Actinomycin D Inhibition of DNA Strand Transfer Reactions Catalyzed by HIV-1 Reverse Transcriptase and Nucleocapsid Protein†

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ABSTRACT: Actinomycin D was found to be a potent inhibitor of HIV-1 reverse transcriptase catalyzed DNA strand transfer reactions. Using an oligonucleotide model system, actinomycin D inhibition of DNA strand transfer was examined to elucidate the mechanism of inhibition and further define the mechanism of DNA strand transfer. Our results show that actinomycin D inhibits HIV-1 reverse transcriptase catalyzed DNA strand transfer without inhibiting RNA-dependent or DNA-dependent DNA polymerase activity. Actinomycin D was found to strongly inhibit annealing of a primary DNA product to the DNA acceptor template, preventing the formation of a key reaction intermediate. The HIV-1 nucleocapsid protein has been shown to participate in catalytic events during reverse transcription including DNA strand transfer. Recombinant nucleocapsid protein was used in conjunction with actinomycin D in this model system to investigate how NC may participate in the mechanism of inhibition by actinomycin D and in DNA strand transfer. The inclusion of nucleocapsid protein was found to partially relieve both DNA annealing and strand transfer inhibition caused by actinomycin D. This study suggests a potential new mechanism for inhibiting retroviral replication by preventing the formation of replication intermediates.

One of the greatest obstacles in the treatment of HIV[1] infection and AIDS is the rapid development of HIV-1 viral drug resistance due to the great variability that develops in the retroviral genome (*1*, *2*). Significant evidence shows that much of this genetic variability is acquired during retroviral reverse transcription, a process catalyzed by the virally encoded enzyme reverse transcriptase (RT). The two principle mechanisms responsible for the rapid evolution of the retroviral genome include nucleotide misincorporation by HIV-1 RT during proviral DNA synthesis, and recombination events occurring during reverse transcription (*3*). HIV-1 RT is involved in both of these processes.

HIV-1 RT is a multifunctional enzyme that can catalyze RNA-dependent DNA polymerization and DNA-dependent DNA polymerization as well as the efficient hydrolysis of RNA from RNA·DNA hybrids (*4*, *5*). The combination of these functions is required for two obligatory DNA strand transfer reactions that occur during the reverse transcription of single-stranded viral RNA into double-stranded proviral DNA. DNA strand transfer is the process by which a nascent DNA strand synthesized by a reverse transcriptase is transferred from one RNA template (donor template) to a second RNA or DNA template (acceptor template).

Reverse transcriptase initiates minus strand DNA synthesis near the 5′-end of the genomic RNA. DNA synthesis continues to the end of the viral RNA genome whereupon the nascent DNA strand (strong-stop DNA) undergoes strand transfer catalyzed by reverse transcriptase and other viral proteins. This strand transfer event is directed by the terminal repeat sequences (r) located on both ends of the RNA and requires the RNase H activity of reverse transcriptase (*6*). Minus strand DNA synthesis is completed by the RNA-dependent DNA polymerase activity of reverse transcriptase, during which the RNase H activity is actively hydrolyzing the genomic RNA strand. Plus strand DNA synthesis is initiated at an RNase H-resistant RNA oligonucleotide called the polypurine tract located near the 5′-end of the minus strand DNA. DNA-dependent DNA polymerization proceeds, and a second DNA strand transfer occurs, facilitated by the removal of the tRNA primer catalyzed by the RNase H function of RT (*2*, *7*).

Although a great deal is known about the process of DNA strand transfer from both enzymological and viral studies, the identities of many of the DNA strand transfer intermediates are largely unknown. One particular model for the first DNA strand transfer describes the process occurring via an RNA·DNA·RNA intermediate facilitated by RT (*6*, *8*). Another model suggests that after pausing, RT must disassociate from the RNA·DNA hybrid, and then rebind after annealing of the nascent DNA to the acceptor template has occurred (*9*). It has also been proposed that DNA strand

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[1] Abbreviations: HIV-1, human immunodeficiency virus type-1; AIDS, acquired immunodeficiency syndrome; RNase H, ribonuclease H; NC, nucleocapsid protein; RT, reverse transcriptase; EDTA, ethylenediaminetetraacetic acid; TBE, Tris−boric acid−EDTA buffer; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; IPTG, isopropyl β-D-thiogalactopyranoside; DMSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis.

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transfer occurs as a multiprotein complex in the presence of HIV-1 nucleocapsid protein (NC) (*10*−*17*) since high concentrations of NC are present in the virion and NC has been shown to modulate RT-catalyzed strand transfer reactions *in vitro* (*13, 15, 18, 19*).

HIV-1 nucleocapsid protein is a major structural protein of the virus which is processed from a polyprotein precursor encoded by the *gag* gene (*20, 21*). NC is a very basic protein of 7 kDa that contains two zinc finger binding domains with the $CysX_2CysX_4HisX_4Cys$ motif (*22*−*24*). NC binds tightly to both single-stranded DNA and RNA with moderate cooperativity (*25, 26*) and has been found to accelerate the annealing of complementary DNA (*27*) as well as catalyze the efficient DNA strand exchange between duplex and complementary single-stranded DNA [(*28*) and W. Davis, unpublished results]. Although the role of NC in viral replication and infectivity is not fully clear, recent evidence suggests that NC does play some role in the catalysis of viral genomic RNA dimerization for encapsidation (*29*−*34*), in annealing the tRNA primer to the primer binding site (PBS) on the genomic RNA (*25, 31, 32, 35, 36*), and in DNA strand transfer (*13, 15, 17*).

To further investigate the mechanism of the DNA strand transfer process, we have begun to identify inhibitors of this reaction by using mass screening methods [(*37*) and unpublished results]. From this screen, we identified actinomycin D, a well-characterized anticancer antibiotic drug (*38*), as a potent inhibitor of DNA strand transfer. The antitumor activity of actinomycin D was originally reported to be caused by direct intercalation of actinomycin D into double-stranded DNA complexes (*39*) with a high affinity for GpC steps (*40*−*45*). Binding to these sites occurs through the intercalation of the phenoxazinone ring into the GpC step with the polypeptide portions of the molecule binding in the minor groove and hydrogen bonding to guanines (*38*). Actinomycin D has been shown to inhibit transcription elongation (*46*−*49*), DNA-dependent DNA polymerization (but not RNA-dependent DNA polymerization) (*50*−*53*), and DNA·DNA and DNA·RNA but not RNA·RNA annealing (*54*). Reports that actinomycin D binds single-stranded DNA with high affinity (*42, 55, 56*) have resulted in a reexamination of its mode of action and suggest that it might inhibit transcription or other types of elongation by binding directly to free portions of single-stranded DNA. It has been shown that actinomycin D is capable of generating pauses during DNA-dependent DNA synthesis by several DNA polymerases, including HIV-1 reverse transcriptase (*57*). These results suggest that the development of molecules that bind single-stranded DNA might be useful as drugs against retroviruses since their replication proceeds through a single-stranded DNA intermediate.

This study focuses on elucidating the mechanism of DNA strand transfer inhibition by actinomycin D. Actinomycin D was found to inhibit DNA strand transfer at much lower concentrations than previously reported for the inhibition of DNA polymerase activity. Actinomycin D inhibition of strand transfer was shown not to involve DNA·RNA or DNA·DNA extension inhibition or pausing. Instead, actinomycin D was found to inhibit annealing of primary DNA product (before transfer) to a DNA acceptor template. We confirmed this observation by the inclusion of HIV-1 nucleocapsid protein in both annealing and strand transfer reactions to determine if NC could rescue these activities. Not only did NC relieve the annealing inhibition but it also relieved the DNA strand transfer inhibition by actinomycin D. These results shed new light on the mechanism of actinomycin D function, the mechanism of DNA strand transfer, and the possible use of DNA binding drugs as new inhibitors of HIV-1 RT catalyzed reverse transcription.

MATERIALS AND METHODS

Materials. HIV-1 NC71 was purified as described previously (*58*). The sequences of oligonucleotides used in the strand transfer and annealing assays are as follows: 22-base DNA primer, 5′-GCATCTGGGGCTCGCAAATTTG-3′; 40-base RNA template, 3′-CGUAGACCCCGAGCGUUUAAACAAUAGUAGAGUGAGUGGA-5′; 43-base DNA acceptor, 3′-ACAATAGTAGAGTGAGTGGATCATGCGTCTTAAGTTCGTCGAG-5′; 40-base primary product DNA, 5′-GCATCTGGGGCTCGCAAATTTGTTATCATCTCACTCACCT-3′.

DNA was synthesized by Gibco/BRL Technologies or the University of Michigan Core DNA Synthesis Facility. Oligonucleotides were purified by denaturing PAGE through 20% acrylamide/8 M urea/TBE (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) gels and eluted from excised gel slices by electroelution. Eluted DNA was concentrated and desalted using C18 Sep-Pak columns (Waters). DNA was ^{32}P-5′-labeled using [γ-^{32}P]ATP (NEN) and polynucleotide kinase according to standard procedures (*59*). RNA synthesis was performed using standard phosphoramidite solid-phase chemistry methods on an Applied Biosystems nucleic acid synthesizer (*60*). The RNA template was deprotected (*60*) and then purified as described above for DNA except that the RNA was eluted from excised gel pieces with 0.5 M sodium acetate, pH 5.2. Oligonucleotides were quantitated by their absorbance at 260 nm based on their calculated extinction coefficients.

All buffers were prepared from RNase-free reagents that were of the highest quality obtainable. A solution of actinomycin D (Sigma, A-1410) was diluted in DMSO and the concentration determined by its absorbance at 440 nm using the extinction coefficient $\epsilon = 24\,450\ M^{-1}$ (*61*).

Purification of HIV-1 RT. The p66 and p51 subunits of HIV-1 RT were separately expressed in *E. coli* JM109 using a synthetic gene (N. Santoro, unpublished) for RT under the control of the tac promoter. Cells were grown at 37 °C in 1 L of superbroth supplemented with ampicillin, and grown to an OD_{600} of 0.5−0.8. The cultures were induced with IPTG (0.4 mM) for 4 h at 37 °C. Cells were pelleted, and the p66- and p51-expressing cell pellets were combined during resuspension in 15 mL of lysis buffer (50 mM Tris, pH 8.0, 1 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100). A mixture of 100 units of DNase, 100 μg of RNase, 150 μg of aprotinin/leupeptin, 1 mM PMSF (final), and 100 mg of lyzozyme was added, and the cells were then incubated for 10 min at room temperature. A solution of 5 M NaCl was then added to a final concentration of 0.5 M and centrifuged for 10 min at 13 000 rpm, and the supernatant was removed. The resulting pellet was resuspended in 1 mL of 500 mM EDTA, pH 8.3, and 10 mL of MES extraction buffer (50 mM MES, pH 6.0, 10% glycerol, 2 mM EDTA, pH 8.3, 0.02% hexyl glucopyranoside) and incubated at room tem-

perature for 10 min. The mixture was then centrifuged, combined with the first supernatant, and dialyzed overnight at 4 °C against 2 L of buffer A (50 mM Tris, pH 7.5, 50 mM KCl, 2% glycerol, 1 mM DTT, 0.01% PMSF).

The dialysate was loaded onto a DEAE Sepharose FF column (4 °C) preequilibrated with buffer A and washed with 2.5 column volumes of buffer A. The flow-through fractions containing HIV-1 RT dimers composed of equal quantities of p66 and p51 were pooled. These fractions were loaded onto a 45 mL heparin affinity column preequilibrated with buffer B (10 mM NaP_i, pH 7.3, 1 mM DTT, 1 mM EDTA, pH 8.3, 2% glycerol, 150 mM NaCl). The column was washed with buffer B and eluted with buffer B containing 450 mM NaCl. Fractions containing RT were pooled and concentrated using a Centriprep-50 concentrator (Amicon) and dialyzed overnight in a 15 mL dialysis cassette (Pierce) against 2 L of buffer C (20 mM Tris, pH 7.8, 1 mM EDTA, pH 8.3, 1 mM DTT). Dialysate was then loaded onto a BioScale S-10 column (BioRad) and eluted using a 90 mL gradient of buffer C containing 0−500 mM KCl at a flow rate of 3 mL/min (3 mL fractions). Fractions containing heterodimer HIV-1 RT were pooled and concentrated with Centricon-50 concentrators to 1.0 mL. The concentrate was then loaded onto a Superose-12 column preequilibrated and eluted with buffer C containing 50 mM KCl. Fractions containing pure HIV-1 RT were pooled, and glycerol was added to 50% (v/v). Pure HIV-1 RT, judged homogeneous by SDS−PAGE, was stored at −20 °C.

DNA Strand Transfer Reactions. A reaction mixture containing reaction buffer (63 mM Tris-HCl, pH 7.6, 60 mM KCl, 0.3 mM DTT), 20 nM ^{32}P-5′-end-labeled 22-base DNA primer·40-mer RNA template duplex, 400 nM 43-base DNA acceptor template, 20 nM HIV-1 RT, and varying amounts of actinomycin D (in DMSO, 4% final DMSO concentration) was preincubated at 37 °C for 5 min. Some of the reactions also contained varying amounts of NC71 in the preincubation mixture. Reactions were initiated by the addition of 0.1 mM of each dNTP and 10 mM $MgCl_2$ (final concentrations). Samples were taken at the times indicated, quenched into 50 mM EDTA/denaturing load buffer, and resolved by denaturing PAGE (20% acrylamide/8 M urea/TBE). Bands were visualized using a Molecular Dynamics PhosphorImager and quantitated using Imagequant 1.1 software.

RNA·DNA template-primer extension experiments were performed as described above except the 43-base acceptor template was absent from the reactions. DNA·DNA extension experiments were performed as above except a DNA·DNA template-primer consisting of the 40-base primary DNA product·43-base acceptor template (transfer intermediate, Figure 1) was used instead of the RNA·DNA template-primer system.

Effect of Actinomycin D on HIV-1 RT RNase H Activity. To determine if RNase H activity is inhibited by actinomycin D, the 40-base RNA primary template was ^{32}P-5′-end-labeled, and the rate and pattern of RNA degradation were examined (*6*). The 40-base RNA template was ^{32}P-5′-end-labeled using [γ-^{32}P]ATP and polynucleotide kinase according to standard methods (*59*) except that the labeling reaction was incubated for 5 min prior to heat inactivation to minimize RNA degradation. RNase H activity experiments were performed as described for the DNA strand transfer reactions above except the label was now on the RNA template. Final

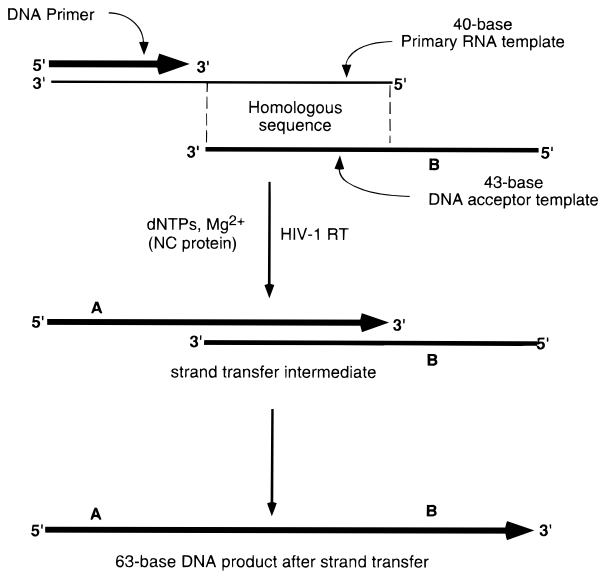

FIGURE 1: DNA strand transfer assay system. A template·primer system consisting of a 22-base primer annealed to a 40-base primary RNA template was used in the presence of a 43-base DNA acceptor that is homologous to the 20 bases at the 5′-end of the primary RNA template, and containing an additional 23 bases. The DNA primer was radioactively labeled at its 5′-end, annealed to the template RNA, and preincubated in the presence of HIV-1 RT and other necessary components as described under Materials and Methods. Reactions were initiated with dNTPs and $MgCl_2$, aliquots were withdrawn, quenched in EDTA, and analyzed with denaturing PAGE. DNA strand transfer is indicated by the production of a 63-base DNA product.

reaction conditions included 20 nM ^{32}P-5′-labeled 40-base RNA template·22-base DNA primer, 20 nM HIV-1 RT, 400 nM 43-base DNA acceptor template, 0.1 mM of each dNTP, 10 mM $MgCl_2$, 0 or 1 μM NC71, and 0, 2.5, or 20 μM actinomycin D. Products were resolved by denaturing PAGE (20% acrylamide/8 M urea/TBE) and quantitated as described previously (*6*) using a Molecular Dynamics PhosphorImager and Imagequant 1.1 software.

DNA·DNA Annealing Reactions. A reaction mixture containing reaction buffer, 20 nM ^{32}P-5′-end-labeled 40-mer DNA primary product, 10 mM $MgCl_2$, and 0.1 mM of each dNTP was preincubated at 37 °C for 5 min. Reactions were initiated by the addition of 400 nM 43-base DNA acceptor, and varying concentrations of actinomycin D and NC71. Actinomycin D was added to the DNA acceptor no more than 30 s before the reactions were initiated. Samples were quenched at reaction times varying from 5 ms to 12 s into a mixture of 2.5 μM nonlabeled 40-base full-length extension product, 0.52% SDS, and 26 mM EDTA. Nondenaturing load buffer was added to the quenched samples, and the products were resolved by 10% nondenaturing PAGE. Duplex DNA was well resolved from the remaining single-stranded DNA. The concentration of duplex DNA was quantitated by phosphoimager analysis. Background annealing rates resulting from trap leakage were determined by the addition of 2.5 μM nonlabeled DNA trap before the reaction was initiated. The resulting background was subtracted from the annealing data. For reactions that required time points of less than 6 s, assays were performed on a Kin-Tek Model RQF-3 Quench-Flow Apparatus at 37 °C. Second-order rate constants for annealing were calculated using the initial rate method (*62*).

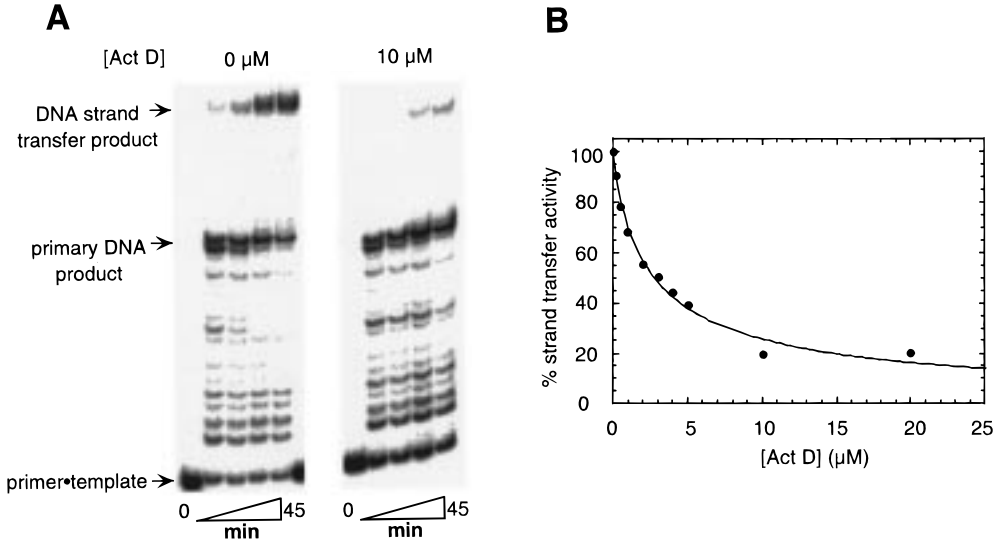

FIGURE 2: Effect of actinomycin D on DNA strand transfer. Reactions were performed as described under Materials and Methods with increasing amounts of actinomycin D at final concentrations of 0, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 10.0, and 20.0 μM. Actinomycin D was added after the addition of all other components during the preincubation. After initiation, samples were withdrawn at 0, 5, 10, 25, and 45 min and analyzed by PAGE. (A) Gel analysis of DNA strand transfer products in the presence of actinomycin D. Representative autoradiogram of two reaction time courses (0 and 10 μM actinomycin D). The amount of DNA strand transfer product was quantitated by phosphoimager analysis, and initial rates were determined. (B) The concentration dependence of DNA strand transfer inhibition by actinomycin D is shown. The percent of DNA strand transfer activity (0 μM actinomycin D representing 100% activity) is plotted as a function of the total concentration of actinomycin D. The data were fit to the equation: % activity $= A_{max}/(1 + \{[\text{Act D}]/IC_{50})\}^n)$. The IC_{50} for actinomycin D inhibition of DNA strand transfer in this experiment was calculated to be 2.7 $\pm$ 0.3 μM.

RESULTS

Effect of Actinomycin D on DNA Strand Transfer Efficiency. A DNA strand transfer model system consisting of DNA and RNA oligonucleotides (Figure 1) was used to quantitate the inhibition of DNA strand transfer by actinomycin D (Figure 2). Figure 2A shows a PAGE gel of a representative DNA strand transfer reaction in the presence and absence of actinomycin D. The concentration dependence of actinomycin D inhibition of DNA strand transfer product synthesis is shown in Figure 2B. DNA strand transfer is slow compared to polymerization (*6*) as indicated by the rapid synthesis of the primary DNA product, representing DNA synthesis out to the end of the primary RNA template. DNA strand transfer product is detected at longer times after the synthesis of primary DNA product. Inhibition of DNA strand transfer by actinomycin D is concentration dependent with an IC_{50} of 2.7 μM, and is capable of inhibiting ~85% of the strand transfer reaction at saturation (Figure 2B). The mechanism of inhibition of DNA strand transfer by actinomycin D does not appear to be the inhibition of polymerase activity, since primary DNA product is made with high efficiency even in the presence of high concentrations of inhibitor (Figure 2A and below). At high concentrations of actinomycin D (>10 μM), some pausing and a slightly lower efficiency of primer extension during DNA polymerization were observed. However, this inhibition was not observed at lower actinomycin concentrations, under conditions where DNA strand transfer production was strongly inhibited.

Effect of Actinomycin D on DNA Extension before and after Strand Transfer. To further examine whether actinomycin D inhibition of DNA extension by the polymerase activity of HIV-1 RT is responsible for the inhibition of DNA strand transfer, DNA polymerase assays were performed in the absence of DNA strand transfer (Figure 3). Two different polymerase reactions are required for the production of DNA strand transfer product: (1) synthesis of primary DNA product using the primary RNA as a template; and (2) extension of the strand transfer DNA primer·DNA acceptor template intermediate (Figure 1). Both DNA extension reactions were examined in the presence of actinomycin D (Figure 3)

In the absence of actinomycin D, full-length extension of the RNA·DNA template·primer (Figure 3A) is complete by the first time point at 5 min. At an actinomycin D concentration of 2.5 μM (~IC_{50} of strand transfer inhibition), extension is also complete within 5 min and is effectively not inhibited (97.5% of the negative control). Even at 20 μM actinomycin D, RNA:DNA extension is only down to 83% of the negative control, suggesting that inhibition of this first extension step contributes very little to the overall inhibition of strand transfer and cannot account for the decrease of strand transfer product observed in Figure 2.

Inhibition of DNA-dependent DNA polymerase activity by actinomycin D was examined using the putative DNA strand transfer intermediate consisting of the 40-base primary DNA extension product annealed to the 43-base DNA acceptor under the same enzyme and nucleotide conditions as used in the strand transfer reactions (Figure 2B). Actinomycin D did not inhibit full-length extension at concentrations of either 2.5 μM or 20 μM. These studies show that inhibition of HIV-1 RT DNA polymerase activity by actinomycin D is not responsible for the observed inhibition of DNA strand transfer.

Effect of Nucleocapsid Protein on the Inhibition of DNA Annealing by Actinomycin D. While the identities of the intermediates during DNA strand transfer have not been completely characterized, strand transfer must include an annealing of nascent primary DNA product to the acceptor template to form the strand transfer intermediate shown in

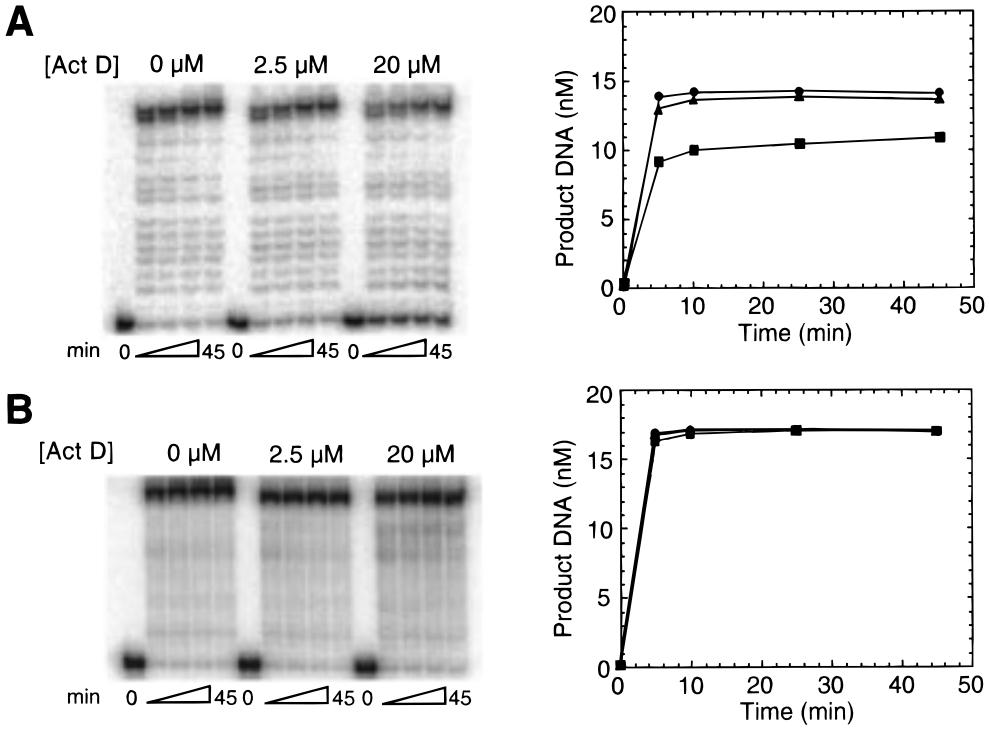

FIGURE 3: Effect of actinomycin D on RNA·DNA and DNA·DNA template·primer extension by HIV-1 RT. (A) PAGE autoradiogram and graphical analysis of RNA·DNA extension products in the presence of actinomycin D. This half-reaction represents DNA synthesis before DNA strand transfer (see Figure 1). Reactions were performed as described under Materials and Methods. Actinomycin D was added to final concentrations of 0 (●), 2.5 (▲), and 20.0 (■) μM. Reaction samples were withdrawn and quenched at 0, 5, 10, 25, and 45 min. (B) PAGE autoradiogram and graphical analysis of DNA·DNA extension products in the presence of actinomycin D. This represents DNA synthesis after DNA strand transfer. Reactions were performed as in (A) with the exception of the template·primer used (see Materials and Methods).

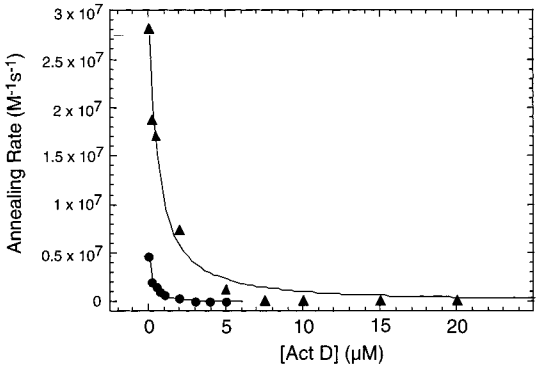

FIGURE 4: Effect of actinomycin D on DNA annealing in the presence or absence of NC. Reactions were performed as detailed under Materials and Methods. Annealing rates were determined from initial annealing rates at increasing concentrations of actinomycin D in the absence (●) or presence (▲) of 1 μM NC. The IC_{50} for actinomycin D annealing inhibition is 0.22 μM in the absence of NC, and 0.64 μM in the presence of NC.

Figure 1. Whether this annealing step occurs in solution or as part of a larger complex with RT and other viral proteins is not known. However, since strand transfer requires this annealing step, it is a possible target for inhibition by actinomycin D. We tested this possibility by examining the annealing of single-stranded primary DNA product to the DNA acceptor template in the presence of inhibitor (Figure 4).

In the absence of actinomycin D, annealing of the complementary DNA was rapid with rates of $\sim 5 \times 10^6$ M^{-1} s^{-1}. Actinomycin D is a potent inhibitor of DNA annealing (Figure 4). Inhibition was dependent on the concentration of actinomycin D, with an observed $IC_{50} = 0.22$ μM. At an actinomycin concentration of 5 μM, the annealing rate was reduced nearly 500-fold (1×10^4 M^{-1} s^{-1}). The IC_{50} of annealing inhibition is approximately one order of magnitude lower than the IC_{50} value for strand transfer inhibition (2.7 μM).

The nucleocapsid protein (NC) of HIV-1 is known to accelerate the annealing of complementary DNA strands (*27*). Since actinomycin D inhibits DNA annealing, we sought to determine if NC could modulate this inhibition. In the presence of NC, a dramatic acceleration of annealing was observed (Figure 4). This annealing was still sensitive to actinomycin inhibition with an $IC_{50} = 0.64$ μM. However, due to the intrinsically fast annealing rates in the presence of NC, much higher concentrations of actinomycin D are required to inhibit the annealing reaction in the presence of NC to those rates observed in the absence of NC.

Effect of NC on DNA Strand Transfer and Inhibition by Actinomycin D. NC is a known enhancer of HIV-1 RT catalyzed DNA strand transfer *in vitro* (*13*, *17*, *19*). To investigate the effect of NC on actinomycin D inhibition of DNA strand transfer, we first found conditions that would optimize the NC-mediated strand transfer enhancement under our assay conditions. Figure 5 shows that NC71 does enhance DNA strand transfer in a concentration-dependent fashion. Strand transfer rates increased linearly with increasing NC up to 1 μM, and additional NC inhibited the reaction slightly. At the optimal concentration of NC, the initial rate of strand transfer was increased ~2-fold compared to the negative control. At the higher concentration of NC (>1.0 μM), NC inhibited extension of the DNA·RNA duplex by

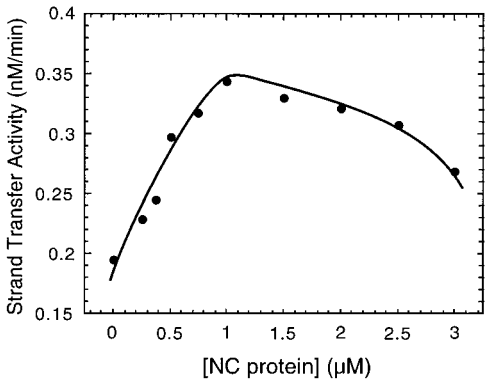

FIGURE 5: Dependence of DNA strand transfer on NC concentration. Reactions were performed as described under Materials and Methods except that increasing concentrations of NC71 were added during preincubation as indicated. DNA strand transfer products were determined as described in Figure 1, and the initial rates were plotted against the total NC concentration.

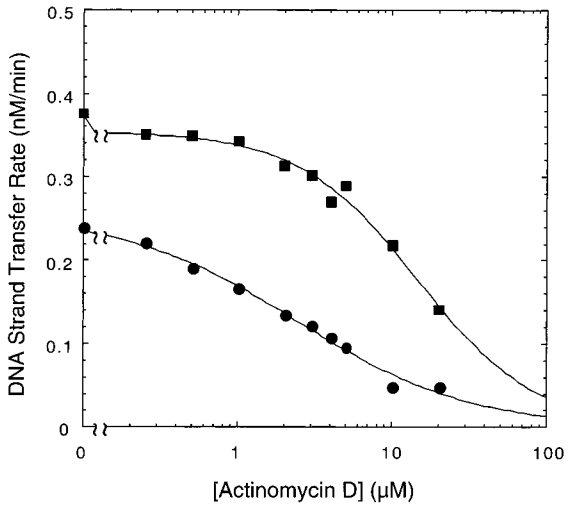

FIGURE 6: Effect of nucleocapsid protein on actinomycin D inhibited DNA strand transfer. Reactions were performed as described under Materials and Methods either in the absence or in the presence of 1 μM NC. Strand transfer products were determined as described in Figure 1. The initial rates of strand transfer were plotted on a semilog plot against actinomycin D concentration, and the IC_{50} concentration was determined for each curve: 2.7 μM in the absence of NC (●), and 13.8 μM in the presence of NC (■).

RT as evidenced by the accumulation of template·primer (data not shown).

Since NC was found to increase DNA annealing rates in the presence of actinomycin D (Figure 4), the ability of NC to rescue DNA strand transfer inhibition was examined. Figure 6 shows the effect NC has on strand transfer inhibition by actinomycin D. When NC was added to the strand transfer reaction in the absence of actinomycin D, the activity was increased by 60%. The inclusion of NC in the DNA strand transfer reaction caused a dramatic shift in the inhibition concentration response curve. DNA strand transfer was significantly less sensitive to inhibition by actinomycin with the inclusion of NC, with an IC_{50} of 13.8 μM compared to 2.7 μM in the absence of NC. These results show that NC can suppress, but not completely eliminate, the inhibitory effect of actinomycin on HIV-1 RT catalyzed DNA strand transfer.

DISCUSSION

In this paper, we describe a new approach for the inhibition of retroviral reverse transcription. While many current therapeutic drugs target the DNA polymerase activity of HIV-1 RT, we show here that DNA strand transfer reactions could also be a target for inhibitor development. Such inhibitors might serve as therapeutic agents, as well as useful reagents to help elucidate the mechanism of this important reaction. We show for the first time that DNA strand transfer reactions can be inhibited in a mechanism independent of DNA polymerase inhibition.

DNA strand transfer during HIV-1 retroviral reverse transcription is obligatory for the production of proviral DNA product (*63*). In addition, due to the diploid nature of the viral genome, strand transfer can occur essentially randomly at internal positions of the genome during minus and plus strand synthesis, resulting in recombinant proviral products (*64*). In combination with the low fidelity of DNA synthesis by reverse transcriptase, these events result in a rapidly changing genome which in turn gives rise to rapid drug resistance and the ability to avoid the host's immune response.

We have developed an automated DNA strand transfer assay which was used to screen a chemical library for compounds capable of specifically inhibiting the HIV-1 RT catalyzed DNA strand transfer reaction [(*37*) and unpublished]. From this screen, we identified several such inhibitors, including the antibacterial/cancer drug actinomycin D (*38*). Since actinomycin D has been shown to inhibit transcription (*46*−*49*), and DNA-dependent DNA polymerization by assorted polymerases (*50*−*53*), it was not surprising that this particular drug might be identified as an inhibitor of HIV-1 reverse transcriptase. However, it appears that the mechanism of inhibition of DNA strand transfer is independent of previously characterized actinomycin D inhibition mechanisms. Actinomycin D could potentially interfere with one or more steps of the DNA strand transfer reaction: (1) the RNA-dependent DNA synthesis reaction that occurs before strand transfer; (2) the RNase H activity of RT, which is necessary for efficient strand transfer to occur (*6*); (3) the annealing or exchange of the nascent DNA strand onto the acceptor template; or (4) the DNA-dependent DNA polymerase synthesis reaction that occurs after DNA strand transfer.

The inhibition of HIV-1 RT DNA polymerase activity by actinomycin D was tested directly using DNA primer extension assays with both DNA and RNA templates (Figure 3). These studies show that actinomycin D exhibits little or no inhibition of DNA synthesis at concentrations less than 10 μM (Figure 3). Both RNA-dependent DNA polymerase and DNA-dependent DNA polymerase extension were examined using concentrations of actinomycin D up to approximately 10 times the observed strand transfer IC_{50}. These two extension reactions represent polymerization steps before and after strand transfer, respectively. Only the RNA·DNA extension reaction showed slight inhibition by high actinomycin D concentrations (15% at 20 μM), inconsistent with the IC_{50} (2.7 μM) observed for strand transfer inhibition. Interestingly, DNA·DNA template·primer extension was not inhibited at all, even at 20 μM actinomycin D. These

extension experiments also show that no polymerase pause sites are generated by inclusion of actinomycin D which would be indicated by strong bands prior to the production of full RNA·DNA or DNA·DNA product. This is in contrast to earlier reports showing actinomycin D inhibition of DNA-dependent DNA polymerase activity (*57*), but these assays were performed under very different reaction conditions. In our assays, much higher concentrations of RT are present, at concentrations more consistent with the viral core where HIV-1 RT is present in large excess over viral RNA (*16*). These higher HIV-1 RT concentrations could serve to bypass polymerase pause sites caused by DNA−actinomycin D interactions. Second, our results show that DNA strand transfer is sensitive to actinomycin D inhibition (1−5 μM) at concentrations significantly lower than those required for DNA polymerase inhibition [>30 μM, (*57*)]. This is also consistent with our polymerase extension assays (Figure 3) which showed DNA polymerase inhibition was observable only at concentrations of 20 μM actinomycin D (Figure 3, unpublished results). So, while other reports have shown that actinomycin D inhibits the DNA polymerase activity of HIV-1 RT, we do not observe this inhibition under our DNA strand transfer inhibition conditions. Inhibition of HIV-1 RT catalyzed DNA strand transfer by actinomycin D is more sensitive than inhibition of RT's polymerase activity.

Since DNA polymerase inhibition was not able to explain the observed inhibition of DNA strand transfer, we sought to determine if actinomycin D interferes with DNA annealing or exchange reactions that must occur for the nascent DNA strand to transfer onto the acceptor template (Figure 1). Actinomycin D has been shown by several laboratories to bind to single-stranded DNA (*54*−*56*, *66*, *67*), exhibiting some specificity for GC bases (*40*−*45*). In addition, an early report suggested that actinomycin D might interfere with annealing of viral RNA and DNA sequences (*54*). We pursued the possibility that actinomycin D could be inhibiting the annealing of the DNA strand transfer primary product intermediate to the acceptor template by examining the kinetics of the annealing reaction directly. Actinomycin D strongly inhibits the annealing of complementary single-stranded DNA, showing a concentration dependence with an IC_{50} of 0.22 μM, approximately 10-fold lower than the IC_{50} value of 2.7 μM for DNA strand transfer. The rate of annealing in the absence of actinomycin D was approximately 4.7×10^6/(M·s), while in the presence of 20 μM actinomycin D this rate decreased to 1.1×10^4/(M·s), a 427-fold decrease. This annealing inhibition correlates well with strand transfer inhibition in that it does not require more actinomycin D to inhibit annealing than strand transfer, suggesting that annealing inhibition might be the target for actinomycin D inhibition of strand transfer. Also, the difference in IC_{50} concentration and magnitude of rates seen between annealing and strand transfer is probably due to the fact that the annealing model assay assumes that 100% of the first DNA extension product is immediately available for annealing to the acceptor DNA. During the reaction, this is probably not the case since RNA template degradation by the RNase H activity of RT is required for strand transfer, and results in smaller amounts of the nascent single-stranded DNA available for transfer at any one time.

Additional structural proteins and enzymes are also present in the HIV-1 virion which could influence the effects of actinomycin D inhibition. The HIV-1 nucleocapsid protein (NC) is a basic protein encoded by the *gag* gene and is a major structural protein of the core ribonucleoprotein complex of HIV (*20*, *21*). It contains two Cys_3His Zn(II) retroviral-type zinc finger domains which have been shown to be important in binding the SL3 stem−loop recognition element of the genomic ψ RNA packaging signal (*34*). NC binds cooperatively to single-stranded DNA and RNA and has been shown to increase the rate for the annealing of complementary DNA (*25*, *26*) and is able to catalyze DNA strand exchange (*28*). It is generally accepted that the rate enhancement of DNA annealing is probably one of the key roles of NC in the HIV life cycle. It has also been shown that NC increases the rates of DNA strand transfer in model systems (*13*, *17*). Therefore, if actinomycin D is inhibiting DNA strand transfer by inhibition of DNA annealing, NC might be able to facilitate a reversal or recovery of this inhibition.

NC increases the rate of annealing of single-stranded primary DNA product and acceptor template to levels approaching the rate of free diffusion in solution (3×10^7 M^{-1} s^{-1}). DNA annealing in the presence of NC is sensitive to inhibition by actinomycin D, with an IC_{50} value of 0.64 μM. However, due to the faster intrinsic rates of annealing in the presence of NC, much larger amounts of actinomycin D are required to reduce the annealing rates to those in the absence of NC. Since NC was shown to influence both the rate and inhibition of DNA annealing, strand transfer experiments were performed in order to determine if NC could recover some or all of the strand transfer activity in the presence of actinomycin D. This would be a strong indication that actinomycin D is inhibiting DNA strand transfer by interfering with the annealing of DNA intermediates. This was found to be the case. While DNA strand transfer in the presence of NC was still sensitive to actinomycin D, the IC_{50} has now been shifted to 13.8 μM.

There are several possible mechanisms as to how NC could reduce the sensitivity of DNA strand transfer to inhibition by actinomycin D. First, NC could displace actinomycin D from the DNA substrates by occupying potential actinomycin D binding sites. However, we conducted order of addition experiments (data not shown) with NC and actinomycin D and found no significant difference in reactivity regardless if actinomycin D was added before or after NC. Also, DNA annealing in the presence of NC is still sensitive to actinomycin D inhibition, suggesting it can remain bound in the presence of NC binding. Together, this suggests that NC displacement of actinomycin D is probably not occurring, or that actinomycin D and NC binding can equilibrate under the reaction conditions.

A second possible mechanism is that NC and actinomycin D bind simultaneously to the DNA substrates, but NC relieves its inhibitory activity by alleviating conformational or structural restrictions brought about by actinomycin D. The corresponding inhibition of DNA strand transfer and annealing, and the effect of NC on this inhibition, suggests that the primary role of NC in strand transfer is in inducing an acceleration of DNA annealing steps. It is possible that actinomycin D inhibits strand transfer by binding RT directly, or could potentially bind higher order nucleic acid/RT complexes (*6*, *8*), or uncouple the polymerase and RNase H activities of RT (*68*). The apparent difference in IC_{50} for

inhibition of DNA annealing and DNA strand transfer suggests a possible change in the rate-limiting step for DNA strand transfer in the presence of actinomycin D. Previous reports have suggested that the polymerase-independent RNase H activity of RT is the rate-limiting step in DNA strand transfer (*6*), and it is possible that actinomycin D inhibits this activity. We examined the rate and cleavage pattern on the primary RNA template by HIV-1 RT associated RNase H activity under DNA strand transfer reaction conditions and found essentially no change in either the cleavage pattern or the rate of RNase H cleavage at actinomycin concentrations as high as 20 μM. When 1 μM NC was included in the reaction, a detectable level of RNase H inhibition was observed at a concentration of 20 μM actinomycin (~40% inhibition compared to the negative control lacking actinomycin D; data not shown). However, this inhibition correlated with the small amount of inhibition of DNA polymerase activity associated with these concentrations of actinomycin D (see Figure 3A) since the rate of the polymerase-dependent RNase H activity will be influenced by inhibition of the DNA polymerase activity (*37*). The high levels of actinomycin D required to see this relatively small effect on RNase H activity are well above the observed IC_{50} for inhibition of DNA strand transfer (2.7 μM). Therefore, we conclude that the observed inhibition of DNA strand transfer by actinomycin D is not due to a pronounced inhibition of RNase H activity. Further, the strong correlation between strand transfer and annealing inhibition by actinomycin D points to the formation of annealed intermediates as the target of inhibition. Before DNA annealing rates could become inhibitory, they must become slower than the RNase H activity. Thus, actinomycin D concentrations above the IC_{50} for inhibition of DNA annealing might be required before they manifest an inhibitory effect on DNA strand transfer.

The current observation of DNA strand transfer inhibition by actinomycin D correlates well with early studies examining the effect of actinomycin D on detergent-disrupted retroviral replication. Novak et al. (*69*) reported that actinomycin D inhibited the DNA elongation beyond the 5′-end of the viral RNA template in detergent-disrupted avian sarcoma virus. That is, actinomycin D allowed for the synthesis of minus strand strong-stop DNA, but appeared to prevent the DNA elongation following the DNA strand transfer step. In subsequent studies (*54*), it was shown that actinomycin D was able to inhibit DNA·DNA and DNA·RNA hybridization equilibrium using viral DNA and RNA. The results presented here confirm and further elaborate on this finding to show that a principle mechanism of actinomycin D inhibition of reverse transcription is the inhibition of DNA strand transfer, and show a direct correlation between the rate of strand transfer and DNA annealing inhibition by actinomycin D.

It is important to consider that many of the previous studies done on actinomycin D polymerization inhibition have been performed at high concentrations of drug and generally low concentrations of enzyme, both of which may be important in determining how and if actinomycin D inhibits either DNA·RNA or DNA·DNA extension under a given set of reaction conditions. Our studies were performed under conditions of excess HIV-1 RT over RNA template in an attempt to mimic viral conditions where RT is present in multiple copies within the viral core containing the diploid genome. Since our inhibition studies were performed at high enzyme concentrations and low concentrations of actinomycin D, it is possible that using a similar strategy of DNA strand transfer inhibition, other new drug analogues could be developed that offer increased target specificity and even greater potency. Importantly, this new class of drug would have a different target and specificity than current HIV-1 RT inhibitors. DNA strand transfer inhibitors, in combination with other known inhibitors of NC function (*70*), might provide a novel means for combating HIV infection.

ADDED IN PROOF

During the revision of this paper, an independent examination of the effect of actinomycin D on DNA strand transfer has appeared (*65*). The results presented in Guo et al. (*65*) are consistent with the results shown here, and include analysis of actinomycin D inhibition of endogenous HIV-1 RT reactions. Together, these results demonstrate the feasibility of inhibiting retroviral replication using inhibitors targeting DNA strand transfer events. We thank Dr. Judith Levin for sharing her results with us prior to publication.

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