Kinetic Analysis of the Effect of HIV Nucleocapsid Protein (NCp) on Internal Strand Transfer Reactions†

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ABSTRACT: The mechanism of HIV reverse transcriptase (RT) catalyzed strand transfer synthesis (i.e., switching of the primer to a new template) from internal regions on RNA templates in the presence and absence of HIV nucleocapsid protein (NCp) was investigated. Two different systems each consisting of DNA-primed RNA donor (on which primer extension initiated) and acceptor (to which DNAs initiated on the donor could transfer) templates were used to determine kinetic parameters of strand transfer. The donor and acceptor shared an internal region of homology where homologous strand transfer could occur. The rate of strand transfer at various acceptor concentrations was determined by monitoring the production of transfer products over time. These rates were used to construct Lineweaver−Burk plots. In each system, NCp increased the V_{max} about 3-fold while the K_m for acceptor template was decreased severalfold. NCp's effects on RT extension ranged from no effect to inhibition depending on the primer-template used. The lowered K_m shows that NCp increases the affinity of the acceptor template for the transferring DNA. V_{max} increases despite the inhibition of RT extension. The increased V_{max} implies a stimulatory mechanism that cannot be mimicked by high acceptor concentrations. Therefore, NCp does not act by merely increasing the effective concentration of nucleic acids.

The human immunodeficiency virus (HIV),[1] a member of the Retroviridae family of viruses, contains a diploid RNA genome and replicates through a DNA intermediate. Retroviruses are known to undergo recombination during replication (*1*−*4*). Recombination between the two RNA genomes can occur by a process referred to as strand transfer (also called template switching or strand jumping). Strand transfer, as it relates to retroviruses, describes the transfer of DNA between the two RNA genomes or between different regions of the same genome. When transfer occurs between genomes, the process results in recombination. Since the two copies of parental genomic RNA are not necessarily 100% homologous, strand transfers between the RNA templates can result in a chimeric proviral DNA that transcribes a unique progeny genome.

In the current model for retroviral replication there are two essential strand transfer events (*5*, *6*). The first occurs when the minus strand strong-stop DNA transfers from the 5′ end of the viral RNA to the 3′ end of either the same RNA or the other RNA in the virion. The second essential transfer event occurs during the synthesis of the plus strand DNA. The plus strand strong-stop DNA, which is initiated near the 5′ end of the minus strand DNA, transfers to the 3′ end to complete synthesis. These two transfer events occur at the terminal regions of nucleic acid templates.

Strand transfer events have also been shown to occur from internal regions of RNA both in vitro (*7*, *8*) and in vivo (*2*, *3*). This type of transfer event can occur from any point on the genome. Internal strand transfers result in recombination between homologous regions of the genomic RNA. Nonhomologous recombination between RNAs also occurs but at 1/100th to 1/1000th the frequency of homologous recombination (*9*).

There are two proposed mechanisms of strand transfer. They are the strand dissociation and the acceptor-facilitated models. In the strand dissociation model, the nascent DNA being synthesized on the RNA dissociates prior to binding to the second RNA template. In this model, the 3′ end of the DNA is free of both RNA templates. In the acceptor-facilitated model, the second RNA (acceptor) actively displaces the DNA from the original template. In such a model the 3′ end of the DNA is always hybridized to either the original template or the second template or both. Apart from the ability of complementary nucleic acids to hybridize, strand transfer may be influenced by some host or viral factors (*2*). One such factor is the viral nucleocapsid protein (NCp). Experiments in vitro have shown that NCp accelerates homologous strand transfer from template termini (*10*, *11*) and internal regions of RNA (*12*).

Nucleocapsid protein (NCp) is encoded by the 3′ region of *gag* and is well conserved among retroviruses (*11*). NCp 7 is produced from in vivo processing of NCp 15 and retains full nucleocapsid protein activity in vitro (*11*). Within the viral nucleocapsid, NCp is closely associated with the viral genome (*13*). NCp 7 is a highly basic protein containing two zinc fingers and has a strong affinity for nucleic acids (*13*). It has been shown that the conserved basic residues flanking the zinc fingers are important for nucleic acid binding activity (*11*). However, mutations in the zinc finger motifs produce mutant viral particles that encapsidated less viral RNA than

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[1] Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; NCp, nucleocapsid protein; BSA, bovine serum albumin.

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the wild-type particles, suggesting an important role for the zinc fingers (*14*). NCp has been shown in vitro to promote the binding of primer tRNA to the primer binding site (pbs) on the viral RNA and also promotes dimerization of viral RNA fragments containing specific packaging signals (*15*). The latter activity is consistent with NCp's proposed role in the dimerization of the two RNA strands (*16*−*18*). NCp accelerates the binding of complementary nucleic acids and can catalyze strand exchange in which a more thermodynamically stable hybrid forms at the expense of a less stable hybrid (*13*). The enhancement of binding is consistent with the promotion of strand transfer by NCp.

Previously, a kinetic analysis of the catalysis of internal strand transfer performed in the absence of NCp indicated that transferring DNAs were rapidly released from the RNA template on which synthesis was initiated (donor). Association of these DNAs with a second RNA template (acceptor) was relatively slow and rate-limiting with respect to the production of strand transfer products (*8*). Kinetic constants (V_{max} and K_m) for the strand transfer reaction were determined and showed that V_{max} for the formation of transfer products was about 20-fold lower than the rate of product formation on the donor template. Although NCp increases the velocity of strand transfer by accelerating the binding of the transferring DNA to the acceptor template (*10*, *12*), the effect of NCp on kinetic constants for strand transfer has not been evaluated. In this report we show that NCp lowers the K_m for acceptor template and increases the V_{max} of strand transfer reactions. The lower K_m implies that NCp increases the affinity of the transferring DNA for the acceptor. The increase in V_{max} indicates that the effects of NCp cannot be completely mimicked even by increasing the level of acceptor template to a theoretically "infinite" concentration. The latter finding suggests that NCp alters the characteristics of the nucleic acids involved in transfer such that rapid and accurate association can occur.

MATERIALS AND METHODS

Recombinant HIV-RT, having properties as described (*19*), was graciously provided to us by Genetic Institute (Cambridge, MA). This enzyme had a specific activity of approximately 40 000 units/mL [1 unit of RT is defined as the amount required to incorporate 1 nmol of dTTP into nucleic acid product in 10 min at 37 °C using poly(rA)−oligo(dT) as template-primer]. The enzyme preparations contained very low levels of single-stranded nuclease activity (*7*). This activity was inhibited by including 5 mM AMP in the assays. The AMP, at this concentration, did not affect the polymerase or RNase H activities of RT. Aliquots of HIV-RT were frozen at −70 °C, and a fresh aliquot was used for each experiment. HIV-1 nucleocapsid protein (NCp) was obtained from Enzyco (Denver, CO). This 55-amino acid protein corresponded to the p7 portion of HIV-1 MN (*20*). T4 polynucleotide kinase was from United States Biochemical. T7 and T3 RNA polymerases, *Mva*I, *Pvu*II, *Bgl*I, RNase DNase free, placental RNase inhibitor, rNTPs, and dNTPs were obtained from Boehringer Mannheim. Deoxyoligonucleotides were synthesized by Genosys Inc. (The Woodlands, TX). All other chemicals were from Fisher Scientific or Sigma. Radiolabeled compounds were from New England Nuclear.

Strand Transfer Assay. In the standard reaction, primer-donor template (5 nM) and acceptor template at varying concentrations (as indicated in Figure 2) were preincubated for 5 min in the presence or absence of HIV nucleocapsid (one molecule of NCp for every two bases of nucleic acid, including primer, donor, and acceptor template) in a volume of 26.5 μL at 37 °C. Reactions were initiated by the addition of 2 units of HIV-RT in a 5 μL volume in 50 mM Tris-HCl (pH 8.0), 1 mM dithiotreitol, and 80 mM KCl. The final concentrations of reaction components were 50 mM Tris-HCl (pH 8.0), 5 mM AMP, 6 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA (pH 8.0), 100 μM dNTPs, 100 μM $ZnCl_2$, 0.1 μg/μL BSA, and 80 mM KCl. Five microliter aliquots were removed at the indicated times and terminated by the addition of 5 μL of gel loading buffer [90% formamide, 10 mM EDTA (pH 8.0), 0.1% xylene cyanol, 0.1% bromophenol blue] containing 0.25 unit of DNase-free RNase. The samples were treated at 65 °C for 10 min to digest the RNA and then for 2 min at 90 °C before subjecting to gel electrophoresis on 6% or 8% denaturing polyacrylamide gels containing 7 M urea. Gels were dried and subjected to autoradiography and then scanned with a phosphorimager to quantify transfer and donor-directed extension products.

Production of RNAs by Runoff Transcription. Runoff transcription was done as described in the Promega Protocols and Application Guide (1989). For the donor template from System 1 (see Figure 1), plasmid pBSM13+(Δ) prepared as described previously (*21*) was cleaved with *Bgl*I, and T3 RNA polymerase was used to prepare runoff transcripts 189 nucleotides in length. For the acceptor template, plasmid pBSM13+ was cleaved with *Pvu*II, and T3 RNA polymerase was used to prepare runoff transcripts 180 nucleotides in length. For the donor template from System 2, plasmid pBSM13+(Δ) was cleaved with *Pvu*II, and T7 RNA polymerase was used to prepare runoff transcripts 225 nucleotides in length. For the acceptor template, plasmid pBSM13+ was cleaved with *Mva*I, and T7 RNA polymerase was used to prepare runoff RNA transcripts 189 nucleotides in length. Reactions were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol. The precipitates were resuspended in formamide dye. The RNAs were gel purified on denaturing polyacrylamide gels, located by ultraviolet shadowing, and recovered as previously described (*8*). The amount of recovered RNA was determined spectrophotometrically from the optical density.

RNA−*DNA Hybridization.* Hybrids for the strand transfer reactions were prepared by mixing the RNA donor template and the specific primer deoxyoligonucleotides that were 5′-^{32}P-labeled using T4 polynucleotide kinase. The hybrids were prepared by mixing primer/template at a 5:1 ratio in 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, and 80 mM KCl. The mixtures were heated to 65 °C for 5 min and then slow-cooled to room temperature.

Gel Electrophoresis and Product Quantification. Denaturing 6% or 8% polyacrylamide sequencing gels (19:1 acrylamide:bisacrylamide ratio) containing 7 M urea were prepared and subjected to electrophoresis as described (*22*). Quantification of strand transfer products was accomplished by phosphorimager analysis of dried gels, using a GS-525 phosphorimager from Bio-Rad.

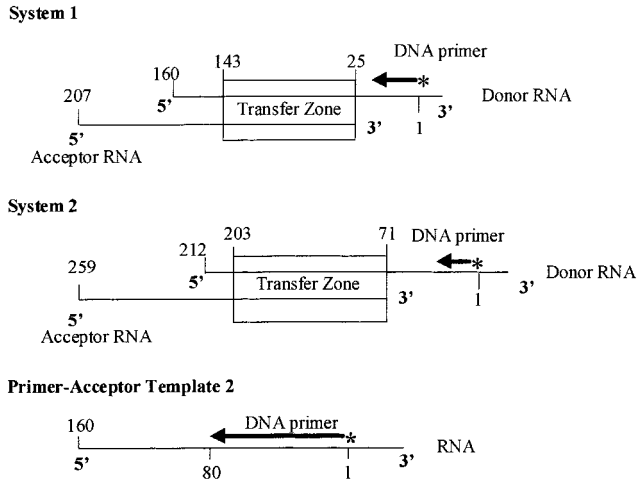

FIGURE 1: Systems used to analyze strand transfer. The two systems consist of a DNA-primed donor RNA, on which DNA synthesis initiated, and an acceptor RNA, to which DNAs initiated on the donor could potentially transfer. In System 1, the donor and acceptor were 189 and 180 nucleotides, respectively, while in System 2 the donor and acceptor were 225 and 189 nucleotides, respectively. The 5′-^{32}P-labeled DNA primers (*) were each 20 nucleotides in length. The boxes indicate the "transfer zone" which corresponds to the region of homology between the donor and acceptor. The numbers indicate the lengths (in nucleotides) of primer extension products that had reached the indicated position on the donor or acceptor template. In Primer-Acceptor Template 2, the acceptor template from System 2 was primed with a DNA primer 80 nucleotides long. The 5′-terminal nucleotide of the primer was complementary to the 27th base from the 3′ end of the acceptor.

RESULTS

Systems Used To Evaluate Strand Transfer. Two separate systems were used to study the effect of NCp on strand transfer (see Figure 1). Both consisted of a DNA-primed RNA "donor" template, on which DNA synthesis was initiated, and an RNA "acceptor" template, to which DNAs initiated on the donor could potentially transfer. The donor and acceptor were homologous over a defined region located within the internal portion of the donor. This design excluded homologous transfer of those products extended to the end of the donor. Therefore, the systems were constructed to examine "internal" strand transfer from the donor to an acceptor. Both of the donor templates were derived from pBSM13+(Δ). However, the nucleotide sequences were different since the System 1 and 2 donors were produced with T3 and T7 RNA polymerase, respectively. The systems were designed so that fully extended donor-directed and transfer products could be easily resolved by gel electrophoresis. In each system, full-length transfer products (Systems 1 and 2, 207 and 259 nucleotides, respectively) were 47 nucleotides longer than full-length donor-directed products (Systems 1 and 2, 160 and 212 nucleotides, respectively). After resolution by gel electrophoresis, the amount of transfer product was determined using a phosphorimager.

Increasing Acceptor Concentration and NCp Enhance Strand Transfer to the Acceptor Template. To determine the effect of acceptor template concentration and NCp on strand transfer, a titration experiment was performed in which the concentration of acceptor template was varied in the presence or absence of NCp. Aliquots were removed at various time points, and the products were resolved by gel electrophoresis (see Figure 2). In the absence of NCp, using 5 nM donor template and increasing acceptor template concentrations, the amount of transfer product (T) increased over time (see Figure 2). This is consistent with other results which indicate that increasing the ratio of acceptor to donor template increases the efficiency of strand transfer (*23*). No transfer product was observed in the absence of acceptor as expected (data not shown). Under these same conditions, in the presence of acceptor templates and NCp, the rate of production of transfer products increased [note that the time points for reactions with or without NCp are different in Figure 2 (see below)]. The amount of NCp used was varied with the amount of template present in the reactions such that one molecule of NCp was present for every two nucleotides of total nucleic acid (including primer, and donor and acceptor templates). This condition was required in order to obtain a linear relationship for $1/V_o$ vs 1/[acceptor] on Lineweaver−Burk plots (see Figures 4 and 5). The ratio of 2 nucleotides per NCp molecule (2:1) is somewhat higher than the about 7:1 ratio present in the virion (*24*). This amount was chosen because maximum levels of transfer products were observed at this ratio of NCp. It is possible that the higher ratio of NCp was required for maximal stimulation due to a portion of the NCp preparation being inactive. It is also possible that conditions required for optimal enhancement of strand transfer in vitro are different from those occurring in vivo. Also, different time points were used for reactions with or without NCp. The time points were chosen to be within the linear range in plots of the amount of transfer product vs time (see Figure 3). In the presence of NCp for System 1, most of the transfer products appeared in a single band migrating approximately at the predicted position for full-length transfer products (207 nucleotides).

The amount of transfer product obtained at each of the time points was quantified using a phosphorimager. These data were used to construct plots of transfer product vs time. A typical plot using an acceptor-to-donor ratio of about 3:1 (15 nM acceptor and 5 nM donor) is shown if Figure 3. Note that the slope of the line, which corresponds to the initial velocity (V_o) of the strand transfer reaction, was considerably greater in the presence of NCp. Nucleocapsid also augmented the rate of strand transfer at other acceptor concentrations (data not shown). Initial velocity values were determined for several acceptor concentrations. These values increased as the concentration of acceptor was increased (see Figure 2 above).

Determination of the Maximum Velocity (V_{max}) and K_m Values of the Strand Transfer Reaction. To determine how NCp affected the rate of transfer, we obtained the V_{max} and K_m values for the strand transfer reaction. The above experiment was repeated 2 additional times, and the initial velocity values were obtained and used to construct Lineweaver−Burk plots (see Figure 4 and Table 1). From these plots, a theoretical maximum velocity of strand transfer (V_{max}) at infinite acceptor concentration in the absence and presence of NCp was obtained. This value represented the femtomoles of primer transferred per minute per reaction (total reaction volume was 31.5 μL; see Materials and Methods), and was approximately 3-fold higher in the presence of NCp than in its absence. The K_m values for the acceptor template in the transfer reaction in the presence and absence of NCp were

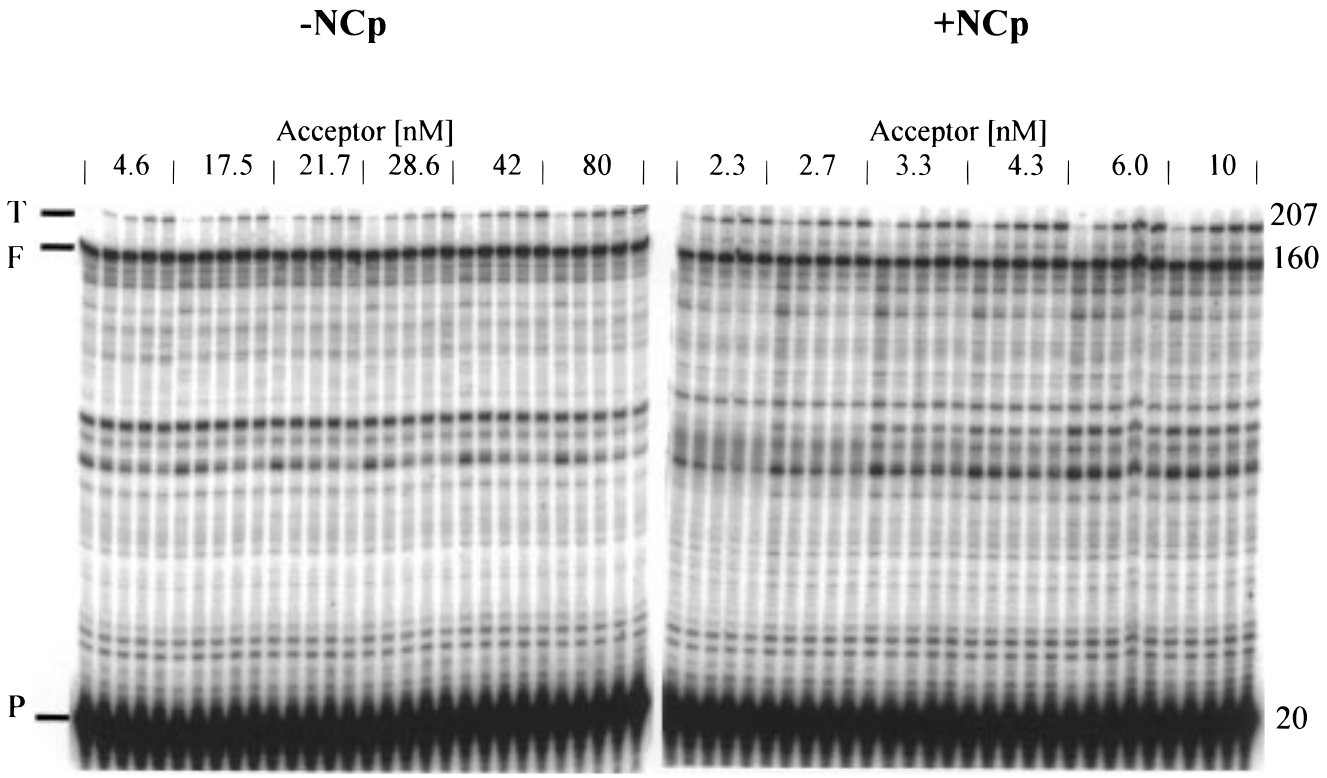

FIGURE 2: Strand transfer in the presence or absence of NCp using System 1 (see Figure 1). This represents an autoradiogram of a time course of strand transfer reactions done in the absence and presence of NCp. The numbers on the right side indicate the lengths of the primer (P), full-length donor-directed (F), and transfer products (T). The acceptor concentrations (in nM) used in the assays are indicated above each set of lanes. Reactions performed in the presence or absence of NCp were terminated at different time points: from left to right for each acceptor concentration, 10, 18, 26, 34, and 42 min or 6, 9, 12, 15, and 18 min, − and + NCp, respectively.

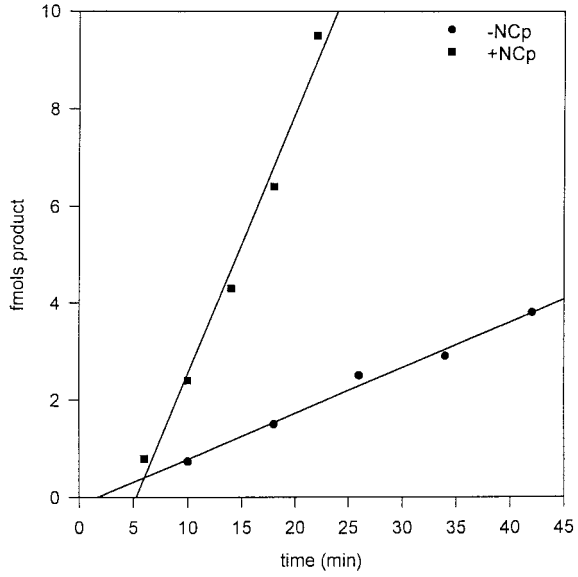

FIGURE 3: Amount of transfer products produced over time in the presence and absence of NCp. A plot of total transfer products (fmol of product) vs time for an assay performed with 15 nM acceptor and 5 nM donor template, in the presence or absence of NCp, is shown. The slope of the lines represents the initial velocity (V_o) of strand transfer. In this particular experiment, the V_o values in the presence and absence of NCp were 0.54 and 0.09 fmol min^{-1} $reaction^{-1}$, respectively.

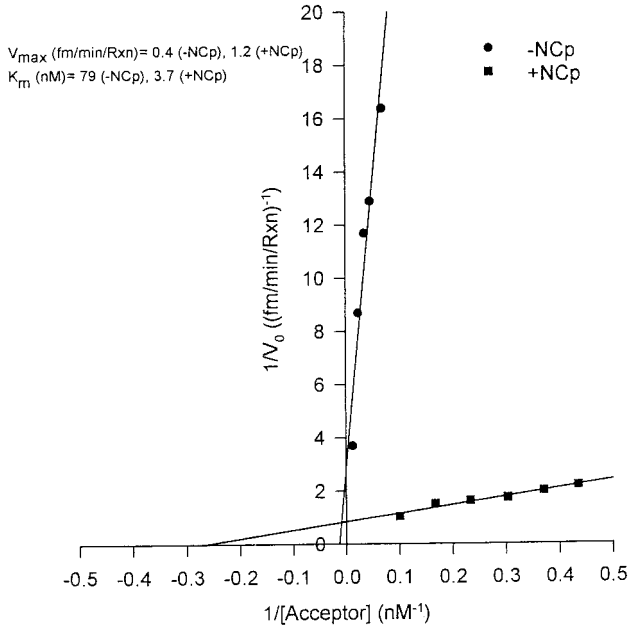

FIGURE 4: Lineweaver−Burk plot for System 1. A representative plot of $1/V_o$ vs 1/[acceptor] for System 1 (see Figure 1), in the presence or absence of NCp, is shown. The V_{max} and K_m values for this particular experiment are indicated on the graph.

also determined from the Lineweaver−Burk plot and are listed in Table 1. Note that for System 1 the K_m value decreased about 17-fold in the presence of NCp. The decreased K_m in the presence of NCp suggested an increased affinity of the acceptor template for the transferring DNA or a complex of which the DNA is a part (see Discussion). These data were further confirmed using a second unrelated template (System 2 in Figure 1). In this case, in the presence of NCp a similar change in V_{max} was observed while the K_m decreased about 4-fold from 35 to 8 nM (Figure 5 and Table 1).

Table 1: Kinetic Constants for Strand Transfer and Primer Extension Results

	K_m (nM)[b]		V_{max} (fmol min^{-1} reaction^{-1})[c]		extension rate (fmol min^{-1} reaction^{-1})[d]	
substrate[a]	−NCp	+NCp	−NCp	+NCp	−NCp	+NCp
system 1 (strand transfer)	71 (21)	4.3 (2.7)	0.35 (0.11)	1.1 (0.1)		
system 2 (strand transfer)	36 (6)	10 (2)	0.60 (0.14)	1.7 (0.1)		
primer-donor template 1					3.5 (0.1)	4.1 (0.1)
primer-acceptor template 2					3.1 (0.6)	0.67 (0.15)

[a] Substrates are described in Figure 1. Primer-donor template 1 is the primed donor template from System 1. Results for System 1 and primer-acceptor template 2 are from three independent experiments while those from System 2 and primer-donor template 1 are from two independent experiments. [b] K_m was determined from Lineweaver−Burk plots with standard deviations in parentheses. [c] V_{max} was determined from Lineweaver−Burk plots with standard deviations in parentheses. [d] Extension rates were calculated from the slope of the linear portion of plots of femtomoles of full-length product vs time (see Figure 6) with standard deviations in parentheses.

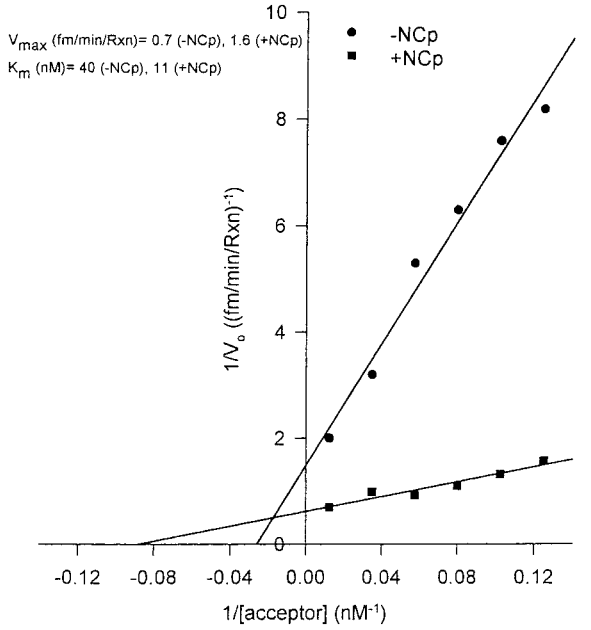

FIGURE 5: Lineweaver−Burk plot for System 2. A representative plot of $1/V_o$ vs 1/[acceptor] for System 2 (see Figure 1), in the presence or absence of NCp, is shown. The V_{max} and K_m values for this particular experiment are indicated on the graph.

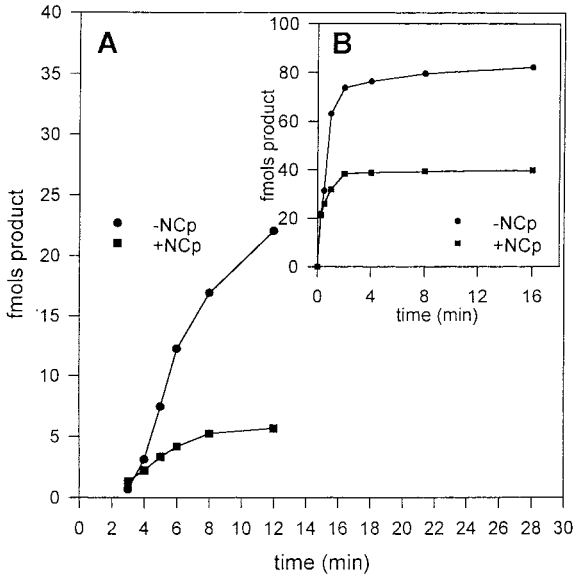

FIGURE 6: Extension on primer-acceptor template 2 in the presence and absence of NCp. A representative plot of femtomoles of product vs time for primer-acceptor template 2 (see legend to Figure 1) is shown. (A) Femtomoles of fully extended (extended to the end of the template) product vs time. (B) Femtomoles total primer extension (fully extended + partially extended) vs time.

Effect of NCp on Extension on the Donor and Acceptor Templates. From the above experiments we observed that NCp increased the rate of production of transfer products. Such an increase could result from an increase in the rate of DNA being transferred to the acceptor or from an increase in the efficiency of DNA extension after transfer to the acceptor. To test the latter, a substrate designed to mimic extension of the transferred DNA on the acceptor was used (Primer-Acceptor Template 2 in Figure 1). The substrate consisted of the acceptor template used in template 2 bound to a complementary DNA 80 nucleotides in length. The DNA used terminated (3′ end) at a position on the acceptor where DNA synthesis pausing had been shown to induce strand transfer (*8*). Extension of the DNA by HIV-RT in the presence or absence of NCp was performed using conditions identical to those used for strand transfer reactions. The amount of DNA extended to the end of the template vs time was plotted, and the linear region of the plot was used to calculate the rate of product formation (Figure 6A). The total amount of primer extended was also determined (Figure 6B). Both the rate of full-length product formation (Table 1) and the total amount of extended primer decreased significantly in the presence of NCp. This is consistent with NCp inhibiting the binding of RT to primer-template as has been observed previously (*13*, *15*, *25*). In contrast, the rate of production of full-length donor-directed products using the primed donor template from System 1 (see Figure 1) in the absence of acceptor was essentially unchanged in the presence of NCp (Table 1). There was also little effect when the System 2 donor template was used (data not shown). Although we have observed inhibition of primer extension by NCp on other primer-templates (data not shown), the extent of inhibition observed on Primer-Acceptor Template 2 was very high. This may have resulted from the primer terminating at a strong pause site (see Discussion). This implies that the inhibitory effect of NCp on RT binding is sequence dependent or can be compensated to some extent by enhancement of processivity (*15*). Overall the influence of NCp on primer extension ranges from essentially no effect to a pronounced inhibition. Therefore, the observed increase in strand transfer in the presence of NCp must result from an increase in the rate of DNA transfer to the acceptor and

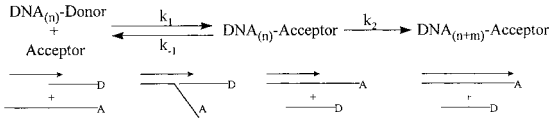

FIGURE 7: Proposed scheme for strand transfer. An overview of the process of strand transfer is presented. In this scenario, DNA of length (*n*) bound to the donor template ($DNA_{(n)}$−Donor) transfers to the acceptor at a rate equal to k_1 (nM^{-1} min^{-1}). Extension of the DNA by (*m*) bases on the acceptor at a rate equal to k_2 (min^{-1}) yields a DNA strand transfer product of ($n+m$) nucleotides. Diagrams below the scenario represent the structures present at different points during strand transfer if the process were occurring by a strand invasion mechanism (see Discussion). The "replication intermediate" is represented by the DNA (arrowhead at 3′ end) bound to the donor template (D, labeled at 5′ end) while the acceptor is labeled "A" (5′ end).

not an increase in the extension of the transferred DNA to the end of the acceptor.

DISCUSSION

In this report, some of the kinetic parameters associated with HIV-RT-catalyzed strand transfer in the presence and absence of NCp were measured. Kinetic parameters have been reported earlier for the catalysis of strand transfer in the absence of NCp (*8*); hence, this report provides additional information to better understand the role of NCp in the mechanism of strand transfer. Several reports have shown that NCp enhances strand transfer (*10*−*12*, *15*, *24*, *26*). By assigning numerical values to specific kinetic parameters, a better understanding of the mechanism of NCp enhancement can be gained.

A simple scenario for strand transfer is presented in Figure 7. Clearly this scenario oversimplifies the process by which the DNA bound to the donor transfers to the acceptor. Previously two mechanisms have been proposed for this step (*24*, *27*, *28*). In one, the DNA first releases from the donor and subsequently binds to the acceptor, while in the second the acceptor "invades" the DNA−donor complex and displaces the donor. Invasion of a substrate designed to mimic a replication intermediate was significantly enhanced in the presence of NCp (Figure 7, see legend) presumably because NCp accelerates the binding of the acceptor to the single-stranded region of the DNA and also destabilizes the interaction between the DNA and donor template (*13*, *15*). To what extent each of these effects enhances strand transfer is not clear. In the scenario shown in Figure 7, the rate constant k_1 would then be the overall rate constant for several steps including the time required for the acceptor and DNA to initially associate, followed by the hybridization step, and then the displacement of the donor. The rate constant k_2 describes the average rate at which the various transferred DNAs are extended to the end of the acceptor template. Although this step is not part of the strand transfer process, the fully extended transfer products are used to monitor the process of strand transfer. Note that "average rate" implies that strand transfer occurs from various places on the donor and the time required to extend the transferred DNA to the end of the acceptor may vary depending on where transfer occurred. Given the sizes of the regions of homology in Systems 1 and 2, it is likely that DNA transfer occurs from several points on the donor. In the presence of NCp, the rate of extension of a specific DNA on the acceptor for System 2 (Primer-Acceptor Template 2, see Figure 1) was decreased although little effect was observed for primer extension on either donor template. The DNA used to prime the acceptor terminated at a strong pause site. Pausing implies that the rate of nucleotide addition is decreased at this site. This may be caused by a lowered affinity of RT or a decrease in the rate constant for nucleotide addition at the site. A combination of these effects and competition with NCp for binding the template may result in the large inhibition of primer extension that was observed (Figure 6). Clearly NCp's inhibitory influence on RT primer extension is sequence dependent since neither primed donor template was significantly inhibited. Since pausing induces strand transfer, it is likely that many transfers occur from pause sites. If the inhibitory effects of NCp were accentuated at pause sites, then k_2 would be lower in the presence of NCp. Therefore, the calculated transfer velocities (V_o) in the presence of NCp relative to those in the absence would underestimate the actual enhancement of strand transfer by NCp. This would result in an underestimate of V_{max} in the presence vs the absence of NCp.

In the scenario presented, $V_o = k_2[DNA_{(n)}-acceptor]$ and $K_m = k_{-1} + k_2/k_1$ (assuming a steady state). Since k_2 either decreases or remains constant (see above), V_o increases due to an increase in the concentration of DNA−acceptor complex. If the magnitude of k_2 decreased, the increase in [DNA−acceptor] would be somewhat greater than the increase in the V_o value in the presence vs absence of NCp. A decrease in k_2 would contribute to an increase in [DNA−acceptor]. However, since V_o increases severalfold in the presence of NCp (Figure 3), changes in k_{-1} and/or k_1 must occur to account for the large increase in DNA−acceptor complex. Such an increase could be produced by a decrease in k_{-1} or an increase in k_1, of which the latter seems more likely. Presumably the magnitude of k_{-1} should not change significantly in the presence of NCp. The conversion of the DNA−acceptor complex back to free acceptor and DNA−donor complex should be very low once a stable complex has formed. However, due to RT RNase H activity, some of the DNA may be released from the acceptor prior to extension. During this process, the acceptor would be cleaved and the DNA would be released in free form, not as a complex with the donor template. How NCp would affect this step is not clear, although less total extension occurred on the acceptor in the presence of NCp (Figure 6B). This could be due to an increase in the release of DNA from the acceptor either resulting from cleavage of the acceptor or destabilization of the complex between the DNA and acceptor. Consistent with the latter, NCp has been shown to decrease the thermostability of the replication intermediate substrate (*13*). So if k_{-1} is taken as anything that causes dissociation of the DNA−acceptor complex and not strictly as the reverse of the reaction shown in Figure 7, the magnitude of k_{-1} may actually increase in the presence of NCp. This and the large increase in V_o imply that the increase in [DNA−acceptor] complex results from a substantial increase in the magnitude of k_1. Such an increase is consistent with NCp's ability to catalyze rapid strand exchange between an acceptor and a replication intermediate (*13*).

On the two systems used in these experiments, there were approximately 3-fold increases in the V_{max} values in the presence of NCp while in both cases the K_m values decreased

to different extents (Table 1). Since $K_m = k_{-1} + k_2/k_1$, any combination of decreases in k_{-1} and k_2 and an increase in k_1 would lead to a decrease in K_m. Results indicated that NCp may decrease k_2 while an increase in k_{-1} is suggested. Since the changes in k_{-1} and k_2 would tend to decrease the level of strand transfer, the observed increase with NCp implies an increase in k_1 that far exceeds the effects of changes in the other rate constants. All factors considered, the large decrease in K_m in the presence of NCp indicates that the apparent affinity of the acceptor for the transferring DNA increases and this results in a faster binding of the DNA to the acceptor. The variations in the K_m values with the different templates suggest that the sequences of the acceptor and transferring DNA influence the magnitude of the NCp-induced changes in the rate constants.

Questions remain as to how NCp increases the rate at which complementary nucleic acids bind. It has been proposed that NCp destabilizes secondary structures within the single-stranded nucleic acids, allowing for complementary regions to associate more rapidly (*15*). Not all secondary structures are susceptible since some hairpins are stable in the presence of NCp (*29*). Nucleocapsid protein also favors the formation of more stable hybrids at the expense of less stable ones (*30*, *13*). This may be important in the initial interactions between complementary strands since NCp could destabilize weak nonproductive interactions involving short regions of complementarity. What is clear from our results is that a mechanism that simply increases the effective concentration of the nucleic acids is not occurring. Such a mechanism would be similar to the effect of poly(ethylene glycol) on nucleic acids. It could occur, for example, if NCp bound the nucleic acid and then brought strands into close proximity by attractions between NCp molecules. This type of mechanism alone should not lead to a change in V_{max} in the presence of NCp. A "proximity" effect could be mimicked by a high concentration of acceptor template in the strand transfer assay. At "infinite" acceptor, the theoretical acceptor concentration at V_{max}, the distance between nucleic acid strands would no longer be a factor in their association. The change in V_{max} in the presence of NCp suggests that the nucleic acids may be altered in a way that promotes binding or that NCp directly affects the mechanism of stable hybrid formation.

In conclusion, we have shown that NCp enhances strand transfer in a manner that can be modeled using Michaelis−Menten kinetic analysis. Both positive (increased k_1) and potentially negative (decreased k_2 and increased k_{-1}) influences of NCp on strand transfer were uncovered. Clearly, the positive effects overwhelm the negative. Proximity effects that serve to concentrate nucleic acids were ruled out as important in the mechanism by which NCp promotes strand transfer.

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