Nucleotide-Induced Stable Complex Formation by HIV-1 Reverse Transcriptase†

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ABSTRACT: Nondenaturing gel electrophoresis was used to study the nucleotide substrate-induced conformational change in reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1). Dead-end complex was formed between HIV-1 RT, dideoxynucleotide chain-terminated primer, and DNA template in the presence of deoxynucleotide triphosphate (dNTP) complementary to the next position on the template. Complexes which form in the absence of the next complementary dNTP were disrupted by adding excess poly(rA)/oligo(dT) or heparin just prior to electrophoresis. Dead-end complex formation by noncomplementary dNTP's or ribonucleotides was at least 2000-fold less efficient than with the complementary nucleotide. When dA was the next nucleotide on the template, analogues of dTTP supported dead-end complex formation with increased apparent K_d (dTTP < dideoxy-TTP ≈ α-thio-dTTP < dUTP < 3′-azidothymidine triphosphate). A similar relationship was observed for dGTP analogues across from dC on the template (dGTP < dideoxy-GTP < α-thio-dGTP << dITP < dideoxy-ITP). The optimal length of the primer/template duplex region for dead-end complex formation was between 20 and 32 base pairs. Primer-template with a mismatched primer terminus did not support dead-end complex formation, and primer terminated with 3′-azidothymidine formed dead-end complex with 25-fold elevated apparent K_d. By contrast, dead-end complex formation on primer terminated with dideoxy-IMP base paired with dC on the template was more efficient than on primer terminated with dideoxy-GMP. Implications for the mechanisms of discrimination between nucleotide analogues by HIV-1 RT are discussed.

DNA synthesis by the reverse transcriptase (RT)[1] of human immunodeficiency virus type 1 (HIV-1) is distinguished by low fidelity and an increased frequency of extension of mismatched primer termini (Takeuchi et al., 1988; Preston et al., 1988; Roberts et al., 1988; Weber & Grosse, 1989; Bebenek et al., 1989; Perrino et al., 1989; Yu & Goodman, 1992; Zinnen et al., 1994). HIV-1 RT also incorporates chain-terminating nucleotide analogues more readily than cellular DNA polymerases, suggesting that the enzyme can tolerate structural variability at the 3′ position of the nucleotide substrate (Furman et al., 1986; St. Clair et al., 1987; Matthes et al., 1987; Cheng et al., 1987; Huang et al., 1990; Chen et al., 1993). This feature has made the enzyme a target for inhibition by nucleoside analogues.

A strictly ordered reaction sequence for DNA synthesis by HIV-1 RT has been proposed which includes a two-step binding mechanism for nucleotide triphosphate substrates (dNTPs) (Majumdar et al., 1988; Kedar et al., 1990; Reardon & Miller, 1990; Kati et al., 1992; Reardon, 1992, 1993; Hsieh et al., 1993; Rittinger et al., 1995) and is given by the equation:

$$E + D_n \rightleftarrows E{\cdot}D_n + N \rightleftarrows E{\cdot}D_n{\cdot}N \rightleftarrows E'{\cdot}D_n{\cdot}N \rightleftarrows E{\cdot}D_{n+1} + PP_i$$

where E = enzyme, D_n = template/primer (T/P), N = dNTP, E′ = enzyme after conformational change, D_{n+1} = T/P after extension by one base, and PP_i = inorganic pyrophosphate. The dNTP binding depends on correct base pairing between the incoming nucleotide and the next position on the template. The dNTP-induced conformational change in the enzyme−T/P complex is rate-limiting for nucleotide incorporation and provides a second level of accuracy in nucleotide selection preceding phosphodiester bond formation (Mizrahi et al., 1985; Kuchta et al., 1987; Wong et al., 1991; Kati et al., 1992; Johnson, 1992, 1993; Hsieh et al., 1993; Spence et al., 1995; Rittinger et al., 1995). Evidence for the conformational change is mostly indirect. The structural changes that occur in the complex, the mechanism by which they are induced, and the importance of structural features of the nucleotide substrate and the primer terminus for induction of the conformational change in HIV-1 RT are yet to be determined. The conformational change can be studied under conditions where chain extension is prevented. HIV-1 RT complexed with primer/template in which the primer

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[1] Abbreviations: α[S]dNTP, α-thiodeoxynucleoside triphosphate; AZT, 3′-azido-2′,3′-dideoxythymidine; AZTTP, AZT triphosphate; DEC, HIV-1 RT/DNA/dNTP dead-end complex; dNTP, deoxynucleoside triphosphate; ddNTP, dideoxynucleoside triphosphate; fDNA, free duplex DNA; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; T/P, template/primer; Tris, tris(hydroxymethyl)aminomethane.

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lacks a 3′-hydroxyl group (chain-terminated primer) binds the next complementary dNTP and forms a dead-end complex (DEC) that can be identified by its slow rate of dissociation (Müller et al., 1991; Kati et al., 1992; Rittinger et al., 1995). Stable dNTP-dependent enzyme−T/P complexes have been demonstrated for other DNA polymerases including mammalian DNA polymerase α (Fisher & Korn, 1981), herpes simplex virus DNA polymerase (Reardon & Spector, 1989), and *Thermus aquaticus* DNA polymerase (Brandis et al., 1996).

We have taken advantage of the unique stability of the DEC to develop a specific electrophoretic mobility shift assay to measure the formation of this complex in order to investigate the mechanisms of phosphodiester bond formation and error discrimination by HIV-1 RT. Complexes which form in the absence of dNTP are unstable in the presence of competing T/P or heparin, whereas addition of the next correct dNTP renders the complex resistant to dissociation by these competitors. This assay provides a sensitive and quantitative method to monitor enzymatic discrimination between different nucleotide structures in the primer terminus or in the incoming nucleotide and to probe for effects of these substrate modifications on specific steps in phosphodiester bond synthesis.

MATERIALS AND METHODS

Chemicals and Enzymes. Poly(rA)$_{650ave}$, oligo(dT)$_{12-18}$, terminal deoxynucleotidyl transferase, and nucleotides were purchased from Pharmacia LKB Biotechnology Inc. α[S]-dGTP was obtained from Amersham Life Science Inc. and α[S]dTTP from Pharmacia LKB Biotechnology Inc. Both were racemic mixtures of stereoisomers. Concentrations given in this report are calculated at half the concentration of the mixture assuming that only the S_p diastereomer is active (Polesky et al., 1992). AZTTP was prepared by Dr. A. M. Mian (University of Miami, Miami, FL) or purchased from DuPont−New England Nuclear Corp. Bovine serum albumin (monomer standard) was purchased from ICN Biomedicals and was treated at 50 °C for 2 h followed by centrifugation to remove precipitate. [γ-^{32}P]ATP was purchased from DuPont−New England Nuclear Corp. T4 polynucleotide kinase was obtained from New England BioLabs. Recombinant HIV-1 reverse transcriptase p66/p51 heterodimer containing N-terminal hexa-histidine was prepared, cleaved with HIV-1 protease, and purified as previously described (Sharma et al., 1991; Chattopadhyay et al., 1992).

Preparation of Chain-Terminated Primer/Templates and Formation of DEC. DNA oligonucleotides were prepared in the DNA synthesis facility of the University of Miami using an Applied Biosystems 380B DNA synthesizer. Concentrations of oligonucleotides were determined from the absorbance at 260 nm. Primers L_{16}, P_{16}, L_{19}, or L_{20} (see Table 1 for nucleotide sequences) were 5′ end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and the kinase was inactivated at 70 °C for 5 min. The DNA was recovered by ethanol precipitation, redissolved in DNA buffer (10 mM HEPES, pH 7.4, 60 mM KCl), and combined with unlabeled template (WL_{41} for L_{16}, L_{19}, or L_{20}; SL_{41} or SL_{50} for P_{16}) in DNA buffer (molar ratio of primer to template was 1:1 except as indicated). The mixture was heated to 90 °C for 2 min, cooled slowly to room temperature to allow reannealing, and stored at −20 °C prior to use.

Table 1: Oligonucleotide Primers and Templates

template	WL_{41}	3′-GATGATCAAAAGAGGTAGATCATGACAAAAAAAAGAAATAC-5′
primer	L_{20}	5′-CTACTAGTTTTCTCCATCTA-3′
	L_{19}	5′-CTACTAGTTTTCTCCATCT-3′
	L_{16}	5′-CTACTAGTTTTCTCCA-3′
template	SL_{41}	3′-GACTCTGTTGTAGACGACTCCATCCTGAATGGTGTGCTGTG-5′
	SL_{50}	3′-GACTCTGTTGTAGACGACTCCATCCTGAATGGTGTGCTGTGGTGTGCTGT-5′
primer	P_{16}	5′-CTGAGACAACATCTGC-3′

Labeled primer/template was chain-terminated by adding HIV-1 RT at the concentrations specified in the figure legends and 10 μM ddGTP or ddITP (for L_{20}/WL_{41}) or ddATP (for L_{19}/WL_{41}) in RT reaction buffer [final concentrations: 40 mM HEPES, pH 7.0, 20 mM $MgCl_2$, 60 mM KCl, 1 mM dithiothreitol, 80 μg/mL heat-treated bovine serum albumin, and 2.5% (v/v) glycerol] for 5 min at 37 °C and then placed in ice. Free dNTP was added at the concentrations specified for each experiment, and the mixture was incubated for 10 or 15 min at 25 °C and again cooled in ice prior to analysis by the electrophoretic mobility shift assay.

Electrophoretic Mobility Shift Assay. After incubation with or without dNTP, poly(rA)/oligo(dT) (4:1, molar ratio of nucleotides) was added to 0.3 OD_{260} unit/mL [approximately 200 nM oligo(dT) and 50 nM poly(rA)]. The mixture was loaded onto a nondenaturing 6% polyacrylamide gel prepared in TB buffer (89 mM Tris−Borate, pH 8.2) and submitted to electrophoresis at 4 °C for 1 h (200 V). The gel was dried, and the distribution of radioactive species was determined directly from the dried gel using a Molecular Dynamics PhosphorImager SF. Radioactivity in free primer/template and in the complex was determined using the ImageQuant program. The dried gel was exposed to X-ray film for autoradiography. In several experiments, heparin (0.025 unit/mL, grade 1-A; Sigma Chemical Corp.) was substituted for poly(rA)/oligo(dT) with essentially identical results.

Reaction conditions were tested for the presence of nuclease activity by incubating 2.5 nM labeled primer/template with 2.5 nM HIV-1 RT. Labeled primers L_{16} and P_{16} contained significant amounts of shorter DNA species prior to incubation, but incubation in the absence of dNTPs for up to 60 min at 37 °C did not result in increased amounts of these shorter species (not shown).

Estimation of Apparent K_d ($K_{d,app}$). $K_{d,app}$ was estimated from the fraction of duplex DNA detected in complex in the mobility shift assay as a function of dNTP concentration by fitting the data to the equation for single-site ligand binding using Sigma Plot. At saturating dNTP, greater than 70% of the DNA was recovered as DEC.

RESULTS

DEC Formation by HIV-1 RT. We optimized conditions for the electrophoretic mobility shift assay to distinguish between the more labile complex formed by binding HIV-1 RT to T/P (Hsieh et al., 1993; Bakhanashvili & Hizi, 1994; 1996) and the more stable complex formed in the presence of the next complementary nucleotide. Figure 1 shows results with T/P terminated with ddG in which the next position on the template is A. 5′ end-labeled primer (L_{20})

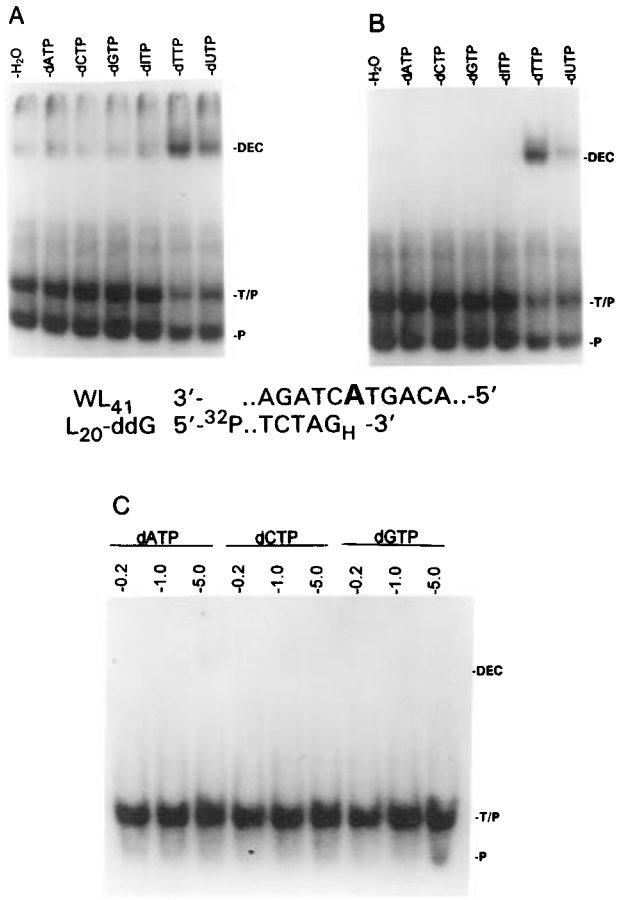


FIGURE 1: Stable complex formation between HIV-1 RT and chain-terminated primer/template. (A and B) 5′-^{32}P-L_{20} (0.5 nM) was annealed to WL_{41} and incubated with HIV-1 RT (5 nM) in RT reaction buffer with 10 μM ddGTP to form ^{32}P-L_{20}-ddG/WL_{41} (shown below the figure; G_H indicates a ddGMP residue at the primer terminus). The mixture was incubated with no nucleotide (H_2O) or with 200 μM dATP, dCTP, dGTP, dITP, dTTP, or dUTP (indicated at the top of each lane) for 15 min at 25 °C and cooled in ice. The mobility shift assays were performed on a 6% nondenaturing polyacrylamide gel at 4 °C in TB buffer as described under Materials and Methods without (A) or with (B) the addition of unlabeled poly(rA)/oligo(dT) (0.3 OD_{260} unit/mL) prior to electrophoresis. (C) 1.4 nM HIV-1 RT was incubated with 0.2 nM ^{32}P-L_{20}/WL_{41} and 10 μM ddGTP to form ^{32}P-L_{20}-ddG/WL_{41}. dATP, dCTP, or dGTP was added at the concentrations indicated (mM) at the top of each lane and the mixtures were incubated for 15 min at 25 °C and cooled in ice. Unlabeled poly(rA)/oligo(dT) was added, and the mobility shift assays were performed at 4 °C as described above. Positions of the complex (DEC), template/primer (T/P), and unannealed primer (P) are shown at the right of each panel.

was annealed to unlabeled template (WL_{41}) and chain-terminated by incubation with HIV-1 RT and ddGTP. The mixture was incubated at 25 °C with or without the addition of various nucleotides and then fractionated by electrophoresis on a nondenaturing polyacrylamide gel at 4 °C. RT−DNA complex was detected as a slow migrating band which became more intense when either dTTP or dUTP was present during the incubation (Figure 1A). The amount of radioactivity recovered in RT−DNA complex in the absence of dNTP or with noncomplementary dNTP was variable. This background binding was removed by adding excess unlabeled poly(rA)/oligo(dT) as a competing primer/template just before electrophoresis (Figure 1B) or heparin (not shown). Complex formed in the presence of complementary dTTP or dUTP was not affected by the addition of poly(rA)/oligo(dT) or heparin. Noncomplementary nucleotides, dCTP and dGTP, failed to induce formation of stable RT−DNA complex even when present at 5 mM (Figure 1C). A small amount of complex was seen with 5 mM dATP (the band is barely visible on the original autoradiogram).

A snapshot of the influence of T/P length and sequence on DEC formation was obtained by allowing HIV-1 RT to interact with a mixture of labeled chain-terminated primer/templates and recovering all of the stable complexes formed as a mixture (Figure 2). A short labeled primer (L_{16}) was annealed to template WL_{41} and incubated with HIV-1 RT and a mixture of all four dNTPs and all four ddNTPs at concentrations previously determined to yield a mixture of chain termination products approximately evenly distributed over the size range from 16 to 41 nucleotides. When the mixture was treated with excess poly(rA)/oligo(dT) and subjected to nondenaturing gel electrophoresis, a discrete band corresponding to the mixture of enzyme−DNA−dNTP complexes (DEC) was observed near the top of the gel (not shown). This band was excised, the DNA component was recovered from the gel fragments, and the size distribution of chain termination products was analyzed by electrophoresis on a DNA sequencing gel (Figure 2A). The free T/P component (fDNA) was also recovered as a well-separated band in the nondenaturing gel and analyzed in the same way. For T/P terminated at each position on the template, the ratio of counts recovered in the DEC band to those recovered in the fDNA band was determined (Figure 2B). The ratio was highest when the duplex portion of the T/P was between 20 and 28 base pairs in length, suggesting that, for this template sequence, DEC is more efficiently formed or more stable once formed if the duplex is at least 20 base pairs in length.

In this experiment, primer/templates with duplex regions greater than 28 base pairs formed DEC very inefficiently, possibly due to the A-rich sequence between positions 27 and 38 on the WL_{41} template. Template regions with stretches of repeated dA residues are known to be replicated inefficiently by HIV-1 RT (Williams et al., 1990). The experiment was repeated with primer (P_{16}) and template (SL_{41}) of different sequences (Figure 3). Again, DEC formed very inefficiently on T/P's with duplex regions less than 20 base pairs in length, and T/P molecules with longer duplex regions were underrepresented in the DEC fraction (the optimum duplex length was between 20 and 32 base pairs). The reduced efficiency of DEC formation on T/P molecules with longer duplex regions was unexpected but has not been investigated further. DEC formation was also studied on a T/P in which the single-strand portion of the template was extended by nine bases (Figure 3C). The DEC/fDNA ratio was similar at comparable positions on the two templates except that DEC formation was impaired on the SL_{41}/P_{16} pair when the template extension was less than four bases beyond the end of the primer. (Compare the two T/P's at positions 39−41 in Figure 3C.)

A product (L_{20}-ddG) that was efficiently incorporated into DEC in the experiment shown in Figure 2 was chosen for further study. Primers one base longer (L_{20}-G-ddT) or one base shorter (L_{19}-ddA) were also used in specific experiments. Figure 4 shows the dependence of DEC formation on free nucleotide concentration during the incubation. With increasing concentration of dTTP, greater amounts of T/P were captured in DEC. The reaction reaches saturation at dTTP concentrations greater than 100 μM when 70−80% of the T/P is incorporated into DEC. Nucleotide analogues

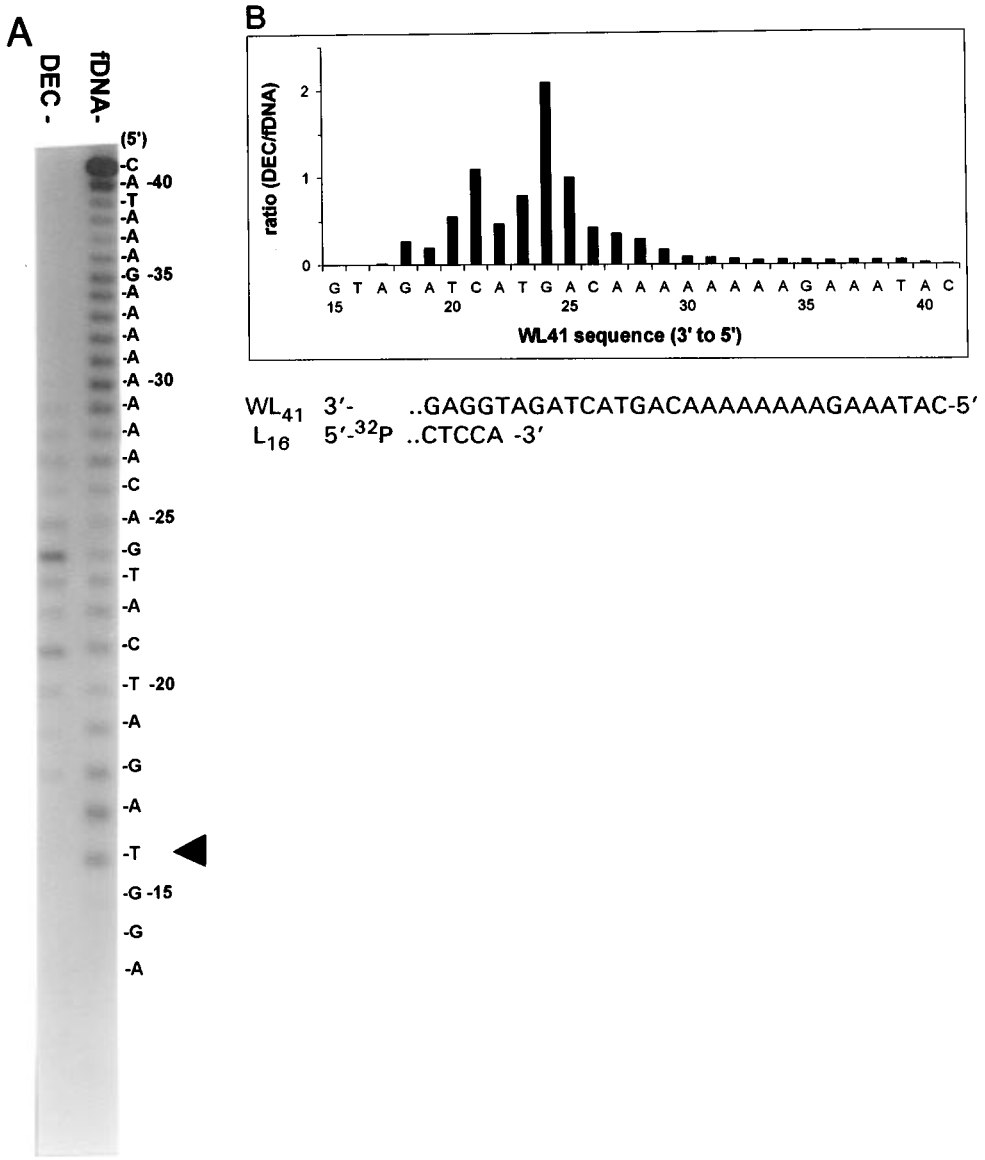


FIGURE 2: Formation of DEC from a mixture of primer extension products terminated at various positions on the template. (A) 2.5 nM ^{32}P-L_{16}/WL_{41} (shown below panel B) was incubated with 2.5 nM HIV-1 RT in the presence of dTTP, dATP, dGTP, and dCTP (30 μM each), and ddTTP, ddATP, ddGTP, and ddCTP (20 μM each), for 15 min at 37 °C to allow primer extension and termination. The mixture was cooled in ice, unlabeled poly(rA)/oligo(dT) was added, and the reaction mixture was fractionated on a 6% nondenaturing polyacrylamide gel at 4 °C for the mobility shift assay as described under Materials and Methods. DNA from the DEC band and DNA from uncomplexed T/P (fDNA) were recovered by electroelution and fractionated on a 10% polyacrylamide sequencing gel containing 7 M urea. Nucleotide positions were identified by comparison with a sequencing ladder. The solid arrowhead indicates the position of unextended L_{16} primer. (B) Radioactivity present in each band in the gel shown in panel A was determined with a PhosphorImager, and the ratio of DEC to fDNA is shown for each position on the template sequence.

Table 2: Dependence of DEC Formation on ^{32}P-L_{20}-ddG/WL_{41} on Concentration of Various Nucleotides

dNTP or ddNTP	$K_{d,app}$[a] (μM)	ratio to $K_{d,app,dTTP}$
dTTP	2.3 ± 0.7	1.0
ddTTP	11 ± 6	4.8
α[S]dTTP	13 ± 5	5.7
dUTP	42 ± 17	18
AZTTP	57 ± 11	25
dATP	>5000	>2000
dCTP	>5000	>2000
dGTP	>5000	>2000

[a] Mean ± average deviation for 2−8 replicate experiments.

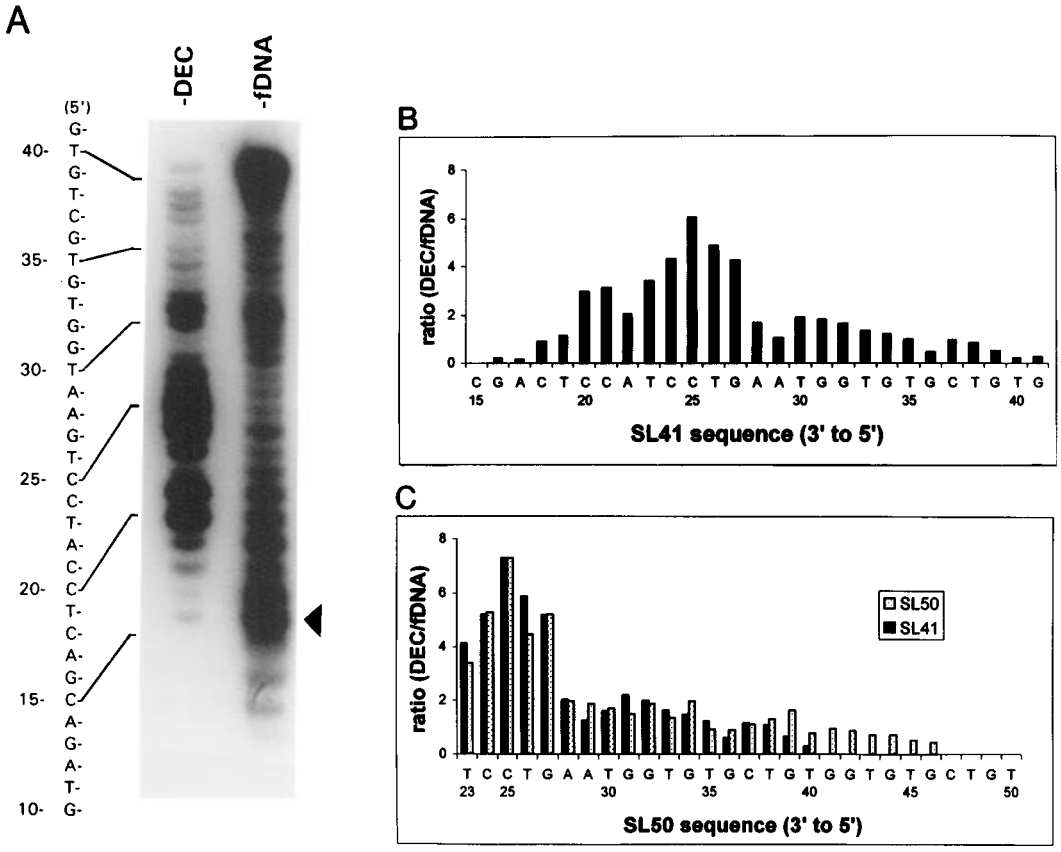
Table 3: Dependence of DEC Formation on ^{32}P-L_{19}-ddA/WL_{41} on Concentration of Various Nucleotides

dNTP or ddNTP	$K_{d,app}$[a] (μM)
dGTP	1.0 ± 0.4
ddGTP	1.7 ± 0.8
α[S]dGTP	2.3 ± 0.4
dITP	120 ± 30
ddITP	390 ± 160
rGTP	>5000

[a] Mean ± average deviation for 2 or 3 replicate experiments.

ddTTP, AZTTP, α[S]dTTP, and dUTP also induced DEC formation at elevated concentrations. Apparent K_d's ($K_{d,app}$'s) for each nucleotide are summarized in Table 2. Primer L_{19}-ddA allowed an evaluation of base-pairing with the C residue at position 21 on WL_{41} (Figure 5). DEC formation depended on addition of dGTP or related nucleotides. $K_{d,app}$'s are given in Table 3.

The Role of Primer Terminus in Formation of DEC with HIV-1 RT. Figure 6A shows a comparison of stable complex formation on T/P with the correctly base paired primer



SL_{41} 3′- ..AGACGACTCCATCCTGAATGGTGTGCTGTG-5′
P_{16} 5′-^{32}P ..TCTGC -3′

SL_{50} 3′- ..AGACGACTCCATCCTGAATGGTGTGCTGTGGTGTGCTGT-5′
P_{16} 5′-^{32}P ..TCTGC -3′

FIGURE 3: Formation of DEC from a mixture of primer extension products on an alternative template. (A) 2.5 nM ^{32}P-P_{16}/SL_{41} (shown below the figure) was incubated with 2.5 nM HIV-1 RT in the presence of all four dNTPs and all four ddNTPs as described in the legend to Figure 2. DEC and fDNA were separated on a 6% nondenaturing polyacrylamide gel, and DNA was isolated and refractionated on a 10% polyacrylamide sequencing gel containing 7 M urea. Nucleotide positions were identified by comparison with a sequencing ladder. The solid arrowhead indicates the position of unextended P_{16} primer. (B) Band intensity was determined from the autoradiogram shown in panel A with a Zeineh soft laser scanning densitometer, and the ratio of DEC to fDNA is shown for each position on the template sequence. (C) A similar experiment was carried out with ^{32}P-P_{16}/SL_{50} (shown below the figure), and the DEC/fDNA ratio is compared for templates SL_{41} and SL_{50}. The values have been adjusted so that the ratios are equal at position 25 for ease of comparison between the two P/T's.

terminus (L_{20}-ddG/WL_{41}) and with complexes formed on T/P's containing mismatched primer termini (L_{20}-ddA/WL_{41} or L_{20}-ddC/WL_{41}). Neither of the T/P's with mismatched primer terminus formed detectable DEC in the presence of complementary or noncomplementary dNTP (Figure 6A). By contrast, Figure 6B shows that primer terminated with ddI (across from dC on the template) (L_{20}-ddI/WL_{41}) formed stable complex even more efficiently than the same primer terminated with ddG (L_{20}-ddG/WL_{41}).

A similar experiment was carried out to compare primer terminated with ddT (L_{20}-G-ddT) or AZT (L_{20}-G-AZT) (Figure 6C). In this case, primer terminated with ddT supported DEC formation with dATP (the next complementary nucleotide) much more efficiently than the same primer terminated with AZT.

DISCUSSION

We have described a quantitative assay for the dead-end complex formed by HIV-1 RT, dideoxynucleotide chain-terminated primer/template, and the next complementary dNTP, which is stable in the presence of excess competing primer/template or heparin. In spite of the low fidelity characteristic of this enzyme, our results show that HIV-1 RT is highly selective for correct base-pairing both for the incoming nucleotide and for the primer terminus and exhibits some selectivity against modified nucleotides.

The strong preference we have observed for dGTP over dITP and for ddGTP over ddITP at a C position on the template is consistent with a major role in DEC formation for stable hydrogen bonding between the next dNTP and the template. Both I and G are complementary to dC, and the structural features of I-C and G-C base pairs are similar; however, the presence of three hydrogen bonds makes the G-C base pair more stable than the I-C base pair which has only two hydrogen bonds (Wells et al., 1970).

HIV-1 RT also discriminated on the basis of other structural features of the dNTP substrate. $K_{d,app}$'s for DEC formation with dTTP analogues increased in the following order: dTTP < ddTTP ≈ α[S]dTTP < dUTP < AZTTP. A similar relationship was seen for analogues of dGTP:

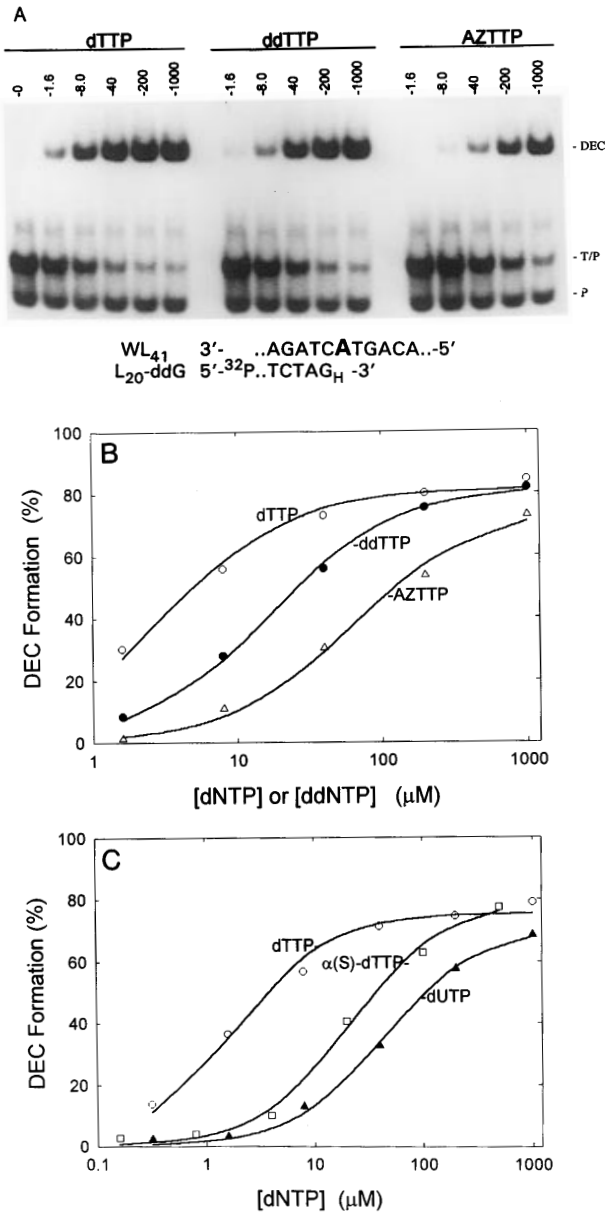
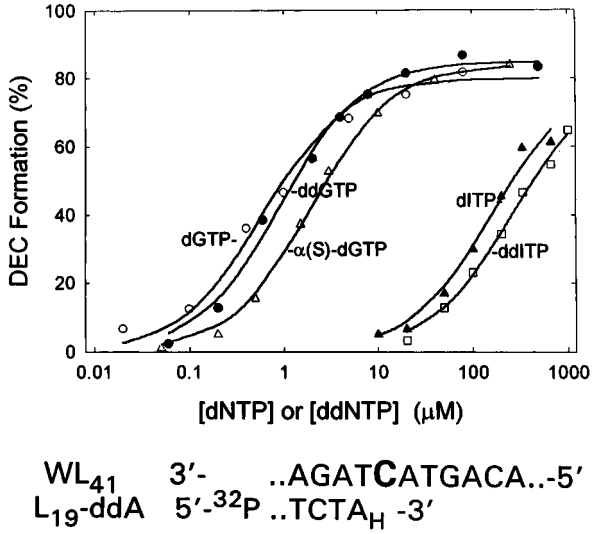


FIGURE 4: Effect of concentration of dTTP and nucleotide analogues on the formation of DEC. (A) HIV-1 RT (10 nM) was incubated with 5′-^{32}P-L_{20}/W_{41} (1.5 nM) and 10 μM ddGTP to form ^{32}P-L_{20}-ddG/WL_{41} (shown below the figure; G_H indicates a ddGMP residue at the primer terminus). The mixture was further incubated for 10 min at 25 °C either with no added nucleotide or with increasing concentrations of dTTP, ddTTP, or AZTTP (concentration, in μM, at the top of each lane). After cooling in ice, poly(rA)/oligo(dT) was added, and the mobility shift assay was performed as described under Materials and Methods. Positions of complex (DEC), template/primer (T/P), and unannealed primer (P) are shown at the right of the panel. (B) Radioactivity in DEC and T/P bands was determined from the dried gel with a Molecular Dynamics PhosphorImager. Percent T/P in DEC is shown as a function of nucleotide concentration for the experiment shown in panel A. (○) dTTP, (●) ddTTP, (△) AZTTP. The solid lines represent the best fit of the data to the single-ligand binding curves and correspond to $K_{d,app}$ = 3.2 μM (dTTP), 17 μM (ddTTP), and 63 μM (AZTTP). (C) HIV-1 RT (20 nM) was incubated with 5′-^{32}P-L_{20}/W_{41} (3 nM) and 10 μM ddGTP to form ^{32}P-L_{20}-ddG/WL_{41}, and then incubated for 10 min at 25 °C with no nucleotide or with increasing concentrations of dTTP (○), α[S]dTTP (□), or dUTP (▲). The mixture was cooled in ice, poly(rA)/oligo(dT) was added, and mobility shift assays were performed as described under Materials and Methods. Percent T/P in DEC is shown as a function of nucleotide concentration. The solid lines represent the best fit of the data to the single-ligand binding curves and correspond to $K_{d,app}$ = 1.9 μM (dTTP), 21 μM (α[S]dTTP), and 44 μM (dUTP).



WL_{41} 3′- ..AGATCATGACA..-5′
L_{19}-ddA 5′-^{32}P ..TCTA$_H$ -3′

FIGURE 5: DEC formation with guanosine and inosine nucleotides. HIV-1 RT (1.2 nM) was incubated with 5′-^{32}P-L_{19}/W_{41} (1.7 nM primer/template formed with a 2-fold excess of template) and 10 μM ddATP to form 5′-^{32}P-L_{19}-ddA/W_{41} (shown below the figure; A_H indicates a ddAMP residue at the primer terminus) and further incubated for 10 min at 25 °C with no additional nucleotide or with increasing concentrations of dGTP (○), ddGTP (●), α[S]dGTP (△), dITP (▲), or ddITP (□). The mixture was cooled in ice, heparin (0.025 unit/mL) was added, and mobility shift assays were performed as described under Materials and Methods. Percent T/P in DEC is shown as a function of nucleotide concentration. The solid lines represent the best fit of the data to the single-ligand binding curves and correspond to $K_{d,app}$ = 0.6 μM (dGTP), 0.9 μM (ddGTP), 2.0 μM (α[S]dGTP), 160 μM (dITP), and 260 μM (ddITP).

dGTP < ddGTP < α[S]dGTP << dITP < ddITP << rGTP. Quantitative comparison between our assay results based on equilibrium binding and kinetic measurements of nucleotide incorporation is difficult; however, our results show that measurement of DEC formation provides a sensitive test of nucleotide discrimination by HIV-1 RT. Where kinetic assays detect only a small preference for dTTP over AZTTP (less than 7.5-fold) (Reardon & Miller, 1990; Parker et al., 1991; Reardon, 1993), the difference is 25-fold in the DEC assay. In addition, α-thio-substituted nucleotides are incorporated by HIV-1 RT at nearly the same rate as unsubstituted nucleotides (Reardon, 1992; Kati et al., 1992; Hsieh et al., 1993; Zinnen et al., 1994), whereas DEC formation was 2.3−5.7-fold less efficient with α[S]dNTPs. A large effect of α-thio substitution (elemental effect) is often taken to indicate a rate-limiting role for the chemical step in phosphodiester bond synthesis (Benkovic & Schray, 1973; Mizrahi et al., 1985; Kuchta et al., 1987; Johnson, 1993; Tan et al., 1994; Zinnen et al., 1994; Lowe & Guengerich, 1996; Kaushik et al., 1996); however, α-thio substitution may also affect other steps in the reaction sequence (Herschlag et al., 1991; Polesky et al., 1992). A large elemental effect would not be expected in our experiments since phosphodiester bond synthesis cannot occur. We observed a modest elemental effect consistent with enzyme−substrate interactions leading up to phosphodiester bond formation.

We observed 1.7- and 4.8-fold preferences by HIV-1 RT for dGTP and dTTP over ddGTP and ddTTP, respectively, in induction of DEC formation. Reports in the literature (Parker et al., 1991; Reardon, 1992) disagree on the degree to which HIV-1 RT discriminates between dNTPs and ddNTPs; however, strong preference for dNTPs over ddNTPs

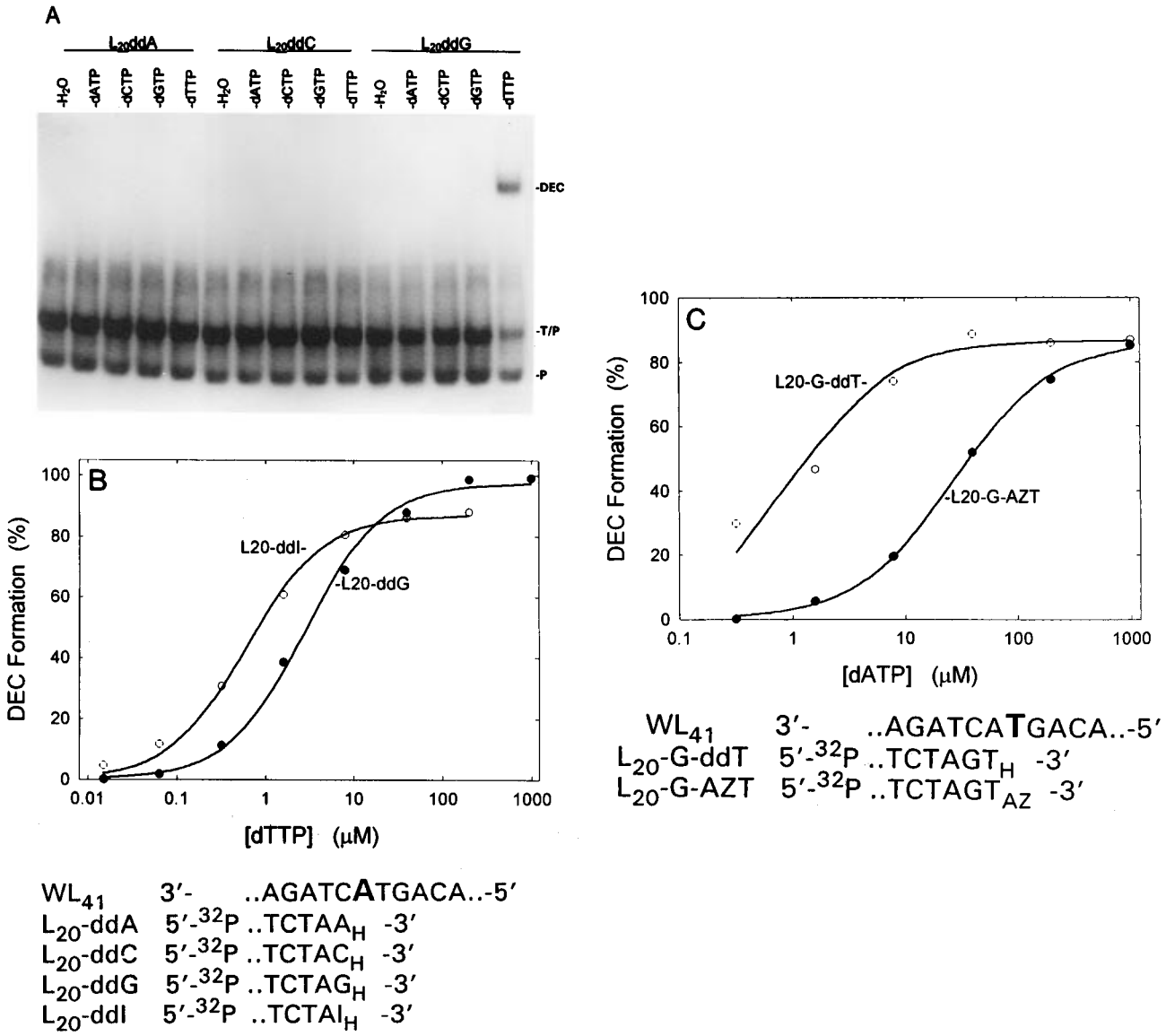


FIGURE 6: Effect of the nucleotide at the primer terminus on formation of DEC. (A) ^{32}P-labeled L_{20} was incubated with terminal deoxynucleotidyl transferase and 2 μM ddATP, 2 μM ddCTP, or 2 μM ddGTP for 30 min at 37 °C (Austermann et al., 1992). DNA was recovered by phenol extraction and ethanol precipitation, dissolved in DNA buffer, and annealed with WL_{41} to form 5′-^{32}P-L_{20}-ddA/WL_{41}, 5′-^{32}P-L_{20}-ddC/WL_{41}, and 5′-^{32}P-L_{20}-ddG/WL_{41} (shown below panel B; G_H, A_H, and C_H indicate ddGMP, ddAMP, and ddCMP residues, respectively, at the primer termini). 10 nM HIV-1 RT was incubated for 15 min at 25 °C with 5 nM of each labeled, chain-terminated primer/template with no added nucleotide (H_2O) or with 200 μM dATP, dCTP, dGTP, or dTTP (indicated at the top of each lane). The mixtures were cooled in ice, poly(rA)/oligo(dT) was added, and the mobility shift assays were performed as described under Materials and Methods. Positions of complex (DEC), template/primer (T/P), and unannealed primer (P) are shown at the right of the panel. (B) 20 nM HIV-1 RT was incubated with 3 nM 5′-^{32}P-L_{20}/WL_{41} and 10 μM ddGTP to form 5′-^{32}P-L_{20}-ddG/WL_{41} (○) or 10 μM ddITP to form 5′-^{32}P-L_{20}-ddI/WL_{41} (●) (shown below panel B; G_H and I_H indicate ddGMP and ddIMP residues, respectively, at the primer termini). The mixtures were further incubated for 15 min at 25 °C with no added nucleotide or with increasing concentrations of dTTP and cooled in ice. Poly(rA)/oligo(dT) was added, and mobility shift assays were carried out as described under Materials and Methods. Percent T/P in DEC is shown as a function of dTTP concentration. The solid lines represent the best fit of the data to the single-ligand binding curves and correspond to $K_{d,app}$ = 2.8 μM for L_{20}-ddG/WL_{41} and 0.6 μM for L_{20}-ddI/WL_{41}. (C) HIV-1 RT (10 nM) was incubated with 1.5 nM 5′-^{32}P-L_{20}/WL_{41}, 8 μM dGTP, and 8 μM ddTTP or AZTTP to form ^{32}P-L_{20}-G-ddT/WL_{41} (○) or ^{32}P-L_{20}-G-AZT/WL_{41} (●), respectively (shown below the figure; T_H and T_{AZ} indicate ddTMP and AZTMP residues, respectively, at the primer termini). The mixtures were incubated for 15 min at 25 °C with no added nucleotide or with increasing concentrations of dATP and cooled in ice. Poly(rA)/oligo(dT) was added, and mobility shift assays were carried out as described under Materials and Methods. Percent T/P in DEC is shown as a function of dATP concentration. The solid lines represent the best fit of the data in this experiment to the single-ligand binding curves and correspond to $K_{d,app}$ = 1.0 μM for L_{20}-G-ddT/WL_{41} and 28 μM for L_{20}-G-AZT/WL_{41}.

has been reported for *Thermus aquaticus* DNA polymerase at a step after the dNTP-induced conformational change (Brandis et al., 1996) which would, presumably, not be detected in our studies.

We also studied the effects of alterations in the primer terminus. Although we showed that DEC formation does not occur on T/P containing a mismatched primer terminus, we have not identified the step in the reaction pathway where discrimination occurs. Pre-steady-state kinetics (Zinnen et al., 1994) and gel mobility shift experiments (Bakhanshvili & Hizi, 1996) have shown that initial binding between HIV-1 RT and T/P is not significantly reduced when the primer terminus is mismatched; however, elongation is very inefficient. It is possible that the binary enzyme−T/P complex

with mismatched primer terminus cannot be converted to the "inner" complex which functions in processive DNA synthesis (Jaju et al., 1995; Pop & Biebricher, 1996), or that a properly base paired primer terminus is essential to induce the conformational changes required for efficient binding of the incoming nucleotide.

A primer terminus in which AZT has been substituted for ddTMP forms DEC with reduced efficiency, suggesting that interactions between the bulky azido group in the terminal AZTMP and either the enzyme or the incoming dNTP interfere with the conformational change in the enzyme−T/P complex.

Substitution of ddIMP for ddGMP at the primer terminus slightly *increased* the efficiency of DEC formation with the next complementary nucleotide. This was unexpected since misincorporation by the Klenow fragment of *E. coli* DNA polymerase I occurs more frequently after G-C than after I-C base pairs (Patten et al., 1984) and misincorporation of 2-aminopurine deoxyribonucleotide is directly correlated with the melting temperature of the last two base pairs produced by the misincorporation (Bloom et al., 1993). DNA polymerases with proofreading exonuclease activity make use of small local differences in duplex stability to direct mismatched primer termini to the exonuclease site for editing (Brutlag & Kornberg, 1972; Lo & Bessman, 1976; Reddy et al., 1992; Beese et al., 1993), a function that is lacking in HIV RT. In the absence of direct comparison between the enzymes, we cannot draw conclusions about the differences between HIV-1 RT and proofreading polymerases in this reaction; however, it is evident from our results that the substantial difference in hydrogen bonding stability between a G-C and an I-C base pair at the primer terminus is not a major factor in the dNTP induction of conformational change in the HIV RT−P/T complex.

The dead-end complex formed by HIV-1 RT on chain-terminated primer/template is a model for the active enzyme complex frozen just prior to phosphodiester bond synthesis. The assay described in this report provides a probe for specific interactions between the enzyme and its substrates leading to the catalytic step. Characterization of the DEC by physical methods, including X-ray crystallography, and of DEC formed by mutant enzymes that have altered ability to interact with either dNTP or primer/template, will help define the structural basis for the dNTP-induced conformational change. Understanding the mechanisms by which HIV-1 RT distinguishes between structural variations in the dNTP substrates and at the primer terminus will be important for rational design of novel nucleosides as antiretroviral agents and may provide new and important insights into the mechanisms by which mutations selected during drug therapy can give rise to drug-resistant forms of the enzyme.

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REFERENCES

Austermann, S., Kruhøffer, M., & Grosse, F. (1992) *Biochem. Pharmacol. 43*, 2581−2589.
Bakhanashvili, M., & Hizi, A. (1994) *Biochemistry 33*, 12222−12228.
Bakhanashvili, M., & Hizi, A. (1996) *Arch. Biochem. Biophys. 334*, 89−96.
Bebenek, K., Abbotts, J., Roberts, J. D., Wilson, S. H., & Kunkel, T. A. (1989) *J. Biol. Chem. 264*, 16948−16956.
Beese, L. S., Derbyshire, V., & Steitz, T. A. (1993) *Science 260*, 352−355.
Benkovic, S. J., & Schray, K. J. (1973) in *The Enzymes* (Boyer, P. D., Ed.) Vol 8, pp 201−238, Academic Press, New York.
Bloom, L. B., Otto, M. R., Beechem, J. M., & Goodman, M. F. (1993) *Biochemistry 32*, 11247−11258.
Brandis, J. W., Edwards, S. G., & Johnson, K. A. (1996) *Biochemistry 35*, 2189−2200.
Brutlag, D., & Kornberg, A. (1972) *J. Biol. Chem. 247*, 241−248.
Chattopadhyay, D., Evans, D. B., Deibel, M. R., Jr., Vosters, A. F., Eckenrode, F. M., Einspahr, H. M., Hui, J. O., Tomasselli, A. G., Zurcher-Neely, H. A., Heinrikson, R. L., & Sharma, S. K. (1992) *J. Biol. Chem. 267*, 14227−14232.
Chen, M. S., Suttmann, R. T., Papp, E., Cannon, P. D., McRoberts, M. J., Bach, C., Copeland, W. C., & Wang, T. S.-F. (1993) *Biochemistry 32*, 6002−6010.
Cheng, Y.-C., Dutschman, G. E., Bastow, K. F., Sarngadharan, M. G., & Ting, R. Y. C. (1987) *J. Biol. Chem. 262*, 2187−2189.
Fisher, P. A., & Korn, D. (1981) *Biochemistry 20*, 4560−4569.
Furman, P. A., Fyfe, J. A., St. Clair, M. H., Weinhold, K., Rideout, J. L., Freeman, G. A., Lehrman, S. N., Bolognesi, D. P., Broder, S., Mitsuya, H., & Barry, D. W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8333−8337.
Herschlag, D., Piccrilli, J. A., & Cech, T. R. (1991) *Biochemistry 30*, 4844−4854.
Hsieh, J., Zinnen, S., & Modrich, P. (1993) *J. Biol. Chem. 268*, 24607−24613.
Huang, P., Farquhar, D., & Plunkett, W. (1990) *J. Biol. Chem. 265*, 11914−11918.
Jaju, M., Beard, W. A., & Wilson, S. H. (1995) *J. Biol. Chem. 270*, 9740−9747.
Johnson, K. A. (1992) *Philos. Trans. R. Soc. London B 336*, 107−112.
Johnson, K. A. (1993) *Annu. Rev. Biochem. 62*, 685−713
Kati, W. M., Johnson, K. A., Jerva, L. F., & Anderson, K. S. (1992) *J. Biol. Chem. 267*, 25988−25997.
Kaushik, N., Rege, N., Yadav, P. N. S., Sarafianos, S. G., Modak, M. J., & Pandey, V. N. (1996) *Biochemistry 35*, 11536−11546.
Kedar, P. S., Abbotts, J., Kovacs, T., Lesiak, K., Torrence, P., & Wilson, S. H. (1990) *Biochemistry 29*, 3603−3611.
Kuchta, R. D., Mizrahi, V., Benkovic, P. A., Johnson, K. A., & Benkovic, S. J. (1987) *Biochemistry 26*, 8410−8417.
Lo, K.-Y., & Bessman, M. J. (1976) *J. Biol. Chem. 251*, 2480−2486.
Lowe, L. G., & Guengerich, F. P. (1996) *Biochemistry 35*, 9840−9849.
Majumdar, C., Abbotts, J., Broder, S., & Wilson, S. H. (1988) *J. Biol. Chem. 263*, 15657−15665.
Matthes, E., Lehmann, C., Scholz, D., Janta-Lipinski, M. V., Gaertner, K., Rosenthal, H. A., & Langen, P. (1987) *Biochem. Biophys. Res. Commun. 148*, 78−85.
Mizrahi, V., Henrie, R. N., Marlier, J. F., Johnson, K. A., & Benkovic, S. J. (1985) *Biochemistry 24*, 4010−4018.
Müller, B., Restle, T., Reinstein, J., & Goody, R. S. (1991) *Biochemistry 30*, 3709−3715.
Parker, W. B., White, E. L., Shaddix, S. C., Ross, L. J., Buckheit, R. W., Jr., Germany, J. M., Secrist, J. A., III, Vince, R., & Shannon, W. M. (1991) *J. Biol. Chem. 266*, 1754−1762.
Patten, J. E., So, A. G., & Downey, K. M. (1984) *Biochemistry 23*, 1613−1618.
Perrino, F. W., Preston, B. D., Sandell, L. L., & Loeb, L. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8343−8347.
Polesky, A. H., Dahlberg, M. E., Benkovic, S. J., Grindley, N. D. F., & Joyce, C. M. (1992) *J. Biol. Chem. 267*, 8417−8428.
Pop, M. P., & Biebricher, C. K. (1996) *Biochemistry 35*, 5054−5062.
Preston, B. D., Poiesz, B. J., & Loeb L. A. (1988) *Science 242*, 1168−1171.
Reardon, J. E. (1992) *Biochemistry 31*, 4473−4479.
Reardon, J. E. (1993) *J. Biol. Chem. 268*, 8743−8751.

Reardon, J. E., & Spector, T. (1989) *J. Biol. Chem. 264*, 7405−7411.
Reardon, J. E., & Miller, W. H. (1990) *J. Biol. Chem. 265*, 20302−20307.
Reddy, M. K., Weitzel, S. E., & von Hippel, P. H. (1992) *J. Biol. Chem. 267*, 14157−14166.
Rittinger, K., Divita, G., & Goody, R. S. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 8046−8049.
Roberts, J. D., Bebenek, K., & Kunkel, T. A. (1988) *Science 242*, 1171−1173.
Sharma, S. K., Evans, D. B., Vosters, A. F., McQuade, T. J., & Tarpley, W. G. (1991) *Biotechnol. Appl. Biochem. 14*, 69−81.
Spence, R. A., Kati, W. M., Anderson, K. S., & Johnson, K. A. (1995) *Science 267*, 988−993.
St. Clair, M. H., Richards, C. A., Spector, T., Weinhold, K. J., Miller, W. H., Langlois, A. J., & Furman, P. A. (1987) *Antimicrob. Agents Chemother. 31*, 1972−1977.
Takeuchi, Y., Nagumo, T., & Hoshino, H. (1988) *J. Virol. 62*, 3900−3902.
Tan, H.-B., Swann, P. F., & Chance, E. M. (1994) *Biochemistry 33*, 5335−5346.
Weber, J., & Grosse, F. (1989) *Nucleic Acids Res. 17*, 1379−1393.
Wells, R. D., Larson, J. E., Grant, R. C., Shortle, B. E., & Cantor, C. R. (1970) *J. Mol. Biol. 54*, 465−497.
Williams, K. J., Loeb, L. A., & Fry, M. (1990) *J. Biol. Chem. 265*, 18682−18689.
Wong, I., Patel, S. S., & Johnson, K. A. (1991) *Biochemistry 30*, 526−537.
Yu, H., & Goodman, M. F. (1992) *J. Biol. Chem. 267*, 10888−10896.
Zinnen S., Hsieh, J.-C., & Modrich, P. (1994) *J. Biol. Chem. 269*, 24195−24202.

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