C with 22.5 μg of trypsin (equal to 2.5% of the weight of RT).

Generation of RT Endoproteinase Asp-N Peptides. The 2-mL [*methyl*-^{3}H]dTTP-cross-linked RT solution was lyophilized, redissolved in 0.2 mL of 0.4 M ammonium bicarbonate and 4 M urea, and then reduced and alkylated as described above. The protein solution was diluted 4-fold with water to reduce the urea concentration to 1 M before the addition of 16 μg of endoproteinase Asp-N protease (equal to 1.7% of the weight of RT). The proteolysis was carried out at 37 °C for 18 h.

Purification of [methyl-^{3}H]dTTP-Cross-Linked RT Peptides. Digests of RT were applied to a Vydac C-18 column (4.6 × 250 mm, The Separations Group) equilibrated in 98% buffer A (20 mM sodium phosphate, pH 6.5) and 2% buffer B (30% buffer A, 70% acetonitrile). Peptides were eluted from the column at a flow rate of 0.5 mL/min using Model 126 Beckman pumps with the following gradient: 0–60 min, 2037.5% buffer B; 60–90 min, 37.75% buffer B; 90–105 min, 75–98% buffer B. Ultraviolet absorbance was monitored at 220 nm with a Beckman Model 166 UV detector. Fractions were collected at 1-min intervals with a Gilson Model 203 fraction collector. Aliquots (5 μL) of each fraction were monitored for ^{3}H in a Packard CA 1900 scintillation counter using 5 mL of Readysafe (Beckman).

The ^{3}H-associated fractions from the C-18 column were further purified by chromatography using a Brownlee RP300 C-8 column (2.1 × 100 mm, Applied Biosystems, Inc.). Prior to rechromatography, fractions were concentrated by vacuum centrifugation to 0.1 mL and then diluted with 1 volume of 8 M urea. The column was eluted at 0.25 mL/min with the following gradient using the same sodium phosphate/acetonitrile buffers as described above: 0–5 min, 5% buffer B; 5–65 min, 5–40% buffer B; 65–80 min, 40–98% buffer B. UV-absorbing materials were fractionated using a Gilson 230 fraction collector with slope detection. Those fractions containing radioactivity were subjected either to amino acid sequence analysis or to further purification.

Fractions to be further purified were concentrated, urea was added to 4M, and the solution was applied to a 2.1 × 250 mm Vydac C-18 column that had been equilibrated in 98% buffer C (0.065% trifluoroacetic acid) and 2% buffer D (0.056% TFA, 80% acetonitrile). The column was eluted as follows: 0–63 min, 2–37% buffer D; 63–95 min, 37–75% buffer D; 95–105 min, 75–98% buffer D. Fractions from this third chromatographic step were collected by UV peak area and were then applied to a Model 477A ABI sequencer connected to an on-line Model 120A PTH–amino acid analyzer.

RESULTS

The results of the UV-induced cross-linking reaction with [α-^{32}P]dTTP and HIV-1 RT are shown in Figure 1. The extent of cross-linking is linearly dependent on the time of irradiation up to 10 min (Figure 1B). It has been demonstrated in previous experiments that cross-linking is half-maximal at 90 μM dTTP (Cheng et al., 1991). Figure 1A shows dTTP incorporation occurs exclusively to the p66 subunit of the heterodimeric RT.

[α-^{32}P]dTTP cross-linked to RT was found to be unstable under the acidic conditions used during HPLC purification,

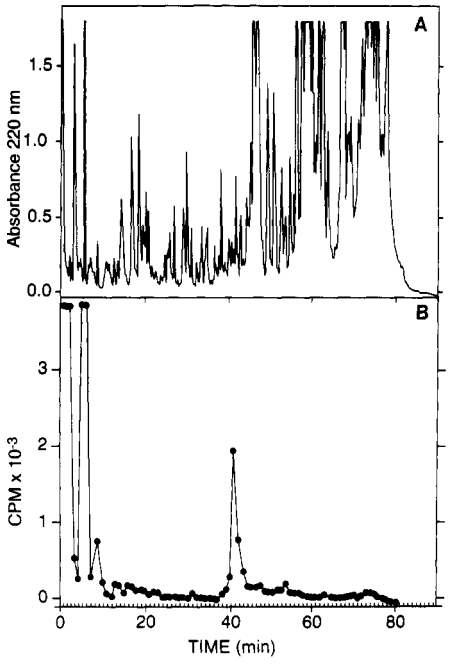

FIGURE 2: Reversed-phase HPLC chromatogram of a total tryptic digest of [*methyl*-^{3}H]dTTP-labeled RT on a C-18 column. The UV absorbance of the effluent was monitored at 220 nm (A). The radioactivity profile was obtained by monitoring aliquots (5 μL) from each fraction which were collected at 1-min intervals. One radioactive peak was detected with a retention time of 41 min (B). The radioactive peaks eluting before 10 min were unincorporated nucleotide.

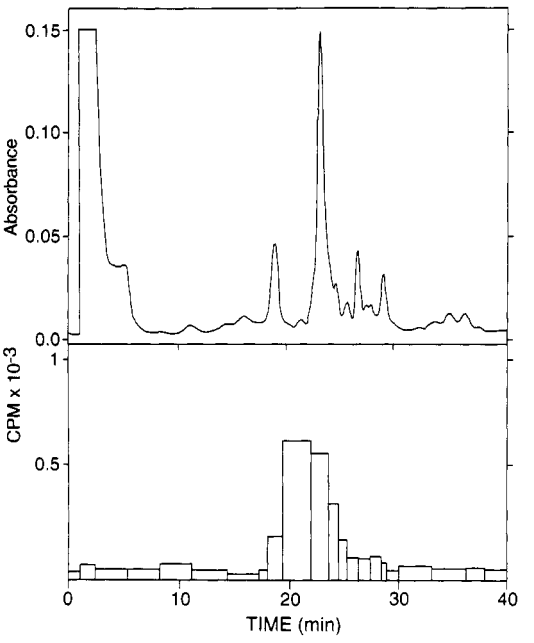

FIGURE 3: Chromatography of partially purified dTTP-cross-linked RT on a C-8 column. Radioactive fractions from Figure 2 were pooled and purified on a C-8 column (A). One radioactive peak was detected at 21 min (B). Fractions were collected on the basis of UV peak absorbance; 5 μL from each fraction was monitored in the scintillation counter to generate the profile of radioactivity.

presumably due to lability of the glycosidic bond of the dTTP. Since the nucleobase is thought to be directly cross-linked to the protein, it was necessary to use the dTTP labeled on the nucleobase ([*methyl*-^{3}H]dTTP). In this case, the cleavage of the glycosidic bond would not result in loss of the radiolabel.

Figure 2 shows a C-18 HPLC chromatogram of radioactivity in a tryptic digest of cross-linked RT. Although the UV profile of this chromatogram was complex (Figure 2A), only one peptide-associated peak of radioactivity was observed, eluting at 41 min (Figure 2B). Radioactive fractions from this C-18 column were pooled and rechromatographed using a C-8 column. The UV profile of this second chromatogram (Figure 3A) was simpler than that shown in Figure 2A. Again, a single radioactive peak was observed, eluting this time at 21 min (Figure 3B). The tritium-containing fractions were pooled and chromatographed a third time using a C-18 column equilibrated with TFA buffer. Radioactivity was eluted at 42 min (data not shown).

The single radioactive peak eluting from this last column was used for amino acid sequencing, which revealed that it consisted of a single peptide with the following amino acid sequence: X-Leu-Val-Asp-Phe-(Arg) (Table I). A gap, denoted by X, occurred in the first cycle of sequencing. When this peptide was compared to the primary sequence of HIV-1 RT (HXB 2), the gap was found to correspond to Lys-73. Since no other gaps were found in the sequencing cycles, it was concluded that Lys-73 was the residue cross-linked to dTTP. Additional data supporting this hypothesis come from the observation that nearly all of the radioactivity in the peptide was released during the first sequencing cycle, in accord with the radioactive label being associated with the first amino acid in the peptide.

Further confirmation of Lys-73 as the site of cross-linking was obtained using a different protease, endoprotease Asp-N, to give a peptide fragment that overlapped with the tryptic fragment. On the basis of the amino acid sequence of RT and

Table I: Amino Acid Sequencing of Tryptic and Endoproteinase Asp-N Peptides

	tryptic peptide			Asp-N peptide		
cycle	amino acid	cpm	residue no.	amino acid	cpm	residue no.
1	X[a]	7065	73	Asp	22	67
2	Leu	2426	74	Ser	35	68
3	Val	1583	75	Thr	377	69
4	Asp	1402	76	Lys	679	70
5	Phe	1013	77	X[b]	843	71
6	(Arg)	389	78	Arg	2815	72
7				X[a]	10307	73
8				Leu	3276	74
9				Val	1234	75

[a] X corresponds to Lys-73. [b] Cycle 5 corresponds to Trp-71. Trp is not identifiable at this level in our sequencer.

the substrate specificity of endoprotease Asp-N, a peptide would be generated with Asp-67 at the amino terminus in which Lys-73 would be the seventh residue. Figure 4A shows the UV profile of a C-18 HPLC chromatogram of the Asp-N digest. The Asp-N digest was complex (Figure 4A), but again only one major peptide-associated radioactivity peak was observed, eluting at 45 min (Figure 4B). The radioactive peaks eluting before 10 min were unincorporated nucleotide. The labeled peptide peak was isolated and further purified by HPLC before being subjected to amino acid sequence analysis. The nine amino acids of this labeled Asp-N peptide, shown in Table I, indicate that it has Asp-67 at its amino terminus, as predicted. A gap at the seventh cycle was observed, coincidental with release of radioactivity (Table I).

To assess the specificity of cross-linking at Lys-73, the concentration of [*methyl*-^{3}H]dTTP in the cross-linking reaction mixture was reduced to 5 μM from 200 μM. The cross-linked RT was treated with trypsin and the reaction mixture purified using the same three HPLC columns as described earlier. Again, a single radioactivity-containing peak was isolated with retention times identical to those of the peptide obtained from cross-linking reactions conducted with 200 μM

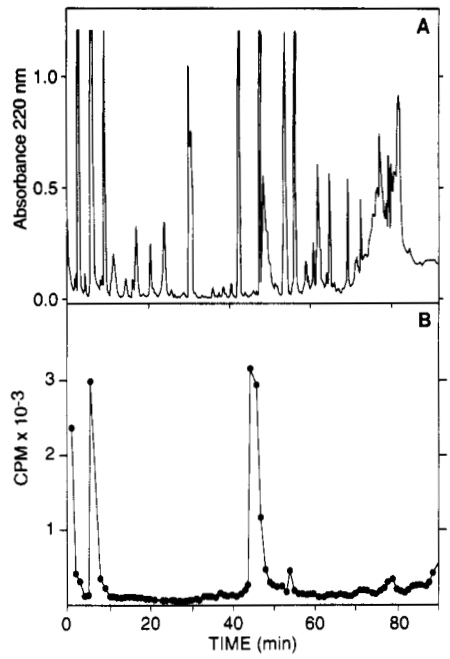

FIGURE 4: Reversed-phase HPLC chromatogram of an endoproteinase Asp-N total digestion of [*methyl*-^{3}H]dTTP-labeled RT. The peptide map is complex (A), but only one major radioactive peptide peak was detected with a retention time of 45 min (B). The radioactive profile was obtained by the same method as in Figure 2. The radioactive peaks eluting before 10 min were unincorporated nucleotide.

[*methyl*-^{3}H]dTTP (data not shown).

DISCUSSION

Nucleotides and their analogs have been used extensively as affinity labels to identify the amino acid residues of polymerases and kinases that are involved in substrate binding (Haley, 1975; Welsh & Cantor, 1984; Evans et al., 1986). Some of the substituted nucleotides, such as those containing 8-azidopurines, are very reactive, and at low UV exposure these analogs are incorporated into the proteins with high efficiency. The underivatized natural nucleotides are much less reactive and are therefore less efficient cross-linkers. However, despite their relative inefficiency, the use of natural nucleotides may be more reliable for identifying residues at the active site since side reactions associated with highly reactive derivatized nucleotide analogs are minimized, and the true dNTP binding site is labeled (Pandey & Modak, 1988).

Upon UV irradiation, the natural RT ligand, dTTP, cross-linked exclusively to the p66 subunit of heterodimeric RT, implying that the p51 subunit lacks a functional nucleotide binding site. This is consistent with findings that the p51 subunit has no polymerase activity in *in situ* gel assays (Hansen et al., 1988; Starnes et al., 1988) and that only one nucleotide binding site per RT dimer was found in Scatchard analysis of fluorescent binding data (Painter et al., 1991). A recently published X-ray crystal structure of HIV-1 RT has shown that, unlike the p66 subunit, the p51 subunit has no catalytic cleft due to the different arrangement of the subdomains that make up the polymerase. As a consequence, the reactive site-(s) is not accessible to dTTP.

Within the p66 subunit, dTTP appears to cross-link to a single site, Lys-73. The evidence is as follows. First, only a single radioactive peptide was isolated when RT was digested by either trypsin or endoproteinase Asp-N. Second, only one gap was observed during amino acid sequencing of each peptide. This gap is thought to be due to the derivatization of Lys-73 by dTTP, which would alter its chromatographic properties so that it did not coelute with any of the known amino acids; the derivitized amino acid is probably Lys because the sequence of the HXB 2 RT gene indicates that a Lys occupies position 73. Third, the gap was associated with [*methyl*-^{3}H]dTTP, since most of the radioactivity in the peptide was released at this cycle of the Edman degradation. We also believe that the cross-linking reaction was specific, since no difference was detected between the elution profiles of radioactive peptide generated from cross-linking reactions having either 5 or 200 μM [*methyl*-^{3}H]dTTP.

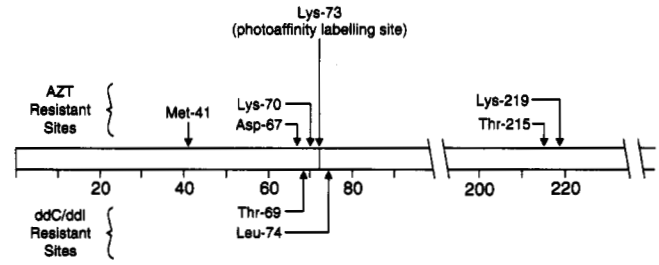

FIGURE 5: dNTP binding site of HIV-1 RT in relation to nucleotide analog resistance sites. Mutations conferring resistance to AZT, ddI, and ddC are indicated, together with Lys-73, in the schematic.

There are additional data on HIV RT that are consistent with Lys-73 being a part of or near the dNTP binding site. First, HIV mutants resistant to the nucleoside analogs AZT, ddI, and ddC have amino acid changes that cluster in two regions, one of which, from Met-41 to Leu-74, encompasses Lys-73. The second cluster of mutants is distal to Lys-73 and consists of Thr-215 and Lys-219 (Figure 5). On the basis of the X-ray crystallographic structure of HIV-1 RT, Kohlstaedt et al. propose that the residues in the clusters play a role in the binding of RT to template–primer (Kohlstaedt et al., 1992). In this model, the p66 subunit of RT is anatomically analogous to a right hand, containing a finger subdomain, a palm subdomain, a thumb subdomain, a connection subdomain, and the RNase H domain. The four subdomains line up, forming a prominent cleft in the polymerase. The cluster of mutants near Lys-73 is in the finger subdomain. The second cluster of mutants is located in the palm domain. The 3′ end of the primer strand, where the next dNTP is to be added, is toward the index finger and the thumb. Lys-73, near the region where the fingers join the palm, is well situated to be a part of the dNTP binding pocket.

Additional structural studies of HIV RT are underway to try to establish a more detailed picture of the topography of the dNTP binding site. A better description of the dNTP binding site of RT, in conjunction with similar information on host-cell polymerases, may make it possible to understand the basis for the selective antiviral activity of nucleoside analogs such as AZT and ddI and to use this information for a more rationally based design of anti-HIV agents.

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