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Effects of 3′-Deoxynucleoside 5′-Triphosphate Concentrations on Chain Termination by Nucleoside Analogs during Human Immunodeficiency Virus Type 1 Reverse Transcription of Minus-Strand Strong-Stop DNA†

ERIC J. ARTS,[1,2] JAMES-PAUL MAROIS,[1,2] ZHENGXIAN GU,[1,3]
STUART F. J. LE GRICE,[4] AND MARK A. WAINBERG[1,2,3]*

McGill AIDS Centre, Lady Davis Institute, Jewish General Hospital[1] and Department of Medicine[3] and Department of Microbiology and Immunology,[2] McGill University, Montreal, Quebec, Canada H3T 1E2, and Division of Infectious Diseases and Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106[4]

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We have compared the effects of nucleoside analogs in quiescent and phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) exposed to human immunodeficiency virus type 1 (HIV-1) with those of their triphosphorylated derivatives in cell-free HIV-1 reverse transcription assays. We observed a substantial decrease in synthesis of early minus-strand proviral DNA products in HIV-1-infected, quiescent PBMC exposed to each of 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxyinosine (ddI), and 2′,3′-dideoxy-3′-thiacytidine (3TC), in comparison with nontreated, infected controls. In contrast, no such diminution was observed when PHA-stimulated, HIV-1-infected PBMC were treated with the same drugs. This result was attributed to previously reported findings that PHA-stimulated PBMC possessed larger deoxynucleoside triphosphate (dNTP) pools than quiescent cells did. To further investigate this subject, a cell-free HIV-1 reverse transcription reaction involving HIV-1 RNA genomic template, recombinant purified HIV-1 reverse transcriptase, all four dNTPs and either $tRNA_3^{Lys}$ or a deoxyoligonucleotide as primer was used to monitor chain termi-nation mediated by 2′,3′-dideoxynucleoside triphosphates (ddNTPs) during synthesis of minus-strand strong-stop DNA. Augmented chain termination was observed with decreasing concentrations of both ddNTP and dNTP when the ratio of dNTP to ddNTP was fixed. We also found that both the number and strength of reverse transcription pause sites were increased at low concentrations of dNTPs and when a deoxyoligonucleotide primer was used in place of the cognate primer, $tRNA_3^{Lys}$. Preferential incorporation of ddATP was observed dur-ing reverse transcription opposite a distinct pause site in a short synthetic RNA template. These results con-firm the notion that the antiviral activities of ddNTP are dependent on both cellular dNTP pools and the state of cellular activation. Pausing of HIV-1 reverse transcriptase during reverse transcription, altered by dNTP concentrations, may be a mechanism that controls the position and extent of incorporation of nucleoside analogs.

Human immunodeficiency virus (HIV-1) infection of $CD4^+$ cells is inhibited during reverse transcription by dideoxynucleoside analogs (ddN), such as 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxyinosine (ddI), 2′,3′-dideoxycytidine (ddC), and the minus-strand enantiomer of 2′,3′-dideoxy-3′-thiacytidine (3TC) (27, 28, 36, 38). ddNs enter cells by either active transport or passive diffusion (26, 43). Once they are in the cytoplasm, nucleoside kinases catalyze the anabolic phosphorylation of these drugs to their respective, biologically active, 5′-triphosphate (ddNTP) forms (9, 12, 22). Different phosphorylation pathways, specific for various nucleosides and their analogs, are controlled by the activation state of the cell (12). Although these drugs have been employed clinically to patient benefit (8, 41), problems of drug resistance have limited their overall utility (34, 40).

Although the precise mechanism whereby ddNs inhibit HIV-1 replication is unclear, it is thought that ddNTPs interfere with the polymerase activities of HIV-1 reverse transcriptase (RT) by both competitive inhibition and chain termination of elongating HIV-1 DNA strands (16, 28, 39). The extent of ddNTP-mediated chain termination is thought to be dependent on both dNTP and ddNTP pool sizes and the state of cellular activation (4, 12, 13, 30, 31). In general, quiescent $CD4^+$ cells have been shown to have lower deoxynucleoside triphosphate (dNTP) and ddNTP pool sizes than activated cells have (12, 30, 31). In this study, we demonstrate an association between dNTP concentrations and efficiency of ddNTPs as chain terminators of RT activity.

To investigate this subject, we compared levels of ddN-mediated chain termination during synthesis of minus-strand strong-stop DNA in both quiescent and phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) with those observed in cell-free RT assays performed in the presence of different concentrations of dNTPs and ddNTPs.

Since HIV-1 RT is known to pause or dissociate from primer-template during DNA polymerization (20), particularly at homopolymeric stretches or regions of RNA secondary structure (1, 4, 15, 23, 37), we investigated the effect of different dNTP concentrations on HIV-1 RT pausing. We found that the strength and number of pause sites were increased at low concentrations of dNTPs in cell-free reactions, as well as under conditions in which a deoxyoligonucleotide primer (dPR) was

* Corresponding author. Mailing address: Lady Davis Institute-Jewish General Hospital, 3755 Chemin Cote Ste-Catherine, Montreal, Quebec, Canada H3T 1E2. Phone: (514) 340-8260. Fax: (514) 340-7537.

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used instead of the cognate primer, $tRNA_3^{Lys}$. In addition, ddNTPs were shown to be more effective chain terminators in reactions with low rather than high dNTP concentrations. To investigate possible preferential chain termination by nucleoside analogs, an RT assay involving a short RNA template with a distinct pause site was used.

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MATERIALS AND METHODS

Nucleoside analogs. AZT and AZT triphosphate (AZT-TP) were generously donated by Wellcome Inc., Research Triangle Park, N.C.; ddI was donated by Bristol-Myers Squibb Inc., Wallingford, Conn.; and 3TC and 3TC-TP were donated by Glaxo Group Research, Greenford, United Kingdom. ddATP, ddITP, and ddCTP were purchased from Sigma Inc., St. Louis, Mo.

Cell preparations and viral infections. PBMC were isolated separately from each of four HIV-seronegative donors by Ficoll-Hypaque density centrifugation, washed twice with serum-free RPMI 1640 medium, and resuspended at a cell density of 10^6/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U of penicillin G per ml, and 100 mg of streptomycin per ml. The cultures were then divided into two equal fractions. One of these was treated with PHA (1 U/ml; Sigma Inc.) and incubated at 37°C under 5% CO_2 for 72 h, after which the cells were washed with serum-free medium and resuspended in the above serum-supplemented medium, also containing 10 U of human recombinant interleukin-2 (Boehringer-Mannheim, Montreal, Canada) per ml. Populations of quiescent and PHA-stimulated PBMC, the latter maintained in interleukin-2, were subdivided into seven fractions of 1.5×10^6 cells (14 preparations in all for each donor). These were pretreated for 12 h with no drug, AZT (0.1 and 1 μM), ddI (10 and 100 μM), or 3TC (5 and 50 μM), after which DNase I-treated HIV-1 was added to the cells at a multiplicity of infection of 0.01 (4). The drug concentrations, in excess of those probably necessary, were chosen to ensure that chain termination would occur in our system. After 1 h of exposure to virus, the cells were washed twice with phosphate-buffered saline and reincubated in medium as described above. After 24 h, the cells were pelleted for lysis and subsequent DNA isolation (see below).

DNA isolation, purification, and PCR amplification. Low-molecular-weight (LMW) DNA was isolated from PBMC pellets by the Hirt lysis and extraction procedure as described previously (4, 18, 21). This material includes all nonintegrated viral DNA, as well as mitochondrial DNA, which served as an internal control (see below). LMW DNA in the cell lysate supernatant was ethanol precipitated and resuspended in 50 μl of water. Viral LMW DNA was identified and quantified in samples by PCR amplifications. The conditions of our PCR and primer pairs have been described previously (4). Briefly, the PCR cocktail (100 μl) contained 5 μl of Hirt LMW DNA, 100 pmol of unlabeled sense primer (S1 or MTS [see below]), 100 pmol of γ^{32}P-labeled antisense primer (A13 or MTA [see below]), 0.2 mM each of the four dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, and 2 U of *Taq* polymerase (Canadian Life Technologies, Burlington, Canada). A13 (positions 635 to 614 in the HXB2-D HIV-1 proviral genome) and S1 (positions 496 to 516) were the oligonucleotide primers used to amplify a 140-bp segment in the U5/R region of the HIV-1 long terminal repeat, corresponding to near-full-length minus-strand strong-stop DNA. The MTA and MTS primers, positions 390 to 370 and 260 to 280, respectively, in human mitochondrial DNA, ampify a 130-bp segment (4). PCR cocktails were subjected to 27 cycles of denaturation for 1 min at 95°C followed by annealing for 1.5 min at 60°C and polymerization for 1 min at 72°C. Serial dilutions (1:10) of the *Xho*I-linearized pHXB2-D plasmid were PCR amplified as above and used as quantification controls. The PCR products were electrophoresed on 7% denaturing polyacrylamide gels (PAG), which were dried, autoradiographed, and analyzed with a Bio-Rad 250 Phosphor-Imager.

Cell-free reverse transcription reactions. HIV-1 primer-binding sequence (PBS) RNA template was produced by in vitro transcription from an *Acc*I-linearized pHIV-PBS plasmid with T7 RNA polymerase as described previously (2, 15). This template (0.5 pmol) was added to a reaction cocktail (20 μl) containing 10 mM Tris-HCl (pH 7.8), 100 mM KCl, 10 mM $MgCl_2$, 1 to 250 μM each of the four dNTPs, and, on occasion, ddITP, ddCTP, ddATP, 3TC-TP, and AZT-TP (at 1/2 to 1/10 the dNTP concentration). The reaction mixtures also contained 2.5 pmol of either human $tRNA_3^{Lys}$, purified from human placenta as described previously (2), or an 18-mer deoxyoligonucleotide (dPR) complementary to the PBS and [α-^{32}P]dCTP and [α-^{32}P]dATP (Amersham) at 1/20 the concentration of dNTP. In some cases, a mutated HIV RNA template, termed HIV RNA ΔPBS, containing an 18-nucleotide (nt) PBS deletion, was used as a negative control.

Reverse transcription cocktails were denatured at 85°C for 2 min, cooled to 55°C for 8 min, and further cooled to 37°C for 2 min prior to the addition of HIV-1 RT (42.5 nM), prepared in our laboratory as described previously (15), and incubation for 1 h at 37°C. A double-stranded DNA-sequencing reaction was performed on the *Acc*I-linearized pHIV-PBS template (0.05 pmol) with the [γ-^{32}P]dPR primer and a *Taq* sequencing reaction kit (Canadian Life Technologies). This sequencing reaction provided a marker for determining sites of ddNTP chain termination in HIV-1 RT reactions primed with cold dPR in the presence of [α-^{32}P]dNTP. Reaction products were purified as described previously (2, 4) and then run on a 5% denaturing PAG, which was later dried and analyzed with a GS-250 Bio-Rad Phosphor-Imager. The percentage of minus-strand strong-stop DNA was quantified as 100 × (amount of minus-strand strong-stop DNA/total amount of product generated).

A second type of reverse transcription assay was used to determine levels of 2′,3′-dideoxynucleoside 5′-monophosphate (dNMP) incorporation at specific sites on an RNA template. A 90-mer of RNA template (0.2 pmol) and a γ-^{32}P-end-labeled 36-mer DNA primer (0.1 pmol) were added to a cocktail containing 10 mM Tris-HCl (pH 7.8) and 50 mM KCl. These RNA and DNA molecules were annealed by incubation at 85°C for 2 min followed by slow cooling to 37°C. HIV-1 RT (10 nM) and $MgCl_2$ (10 mM) were then added at 37°C, 2 min prior to the addition of 1, 10, or 100 μM dNTP, with or without heparin (0.5 mg/ml), ddATP, ddCTP (at 1/2 or 1/10 the concentration of dNTP). After 10 min at 37°C, samples from each reaction mixture were loaded onto a 10% denaturing PAG. Bands representing ddNMP-chain-terminated products were quantified with the GS-250 Bio-Rad Phosphor-Imager by analyzing the radioactivity profiles of relevant lanes. Background levels were those obtained in the absence of ddNTP.

RESULTS

Synthesis of minus-strand strong-stop DNA in HIV-1-infected, nucleoside analog-pretreated quiescent and PHA-stimulated PBMC. The LMW DNA of HIV-1-infected, nucleoside analog-pretreated PBMC was isolated by Hirt extraction (4, 18). By using a HIV-1 specific primer pair (A13-S1), a PCR product representing the majority of HIV-1 minus-strand strong-stop DNA was identified and quantified in untreated and nucleoside analog-pretreated quiescent PBMC and PHA-stimulated PBMC infected with HIV-1 (Fig. 1A). To quantitate differences, the amounts of minus-strand strong-stop DNA PCR-amplified products were standardized to an internal control, i.e., amounts of mitochondrial DNA product amplified by the MTA-MTS primer pair. As a quantitation control, 10-fold serial dilutions of linearized HXB2-D DNA were also PCR amplified with A13-S1 (left-hand portion of Fig. 1A). In the absence of nucleoside analog treatment of PBMC, more minus-strand strong-stop DNA was amplified by PCR in PHA-stimulated PBMC ($>10^6$ copies) (Fig. 1A, lane 1) than in quiescent PBMC ($<10^6$ copies) (lane 8). This difference in levels of reverse transcription in quiescent and PHA-stimulated PBMC has been reported previously (10, 42). All cellular studies were performed with HIV-1 that had been DNase I treated to minimize levels of viral DNA in viral particles; our methods result in the presence of such DNA at $<10^{-6}$ of the level found in infected PBMC (3).

Levels of products obtained with nucleoside analog-treated samples (Fig. 1A, lanes 2 to 7 for PHA-stimulated PBMC and lanes 9 to 14 for quiescent PBMC) were determined by measuring the intensities of relevant bands by phosphor-imaging relative to those of untreated samples (lanes 1 and 8, respectively). In each case, an arbitrary value of 1 was assigned to products generated in the absence of drug (lanes 1 and 8). Standard deviations were calculated on the basis of three independent experiments performed on the PBMC of each of four different donors. For quiescent PBMC, we observed a significant decrease in levels of PCR-amplified minus-strand strong-stop DNA in cells treated with each nucleoside analog compared with untreated cells (Fig. 1B). No such significant differences were seen in comparisons of untreated PHA-stimulated PBMC versus PHA-stimulated PBMC treated with 0.1 or 1 μM AZT, 10 or 100 μM ddI, or 5 or 50 μM 3TC. These findings with PHA-stimulated PBMC are consistent with previous observations (4). We have obtained similar results with concentrations of AZT between 0.001 and 100 μM (data not shown).

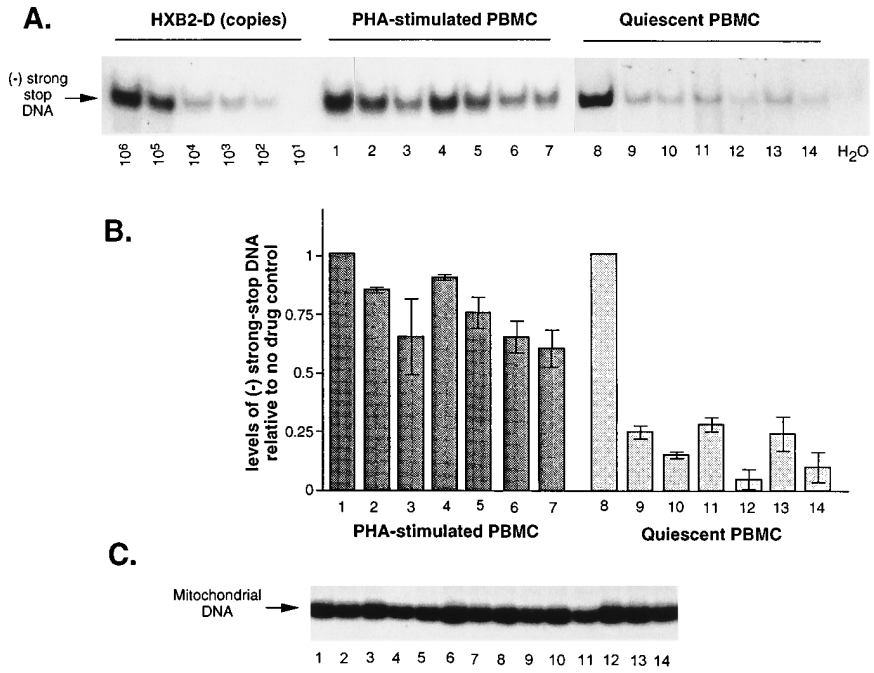

FIG. 1. PCR amplifications of LMW DNA from HIV-1-infected, nucleoside analog-pretreated quiescent and PHA-stimulated PBMC. LMW DNA extracts from quiescent and PHA-stimulated PBMC that had been treated with AZT (0.1 and 1 μM), ddI (10 and 100 μM), or 3TC (5 and 50 μM) were PCR amplified with the A13-S1 and MTA-MTS primer pairs. The former amplifies a 140-bp fragment of minus-strand strong-stop DNA in the HIV-1 genome, while the latter amplifies a 130-bp fragment in the noncoding region of mitochondrial DNA (4). Tenfold dilutions from 10^6 to 10^1 copies of *Xho*I-linearized pHXB2-D plasmid were PCR amplified with the A13-S1 primer pair as a positive quantitation control for experiments performed with PBMC. (A) Products of these reactions on an 8% denaturing PAG. (B) Relative intensities of PCR-amplified minus-strand strong-stop DNA. (C) PCR-amplified mitochondrial DNA as an internal control. (Lanes 1 and 8, no drug; lanes 2 and 9, 0.1 μM AZT; lanes 3 and 10, 1 μM AZT; lanes 4 and 11, 10 μM ddI; lanes 5 and 12, 100 μM ddI; lanes 6 and 13, 5 μM 3TC; lanes 7 and 14, 50 μM 3TC.) Amounts of minus-strand strong-stop DNA in quiescent and PHA-activated nucleoside analog-treated PBMC were adjusted relative to those of untreated infections of PBMC, which were given arbitrary values of 1.

HIV minus-strand strong-stop DNA synthesis in a cell-free reverse transcription reaction. Either $tRNA_3^{Lys}$ or dPR was used to prime RNA-dependent DNA polymerase (RDDP) from an HIV PBS RNA template in reaction mixtures containing recombinant purified HIV-1 RT (Fig. 2) (2, 15). End-labeling studies involving $tRNA_3^{Lys}$ could not be performed because of limited availabilities of this reagent. For this reason, dPR- and $tRNA_3^{Lys}$-primed products were labeled with [α-^{32}P]dCMP and [α-^{32}P]dAMP. The products of these reactions during a time course are shown in Fig. 3A (dPR primed) and B ($tRNA_3^{Lys}$ primed). No minus-strand strong-stop DNA was produced from reactions in which mutated template, i.e., HIV RNA ΔPBS was employed (18-nt PBS deletion), indicating that both primers could initiate synthesis of minus-strand strong-stop DNA only from the PBS (24) (data not shown).

The intensity and number of paused minus-strand DNA products were consistently greater in reactions primed with dPR (Fig. 3A) than in those primed with $tRNA_3^{Lys}$ (Fig. 3B) (experiment repeated three different times). The addition of a heparin trap to reactions primed with dPR limits extensions to the first few pause sites (15) because of an inability of HIV-1 RT to rebind to the primer-template at the pause site and extend synthesis of minus-strand strong-stop DNA. Thus, a fully controlled analysis of the extent of ddNMP incorporation could not be undertaken under steady-state conditions or in the presence of heparin. We observed an increase in the intensity of all bands at 30 and 60 min in reactions primed with each of dPR and $tRNA_3^{Lys}$. This indicates that the increase in synthesis of full-length minus-strand strong-stop DNA at these extended incubation times was not due to a general extension of stall products.

Pause sites during synthesis of minus-strand strong-stop DNA are indicated by letters and are schematically shown on the RNA template in Fig. 3C. Pause sites in reactions primed with end-labeled dPR were identified on PAGs alongside sequencing reactions primed with dPR by using pHIV-PBS plas-

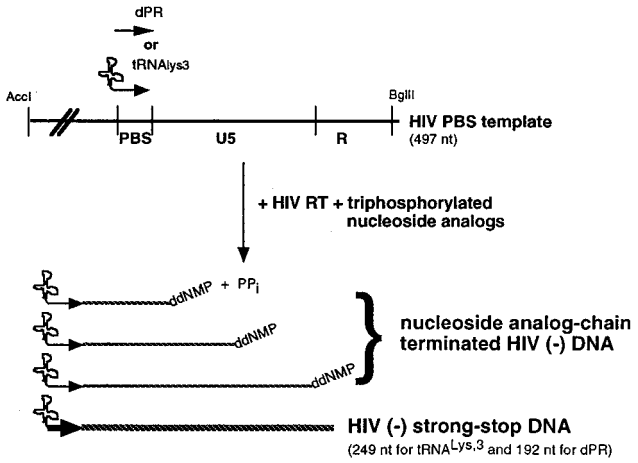

FIG. 2. Schematic representation of in vitro HIV-1 reverse transcription reaction. The two types of primers used, dPR and $tRNA_3^{Lys}$, are shown over the PBS on the HIV RNA PBS template. The addition of RT results in the initiation of RNA-dependent DNA polymerization and the synthesis of minus-strand strong-stop DNA. Because of the low processive potential of RT, we found several minus-strand DNA products that were initiated from the PBS but not extended to full length (not shown). The addition of ddNTP (nucleoside analogs) resulted in chain termination of minus-strand strong-stop HIV-1 DNA. The difference in minus-strand strong-stop DNA length in reactions primed by dPR and $tRNA_3^{Lys}$ is the result of an extra 57 nt found at the 5′ end of $tRNA_3^{Lys}$.

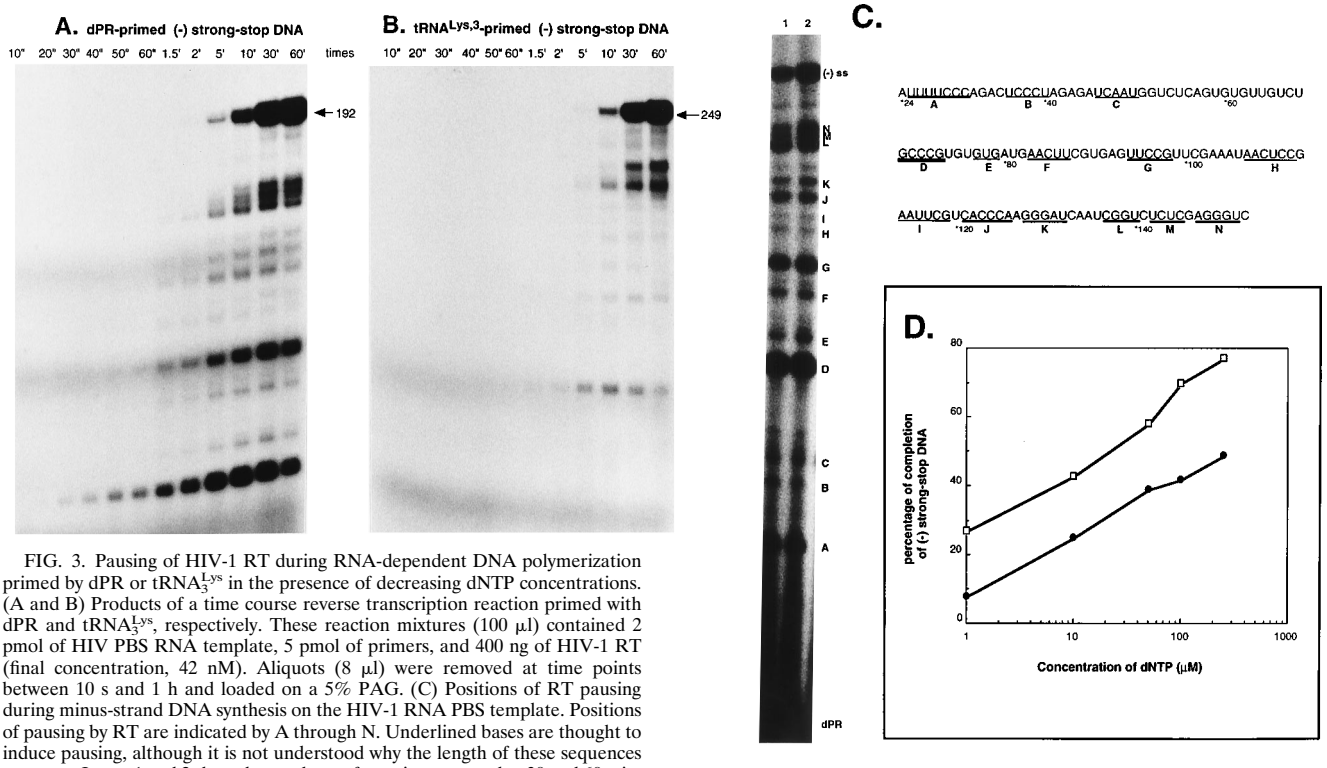

FIG. 3. Pausing of HIV-1 RT during RNA-dependent DNA polymerization primed by dPR or $tRNA_3^{Lys}$ in the presence of decreasing dNTP concentrations. (A and B) Products of a time course reverse transcription reaction primed with dPR and $tRNA_3^{Lys}$, respectively. These reaction mixtures (100 μl) contained 2 pmol of HIV PBS RNA template, 5 pmol of primers, and 400 ng of HIV-1 RT (final concentration, 42 nM). Aliquots (8 μl) were removed at time points between 10 s and 1 h and loaded on a 5% PAG. (C) Positions of RT pausing during minus-strand DNA synthesis on the HIV-1 RNA PBS template. Positions of pausing by RT are indicated by A through N. Underlined bases are thought to induce pausing, although it is not understood why the length of these sequences may vary. Lanes 1 and 2 show the products of reactions stopped at 30 and 60 min, respectively, and containing the same amounts of template and primer. For these experiments, end-labeled [γ-^{32}P]dPR primer was used. (D) The percentage of completion of minus-strand strong-stop DNA synthesis was quantified by measuring the amount of minus-strand strong-stop DNA product over the total amount of product generated in this system. Reactions, primed with either dPR (●) or $tRNA_3^{Lys}$ (□), in the presence of decreasing concentrations of dNTP, are shown in terms of percentage of completion of minus-strand strong-stop DNA synthesis over the concentration of dNTP used.

mid DNA (see Materials and Methods) (data not shown). Pause sites generally preceded homopolymeric stretches in the heteropolymeric HIV PBS RNA template as previously reported (15, 23). Pause sites not corresponding to upstream homopolymeric sequences in the template are probably due to RNA secondary structure (1, 14, 23).

The ratio of incomplete minus-strand DNA products to the total amount of minus-strand DNA product (i.e., both full-length and incomplete minus-strand strong-stop DNA) provides an estimate of the percentage of completion of such DNA. The results in Fig. 3D present a summary of the intensity of product bands, adjusted for levels of incorporation of [α-^{32}P]dAMP and [α-^{32}P]dCMP. Increasing the concentration of dNTP in reactions primed by each of dPR and $tRNA_3^{Lys}$ resulted in an increased percentage of completion of minus-strand strong-stop DNA, i.e., decreased pausing (Fig. 3D).

Chain termination by ddNTP in cell-free reverse transcription reactions. We have previously shown, using a cell-free RT assay, that AZT-TP was preferentially incorporated into elongating DNA, resulting in chain termination after the first template switch in reactions primed with $tRNA_3^{Lys}$ but not dPR (4). Although some chain termination did occur during synthesis of minus-strand strong-stop DNA in reactions primed with $tRNA_3^{Lys}$, it was observed only with high concentrations of AZT-TP and was observed less frequently than when dPR was used as the primer (4). This subject was further investigated by monitoring the synthesis of minus-strand strong-stop DNA in reactions primed with either dPR or $tRNA_3^{Lys}$ in the presence of various ddNTP concentrations. Because of increased pausing with the DNA primer and limited availability of human $tRNA_3^{Lys}$, dPR was used as the primer to study the effects of decreasing dNTP concentrations on ddNTP-mediated chain termination.

Figure 4A displays the products of reverse transcription reactions primed with $tRNA_3^{Lys}$ at 100 μM dNTP. Figure 4B, lanes 1 to 33, shows the products of similar reactions primed with dPR in the presence of three different concentrations of dNTP (i.e., 100 μM [lanes 1 to 11], 10 μM [lanes 12 to 22], and 1 μM [lanes 23 to 33]) and ddNTP (at 1/2 and 1/10 the concentration of the corresponding dNTP). Figure 4A, lane 1, and Fig. 4B, lanes 1, 12, and 23, display the products of reactions performed in the absence of drug. Pause sites are indicated by letters in Fig. 4A and B. Sites of incorporation of ddNMPs, resulting in chain termination, are visualized as nonpause product bands and are not present in Fig. 4A, lane 1, and Fig. 4B, lanes 1, 12, and 23.

Reaction mixtures containing ddNTP at half of the dNTP concentrations showed more chain termination and less synthesis of minus-strand strong-stop DNA than those that contained ddNTP at 1/10 the dNTP concentration. Nonetheless, similar amounts of products (full length, paused, and chain terminated) were obtained in reaction mixtures containing 1/2 and 1/10 ratios of ddNTP to dNTP. However, considerable variation in levels of chain termination was observed in the presence of different nucleoside analogs. AZT-TP, ddATP, and ddCTP caused more chain termination than did ddITP and 3TC-TP (Fig. 4A and B, lanes 8 to 11). These results show that ddITP can be incorporated by RT, resulting in chain termination opposite guanidine residues in the RNA template, even though ddATP is believed to constitute the intracellular active form of ddI (22).

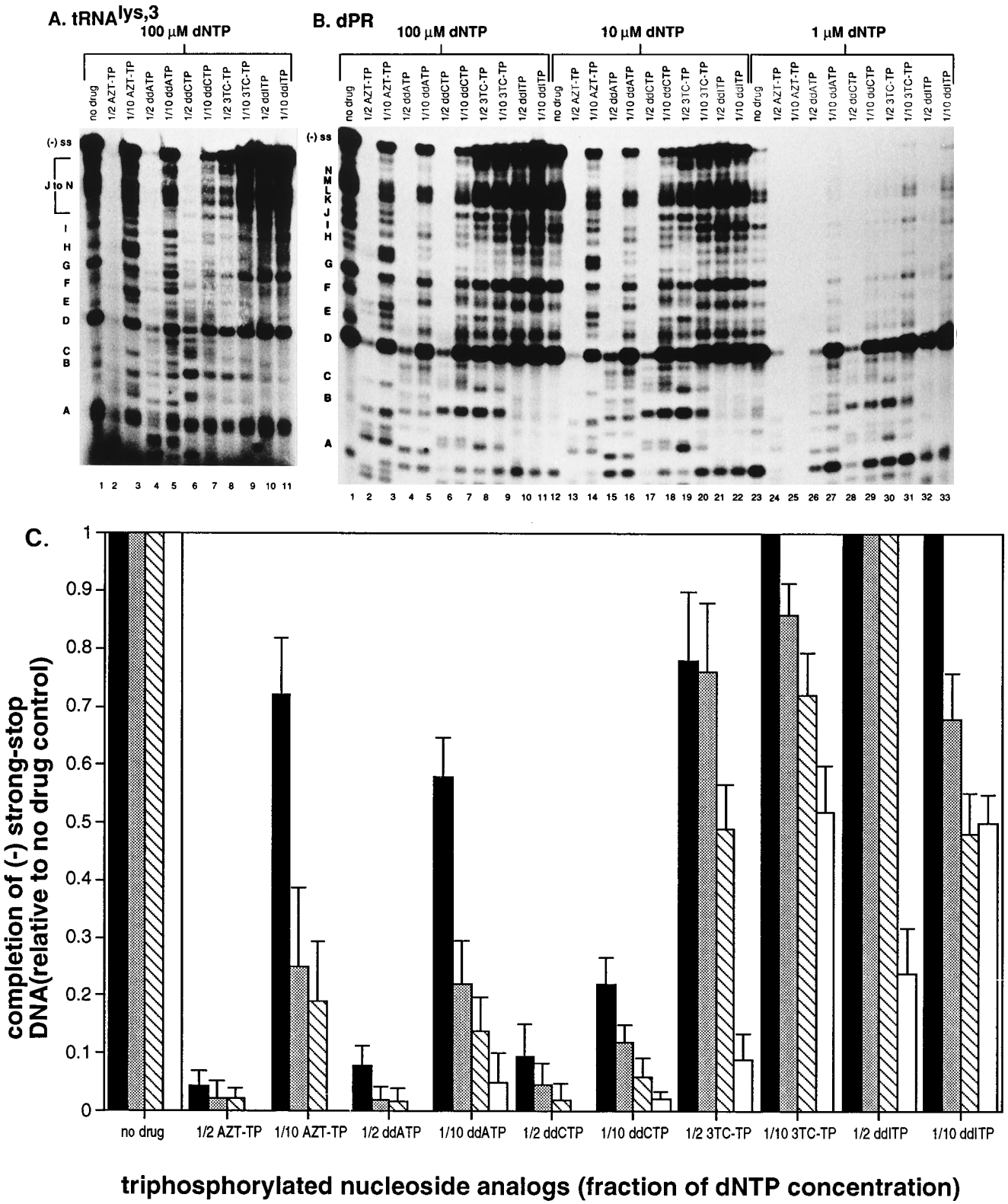

FIG. 4. In vitro HIV-1 reverse transcription reactions in the presence of ddNTP. (A) Products of reverse transcription reactions primed with $tRNA_3^{Lys}$, the cognate primer of HIV-1 reverse transcription. (B) Alternatively, reverse transcription reactions were primed with dPR in the presence of decreasing concentrations of dNTP (100, 10, and 1 μM). AZT-TP, ddATP, ddCTP, 3TC-TP, or ddITP was added to the reaction mixtures at 1/2 or 1/10 the dNTP concentration employed. Pause sites in reverse transcription reactions are indicated by the letters A through N. (C) The amounts of minus-strand strong-stop product shown in panels A and B were determined by phosphor-imaging analysis and plotted. Amounts of minus-strand strong-stop DNA product in untreated samples were arbitrarily assigned values of 1 for reactions primed by $tRNA_3^{Lys}$ (■) and dPR at different dNTP concentrations (▨, 100 μM; ▧, 10 μM; and □, 1 μM).

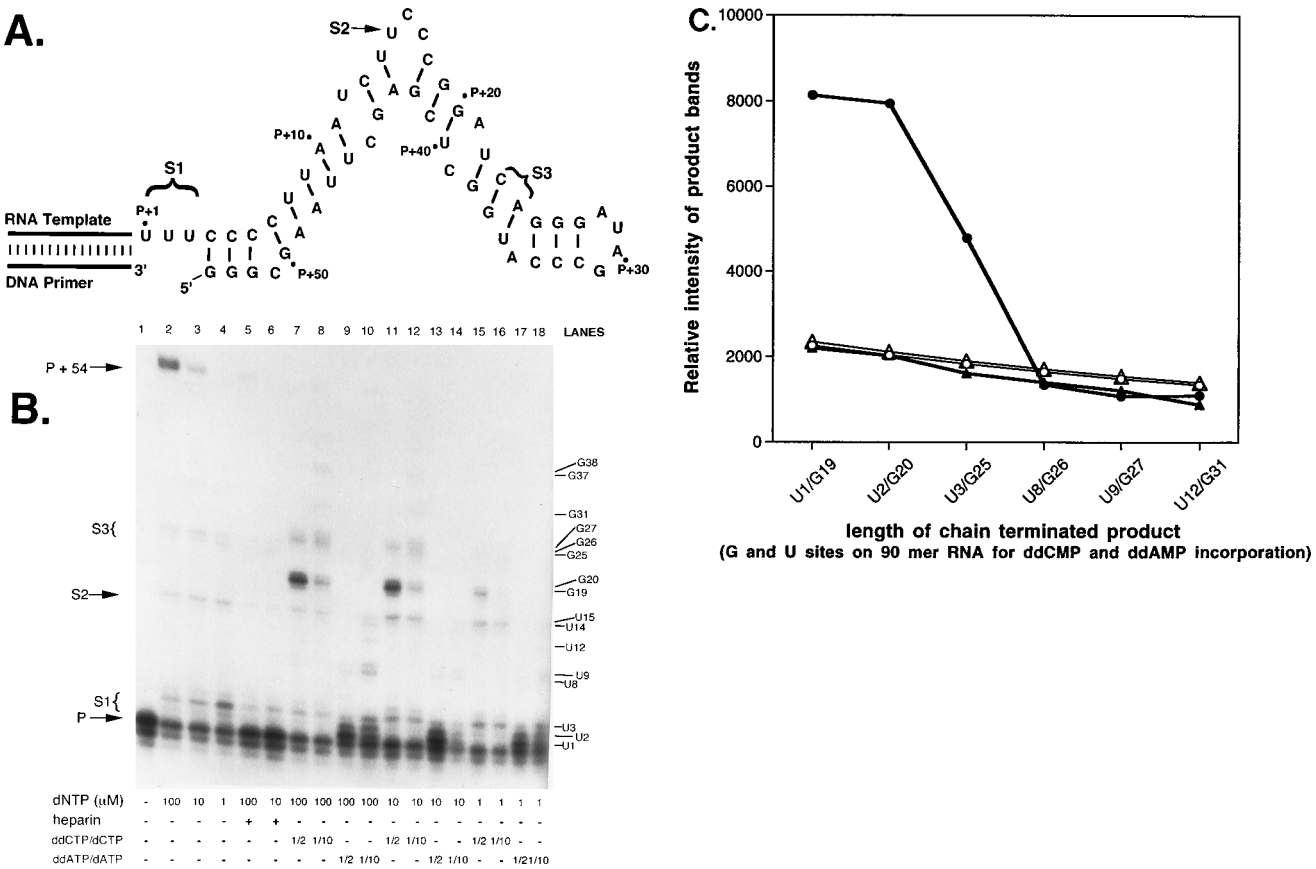

FIG. 5. Site-specific chain termination by ddNMP during RNA-dependent DNA polymerization. RNA-dependent DNA polymerization reactions were primed by a 36-nt DNA primer annealed to a 90-nt RNA template (14). (A) Schematic representation of the sequence and RNA secondary structure of the primer-template. The secondary structure of the RNA template was determined by RNase footprinting (23a). S1, S2, and S3 are the positions of RT pausing during RDDP on the RNA template. (B) Products of RDDP reactions performed in the presence of different dNTP concentrations and ddATP or ddCTP. Lanes: 1, mock reactions performed in the absence of RT; 2 to 4, products of reactions with 100, 10, and 1 μM dNTP, respectively; 5 and 6, reactions performed in the presence of 100 and 10 μM of dNTP plus 1 mg of heparin per ml added as a trap after preincubation of RT with primer-template; 7, 11, and 15, 100, 10, and 1 μM dNTP with ddGTP at 1/2 the dNTP concentration; 8, 12, and 16, 100, 10, and 1 μM dNTP with ddGTP at 1/10 the dNTP concentration; 9, 13, and 17, 100, 10, and 1 μM dNTP with ddATP at 1/2 the dNTP concentration; 10, 14, and 18, 100, 10, and 1 μM dNTP with ddATP at 1/10 the dNTP concentration. (C) Plot of the intensity of ddNMP chain-terminated bands, calculated from results of panel B, lanes 8 and 10 (▲ and ●, respectively) by phosphorimaging, and the predicted intensities of these chain-terminated products in these lanes (△ and □, respectively) at the complementary insertion sites in the RNA template.

Decreased dNTP concentrations and the use of dPR in place of $tRNA_3^{Lys}$ caused augmented chain termination by ddNTP (1/2 and 1/10 the concentration of dNTP) and decreased synthesis of minus-strand strong-stop DNA (Fig. 4B). Figure 4C shows an analysis of levels of minus-strand strong-stop DNA generated in the presence of ddNTP with respect to those produced in the absence of drug. Augmented chain termination was observed with all ddNTPs concomitant with the use of diminished concentrations of dNTP. Increased chain termination under these conditions was most apparent in reactions performed with the weak chain terminators, i.e., ddITP and 3TC-TP (Fig. 4C).

Effect of pausing by HIV-1 RT on chain termination by nucleoside analogs. To assess the effect of pausing on ddNMP-mediated chain termination, we used an RDDP assay with a DNA primer (36-mer) annealed to a short RNA template (90-mer), engineered to contain a defined pause site termed S1 (14). Figure 5A depicts this pause site, which is manifest during polymerization of the first 3 nt and is probably induced by a GC-rich duplex RNA structure that begins 4 nt downstream of the primer terminus (P+4). Three different dNTP concentrations (100, 10, and 1 μM) were used in this experiment (Fig. 5B, lanes 2 to 4). We found that use of low concentrations of dNTP led to increased pausing at each of three distinct pause sites, S1, S2, and S3 (Fig. 5B, lanes 2 to 4). By using a reaction time of 10 min at 37°C, we obtained equivalent levels of primer extension (±10%), independent of dNTP concentrations or the presence of ddATP or ddCTP (1/2 and 1/10 dNTP concentration) (Fig. 5B, lanes 2 to 4). However, the addition of heparin sulfate (1 mg/ml) to reaction mixtures containing 100 or 10 μM dNTP resulted in an accumulation of S1 products (Fig. 5B, lanes 5 and 6), suggesting a dissociation of HIV-1 RT from primer-template at this pause site. Increased levels of unextended primer resulted from a trapping of RT by heparin sulfate, which may have been caused by a dissociation of RT from primer-template.

In this primer extension assay, we also examined the extent of chain termination resulting from incorporation of ddCMP or ddAMP. The first three positions for incorporation of ddAMP on the RNA template (Fig. 5B, U1, U2, and U3) all fell within the S1 pause site. In contrast, positions of ddCMP incorporation, i.e., G19, G20, and G25, were not located near a pause site. More chain termination was effected by ddAMP

at positions U1, U2, and U3 than by ddCMP at positions G19, G20, and G25.

We next analyzed lanes 8 and 10 (reaction mixtures containing 100 μM dNTP and either 10 μM ddCTP or 10 μM ddATP, respectively) by phosphor-imaging. For nearly all ddNTPs, the catalytic efficiency of incorporation (K_{cat}/K_m) and the maximum rate of incorporation (V_{max}) by RT were greatly reduced from the values observed with dNTP (17, 29, 32). For comparison with actual levels of incorporation, a line was fitted for equal efficiencies of incorporation of ddAMP and dAMP or of ddCMP and dCMP (Fig. 5C). Reactions involving lower concentrations of dNTP (10 or 1 μM) were more difficult to quantitate because of reduced processivity of RT as shown above. The use of high concentrations of ddATP and ddCTP (i.e., 50 μM; half the dNTP concentration) resulted in near concordance between expected and actual amounts of chain termination at complementary sites on the RNA template (data not shown). However, when the concentration of ddATP was reduced to 1/10 that of dNTP, preferential chain termination by ddAMP was observed at uridine sites at positions +1, +2, and +3 from the primer terminus (U1, U2, and U3, respectively), i.e., an increase of nearly threefold from expected values (Fig. 5C). Consistent with this, chain termination was reduced slightly from expected values at sites U8, U9, and U12 (Fig. 5B and C). Conversely, no deviations between predicted and actual levels of chain termination were observed for ddCMP. These differences between ddAMP and ddCMP are probably because U1, U2, and U3 overlap a strong RT pause site (S1) on the RNA template whereas sites of ddCMP incorporation (with the exception of G25) are not located in proximity to a pause site.

DISCUSSION

The antiviral activity of nucleoside analogs is directed against the RT of HIV-1 (9, 39). However, phosphorylation of these compounds to their triphosphate form (ddNTP) is required for anti-RT activity, which is attributable to both competitive inhibition with native dNTP for binding to RT and chain termination of elongating DNA as a result of binding of 3′-hydroxyl-deficient ddNTP (19, 39).

We have examined the inhibitory effects of nucleoside analogs on HIV-1 reverse transcription in both quiescent and PHA-stimulated PBMC as well as in cell-free RT assays. We found that nucleoside analogs (AZT, ddI, or 3TC) exerted major reductions in synthesis of minus-strand strong-stop DNA in quiescent but not PHA-stimulated PBMC. This distinction may be attributable to increased dNTP pool sizes in PHA-stimulated cells, relative to quiescent cells, resulting in both increased levels of reverse transcription and length of viral DNA transcripts (10). Similar results were observed with monocyte-macrophage cultures treated with granulocyte-macrophage colony-stimulating factor (30, 31). Others have shown that diminution of dNTP concentrations, enacted via treatment with hydroxyurea, leads to inhibition of reverse transcription as well as to an increase in the antiviral activity of nucleoside analogs (10–12, 25). Hydroxyurea is an inhibitor of ribonucleotide reductase, an enzyme which catalyzes the reduction of ribonucleotides to deoxyribonucleotides.

There appears to be a strong relationship between dNTP concentration, pausing, the extent of reverse transcription, and the inhibitory effects of nucleoside analogs. Others have shown that treatment of PBMC with PHA led to a nearly 10-fold increase in levels of dNTP (5, 9, 10). Addition of nucleoside analogs did not lead to decreased phosphorylation of native nucleosides or to increased dNTP concentrations (5, 9, 10). Furthermore, with the exception of AZT-TP, ddNTP concentrations were only slightly higher in PHA-stimulated than quiescent PBMC, attesting to differences in phosphorylation kinetics between native nucleosides and their analogs (5, 12, 22). Therefore, the increased inhibition of minus-strand strong-stop DNA seen in quiescent PBMC may be due to decreased dNTP levels rather than to changes in levels of ddNTPs.

We also determined the effects of varying the dNTP concentrations on the chain termination effects of nucleoside analogs in cell-free reverse transcription reactions. The use of $tRNA_3^{Lys}$ in place of dPR as primer resulted in decreased pausing during synthesis of minus-strand strong-stop DNA, although similar quantities of dPR and $tRNA_3^{Lys}$ were annealed to the PBS RNA template (2). Diminished utilization of $tRNA_3^{Lys}$ in initiation of polymerization may result from secondary tRNA-RNA template interactions (outside the PBS) (21), which may hinder binding of the 3′ hydroxyl of $tRNA_3^{Lys}$ by RT. Decreased pausing with $tRNA_3^{Lys}$ as primer may also result from specific interactions between $tRNA_3^{Lys}$ and RT (6). We have observed threefold increases in the efficiency of the first template switch in reactions primed with $tRNA_3^{Lys}$ rather than dPR, suggesting that $tRNA_3^{Lys}$ may exert allosteric effects during early reverse transcription events (2). Finally, decreased dNTP concentrations resulted in both increased pausing and decreased levels of minus-strand strong-stop DNA. Decreased dNTP concentrations may result in a lower polymerization constant (k_{pol}), decreased processivity, and increased dissociation of enzyme from primer-template (k_{off}). Diminished synthesis of minus-strand strong-stop DNA is probably due to decreased processivity and k_{pol} of RT. Augmented pausing by HIV-1 RT, as a result of low dNTP concentrations, has been observed previously (20).

We have observed chain termination in cell-free RT assays in the presence of each of ddCTP, ddATP, AZT-TP, 3TC-TP, and ddITP at 1/2 and 1/10 of native dNTP concentrations; however, 3TC-TP and ddITP resulted in lower efficiencies of chain termination than the others did (15). ddI is deaminated and phosphorylated by adenosine deaminase and purine nucleoside kinases to yield ddATP; ddIMP is thought to be a poor substrate for further phosphorylation and for the intracellular synthesis of ddITP (22). The data obtained for 3TC-TP in cell-free assays are in contrast to results showing high levels of inhibition of minus-strand strong-stop DNA in quiescent PBMC (15, 16). Increased anti-RT activity of 3TC in quiescent PBMC may be due to several factors including uptake of ddN by cells (i.e., passive diffusion or active transport), stability of the compound in the cell, relative rate of phosphorylation to ddNTP, degradation of ddNTP, and relative binding efficiency of ddNTP to RT. In general, therefore, effective antiviral concentrations of unphosphorylated nucleoside analogs in tissue culture may not always correspond to effective anti-RT activities of corresponding ddNTPs in cell-free reverse transcription reactions.

Decreased concentrations of dNTP resulted in augmented chain termination by ddNMP, which was most apparent with the weak chain terminators (i.e., ddITP and 3TC-TP). In addition, increased chain termination by ddNMP was observed when reactions were primed with dPR in place of $tRNA_3^{Lys}$. In both these cases, we found an increase in pausing by HIV RT. Incorporation of ddNMP by RT and subsequent chain termination were found to be increased at a strong pause site during polymerization, suggesting an association between pausing, binding of ddNTP, and incorporation of ddNMP by HIV-1 RT.

The catalytic efficiency (K_{cat}/K_m) and maximal rate (V_{max}) for incorporation of a dNMP are significantly greater than those for incorporation of a ddNMP (analog) (17, 29). At

submaximal rates of polymerization (i.e., pausing), V_{max} values for incorporation of ddNMP or dNMP no longer apply. Under these conditions, incorporation of dNMP or ddNMP is probably determined by other parameters, e.g., binding affinities of dNTP and ddNTP to RT and efficiencies of phosphorolysis of bound dNTP or ddNTP. Previous studies on K_m and V_{max} values of ddNTP and dNTP did not employ heteropolymeric templates with a distinct pause site (17, 32, 39). Interestingly, pausing induced by homopolymeric sequences occurred at substitution mutations within the HIV-1 RNA genome (7). Thus, conformational changes in HIV-1 RT may lead to indiscriminate binding of incorrect dNTPs or ddNTPs.

Decreased dNTP concentrations also resulted in increased pausing by HIV-1 RT. Many pause sites are also sites of primer-template dissociation. Thus, it is not surprising that increased HIV DNA synthesis occurred in PHA-stimulated PBMC compared with quiescent PBMC grown in high concentrations of dNTPs. If chain termination by nucleoside analogs is directly related to pausing by HIV-1 RT, it follows that increased pausing might augment such events. Hydroxyurea has been shown to decrease intracellular concentrations of dNTP and to act synergistically with nucleoside analogs to promote inhibition of HIV-1 replication (11, 25). Drugs that disrupt primer–template–HIV-1 RT complexes may also increase pausing by RT during polymerization and show synergy with nucleoside analogs. Nevirapine, synergistic with AZT in inhibition of HIV-1 replication, is thought to inhibit the binding of RT to the primer-template (33).

We have previously observed that nucleoside analog-mediated chain termination occurred preferentially after the first template switch in HIV-1-exposed Jurkat cells (4). Synthesis of minus-strand HIV-1 DNA after this switch involves a second RNA-dependent DNA polymerization priming event that utilizes minus-strand strong-stop DNA as a primer. In this study, we have shown that dPR, when used as primer, initiated a less processive reaction than did $tRNA_3^{Lys}$. Priming of minus-strand HIV-1 DNA by minus-strand strong-stop DNA can be likened to a dPR priming event from the PBS. This would explain the selective incorporation and chain termination by nucleoside analogs following the first template switch in HIV infection of activated $CD4^+$ lymphocytes.

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