Secondary Structure in the Nucleic Acid Affects the Rate of HIV-1 Nucleocapsid-Mediated Strand Annealing†

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ABSTRACT: We studied the effects of human immunodeficiency virus type 1 (HIV-1) nucleocapsid (NC) protein on the kinetics of annealing of nucleic acids using model substrates derived from the 3′ end of the HIV-1 minus-strand strong-stop DNA (−sssDNA). We used HIV-1 reverse transcriptase (RT) to monitor the annealing reaction. Using several different DNA primers and acceptor oligonucleotides, we found that the rate of annealing increased with the size of the complementary region of the primer and the acceptor strands and decreased when secondary structures could be formed in either the primer or the acceptor strands. The secondary structure had a larger effect on the rate of annealing if the secondary structure extends to the 3′ end of the nucleic acid(s). NC protein reduced the rate of annealing between strands with short homologies. NC had no major effect on the rate of annealing when there were at least 13 bases of complementarity between the primer and the acceptor strands and neither strand could form a stable secondary structure. NC increased the rate of annealing when the primer and/or the acceptor strand could form a secondary structure in the region of complementarity. When two strands were in competition as acceptors in an annealing reaction, the specificity of the annealing was determined by the length of the complementarity between the primer and the acceptor strands, the presence or the absence of secondary structures in the primer and/or the acceptor strand, and the presence or the absence of NC in the reaction. This suggests that NC facilitates strand transfer where the nucleic acids have considerable secondary structure (for example, the first strand transfers for viruses whose genomes have considerable secondary structure at their 3′ ends). However, NC also appears to increase the fidelity of recombination by reducing strand transfers between segments that have limited complementarity.

Retroviral reverse transcriptases (RTs)[1] convert the single-stranded RNA found in virions into double-stranded DNA. The RNA is plus strand; RTs synthesize viral minus-strand DNA, using the RNA genome as a template, and plus-strand DNA, using minus-strand DNA as a template. Minus-strand DNA synthesis is initiated from a tRNA primer bound to the primer-binding site (PBS) on the viral RNA (*1*−*3*); the U5 and R regions at the 5′ end of the RNA are the first to be copied. The RNase H activity of RT degrades the RNA strand after it has been copied (*4*−*6*). When RT reaches the 5′ end of the genomic RNA, the nascent single-stranded DNA, which is called minus-strand strong-stop DNA (−sssDNA), is transferred to the R region at the 3′ end of the RNA genome (first strand transfer), and the synthesis of the minus-strand DNA resumes, accompanied by RNase H degradation of the template strand (*7*, *8*). A short polypurine tract (PPT), which is relatively resistant to RNase H degradation, is used to prime plus-strand DNA synthesis. Plus-strand synthesis proceeds until a portion of the tRNA primer is reverse transcribed, yielding a DNA intermediate called plus-strand strong-stop DNA (+sssDNA). RNase H removes the tRNA primer, and the 3′ end of +sssDNA is transferred to the 3′ end of the minus strand (second strand transfer). Plus-strand and minus-strand syntheses are then completed with the minus and the plus strands each serving as the template for the synthesis of the other strand (*3*). The two strand transfers are key steps of the reverse transcription of retroviral genome.

There is extensive recombination during retroviral replication; recombination allows reverse transcriptase to produce a complete DNA copy from genomic RNAs that have been nicked. For the genome to be faithfully copied, both the strand transfers and the recombinations must be accurate. The virus wants to facilitate annealing reactions that involve

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[1] Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HIV-1, human immunodeficiency virus type 1; HIV-2, human immunodeficiency virus type 2; HTLV-1, human T cell leukemia virus type 1; MoMLV, Moloney murine leukemia virus; MTO, multiple turnover; NC, nucleocapsid protein; PBS, primer-binding site; PPT, polypurine tract; -R ssDNA, single-stranded DNA molecule complementary to the sequence of the R region of retroviral genomic RNA; RSV, Rous sarcoma virus; RT, reverse transcriptase; SDS, sodium dodecyl sulfate; ssDNA, single-stranded DNA; −sssDNA, minus-strand strong-stop DNA; +sssDNA, plus-strand strong-stop DNA; STO, single turnover; TBE, Tris−borate−EDTA buffer; TE, Tris−EDTA buffer; Tris, tris(hydroxymethyl)-aminomethane.

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FIGURE 1: Sequences of the DNA oligonucleotides used in this study. (A) DNA oligonucleotides used as acceptor strands. The 5′ end of the oligonucleotide that is not complementary to the DNA primer strand (5′-end extension) is shown in bold. (B) DNA oligonucleotides used as primer strands. For the strand A short-modified oligonucleotide, the bases that differ from the strand A oligonucleotide are shown in bold.

substantial regions of complementarity and, at the same time, decrease (or destabilize) annealing that involves short segments of complementarity. Single-stranded DNAs (ssDNAs), whose sequences are complementary to part of the R region of the genomic RNA (-R ssDNA) of the human immunodeficiency virus type 1 (HIV-1), human immunodeficiency virus type 2 (HIV-2), and human T cell leukemia virus type 1 (HTLV-1), can self-prime in vitro. Self-priming prevents strand transfer. In vitro, self-priming of -R ssDNA can be prevented by annealing a DNA oligonucleotide to the 3′ end of -R ssDNA in the presence or the absence of the HIV-1 nucleocapsid (NC) protein (*9*, *10*). However, when such DNA oligonucleotides were added in large excess, nonspecific strand transfer could occur. At relatively high dNTP concentrations, nontemplated nucleotides could be added to a blunt-ended DNA substrate. If the nontemplated nucleotide could base pair with the 3′ end of a single-stranded DNA acceptor, there was strand transfer (*10*, *11*). Although such events are rare at in vivo dNTP concentrations, one-base-pair strand transfer could explain some of the insertions and deletions seen in retroviral genomes (*12*). We reported that while HIV-1 NC does not prevent nontemplated nucleotide addition in vitro (*12*), it does reduce the efficiency of one-base-pair strand transfer reactions in vitro (*11*).

In the present report, we used the ability of HIV-1 RT to extend a DNA primer to measure the rate of annealing of single-stranded DNAs. The rate of annealing was affected by the size of the region of complementarity between the primer and the acceptor strands, the presence of secondary structure in the primer and/or the acceptor strands, and the presence of HIV-1 NC. In the absence of secondary structure, the rate of annealing increased with the size of the region of complementarity and decreased when at least one of the strands could form a secondary structure in the region of complementarity. HIV-1 NC differentially affected strand transfer as a function of the length of the complementarity between the primer and the acceptor strand and the presence or absence of secondary structure in the two strands. HIV-1 NC reduced the efficiency of transfer when the region of complementarity between the primer and the acceptor strands was shorter than 13 bases. For longer regions of complementarity, NC increased the rate of annealing only when secondary structures were present in either the primer or the acceptor strands; NC had a slightly negative effect on the rate of annealing in the absence of secondary structure. These results explain the role of NC in facilitating the first strand transfer where there is secondary structure in the 3′ end of genomic RNA; it also suggests that NC has an important role in increasing the accuracy of recombination during reverse transcription.

MATERIALS AND METHODS

HIV-1 RT and DNA Oligonucleotides. Wild-type HIV-1 RT (p66/p51) was expressed in *Escherichia coli* and purified as described previously (*13*). HIV-1 nucleocapsid protein (p7 Zn^{2+} NC) was generously provided by Drs. Robert Gorelick, Louis Henderson, and Larry Arthur (SAIC, Frederick). A 30 μM NC solution was prepared by dissolving lyophilized NC in RT binding buffer [50 mM Tris-HCl, pH 8.3, 80 mM KCl, 0.1 mg/mL bovine serum albumin (BSA)] containing 20% glycerol. NC was stored in 4 μL aliquots in 150 μL tubes at −80 °C. Fresh aliquots were thawed immediately prior to use.

DNA oligonucleotides were purchased from Gibco BRL, Integrated DNA Technologies, and BIOSOURCE International. 5′-End labeling of DNA oligonucleotides was performed using [γ-^{32}P]ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase (New England Biolabs). Labeled oligonucleotides were purified on a 12% denaturing polyacrylamide gel. The appropriate band was cut out of the gel and the oligonucleotide eluted overnight in water in the presence of proteinase K. After phenol extraction, the oligonucleotide was precipitated and dissolved in TE (10 mM Tris-HCl, pH 7.0, 1 mM EDTA) buffer.

DNA Primer and Acceptor Strands. Sequences of the DNA oligonucleotides were derived from the 3′ end of the HIV-1 −sssDNA (*14*) (Figure 1). The "H*X*/+*Y*" DNA acceptor oligonucleotide had *X* bases complementary to the 3′ end of one of the two DNA primers (strand A or strand A short modified) and a *Y*-base extension at the 5′ end. The strand B oligonucleotide had 21 bases of perfect complementarity to the strand A oligonucleotide, a five-base extension at the 5′ end, and seven bases at the 3′ end that were only partially complementary to the strand A oligonucleotide (*12*).

DNA Secondary Structure Predictions. DNA secondary structure predictions were performed using mfold version 3.0 software available on the Zuker and Turner web site (http://bioinfo.math.rpi.edu/~zukerm/export/) (*15*) (J. SantaLucia, Jr., M. Zuker, A. Bommarito, and R. J. Irani, unpublished data). Calculations were performed at 37 °C with oligomer corrections and 80 mM NaCl and 6 mM $MgCl_2$, which is equivalent to the salt composition of the buffer used in the annealing experiments.

Kinetics of Formation of Strand Transfer Products. Annealing rates were obtained by measuring the extension of the 3′ end of a DNA primer by HIV-1 RT after it was annealed to a DNA acceptor with a 5′-end extension. Typically, the DNA primer [either the strand A or the strand A short modified oligonucleotide (20 nM)] was mixed with 200 nM HIV-1 RT, 1 mM fresh DTT, and 10 μM dNTP (or 500 μM ddCTP for the experiments performed with the strand B oligonucleotide) in RT binding buffer. HIV-1 NC was incubated with the primer for 5 min at 37 °C when specified. Reactions were started by the addition of a mixture (preincubated at 37 °C) composed of $MgCl_2$ (6 mM final), the acceptor strand oligonucleotide, and HIV-1 NC (when specified) and performed at 37 °C (two acceptor oligonucleotides were present in the competition experiments). Aliquots were removed from the reactions after appropriate incubations and transferred to a tube containing an equal volume of 2× formamide stop solution (90% formamide, 2× TBE, bromophenol blue, and xylene cyanol) and 50 mM EDTA. When the HIV-1 NC was present in the reaction, the 2× formamide stop solution also contained 4 μg/mL plasmid DNA and 1% SDS. Samples were then denatured at 90 °C for 5 min and loaded on either a 12% or a 16% (for the reactions performed with the strand A short-modified oligonucleotide) denaturing polyacrylamide gel. When NC was present in the reaction, the gel also contained 0.5% SDS. After electrophoresis, gels were dried under vacuum and exposed to an X-ray film (Kodak) or a PhosphorImager screen (Molecular Dynamics). Unless otherwise specified, the reactions contained 20 nM primer strand and 200 nM acceptor strand. For reactions involving the H7/+8 and the strand A oligonucleotides, the concentrations were 10 μM and 20 nM, respectively. For reactions performed with the H15/+2, the H21/+15, or the H21/+10 unstable oligonucleotides and the strand A oligonucleotide, the concentrations were 75 and 20 nM, respectively; for the strand B and the strand A oligonucleotides, 300 and 20 nM, respectively; for the H13/+15 or the H21/+9 stable oligonucleotides and the strand A short modified oligonucleotide, 40 and 10 nM, respectively; and for the H21/+10 unstable and the strand A short modified oligonucleotides, 3 and 1 nM, respectively.

Quantitation of the Gels. Radioactivity on the gels was quantitated by phosphorimaging. Results were analyzed using ImageQuant software, version 5.0. For each time point, the amount of unreacted substrate and the amount of extended DNA primer were quantitated. The percentage of extended DNA donor was calculated, and the results were plotted and fit as a function of time (variable *t*) using KaleidaGraph software.

RESULTS

Measurements of the Kinetics of Annealing. We used DNA oligonucleotides to measure the effects of secondary structure and HIV-1 NC on the rate of annealing. Primers and acceptors were created that have different sequences and different lengths of complementarity. The strand used as the primer was 5′-end labeled with ^{32}P. The 3′ end of the strand used as the acceptor was complementary to the 3′ end of the primer strand and had a short 5′ extension. After the 3′ end of the primer strand annealed to the acceptor strand, HIV-1 RT extended the primer strand using the 5′ extension of the acceptor strand as a template. Although RT can also extend the acceptor strand, this has no effect on the rate of annealing because it happens after the oligonucleotides have annealed. The products of the reaction were resolved on a denaturing polyacrylamide gel, and the amount of the strand transfer product was quantitated (Materials and Methods).

Table 1: Formation of Secondary Structures in the DNA Oligonucleotides Used as Primers or Acceptors in the Annealing Reactions

oligonucleotide	ΔG of the most stable conformation[a]	secondary structure at the 5′ end?[b]	single-stranded bases at the 5′ end[c]
strand A	−3.5	N/A	N/A
strand A short modified	no stable structure	N/A	N/A
strand B	−7.2	no	3
up to H15/+2	no stable structure	N/A	N/A
H18/+12	−2.1	yes	0
H21/+15	−3.4	no	6
H21/+10 unstable	no stable structure	N/A	N/A
H21/+9 stable	−3.5	yes	0

[a] In kcal/mol. Calculations were performed at 37 °C in the presence of 6 mM $MgCl_2$ and 80 mM NaCl using the SantaLucia algorithm available on the Zuker and Turner web site (http://bioinfo.math.rpi.edu/~zukerm/export/) (*15*). [b] Is the nucleotide at the 5′ end of the complementary region in the acceptor involved in a secondary structure or not? [c] How far (in nucleotides) is the secondary structure in the acceptor strand from the 5′ end of the region of complementarity before it anneals to a complementary oligonucleotide?

In our experiments, formation of the strand transfer product is a two-step reaction. First, there is the annealing of the 3′ end of the primer strand to the 3′ end of the acceptor strand, k_{ann}. The ternary complex composed of the nucleic acid, RT, and the incoming dNTP is formed, and the primer strand is extended by copying the acceptor strand. We have shown, with conditions used in the experiments described here, that the formation of the ternary complex is faster than the rate of the templated polymerization, k_{pol} (*12*). However, the rate of templated polymerization is also relatively fast, typically higher than 10 s^{-1} (*16*). If the annealing reaction is slow in comparison to the polymerization reaction, then the rate of the formation of strand transfer products depends directly on the rate of the annealing.

Choice of Primer and Acceptor Strands. The sequences of the primer and acceptor strands used in this study were based on the sequence of the 3′ end of HIV-1 minus-strand strong-stop DNA (−sssDNA) [GenBank accession number GI328415 (*14*); see also Figure 1]. Two different primer strands were used. The strand A oligonucleotide is able to fold back on itself and form a hairpin (Table 1, Figure 2). When strand A oligonucleotide (20 nM) was incubated with 10 μM dNTPs and HIV-1 RT (Materials and Methods), it was not extended, showing that there was no detectable self-priming under our experimental conditions (data not shown), presumably because the secondary structure does not extend to the 3′ end of the oligonucleotide (Figure 2). Deletions and mutations were introduced in the 5′ end of the strand A oligonucleotide to create an oligonucleotide which would not form a stable secondary structure (strand A short modified oligonucleotide; Figure 1, Table 1).

Ten different acceptor strands (Figure 1) were prepared which had different lengths of complementarity to the strand A and strand A short-modified oligonucleotides. The "H*X*/

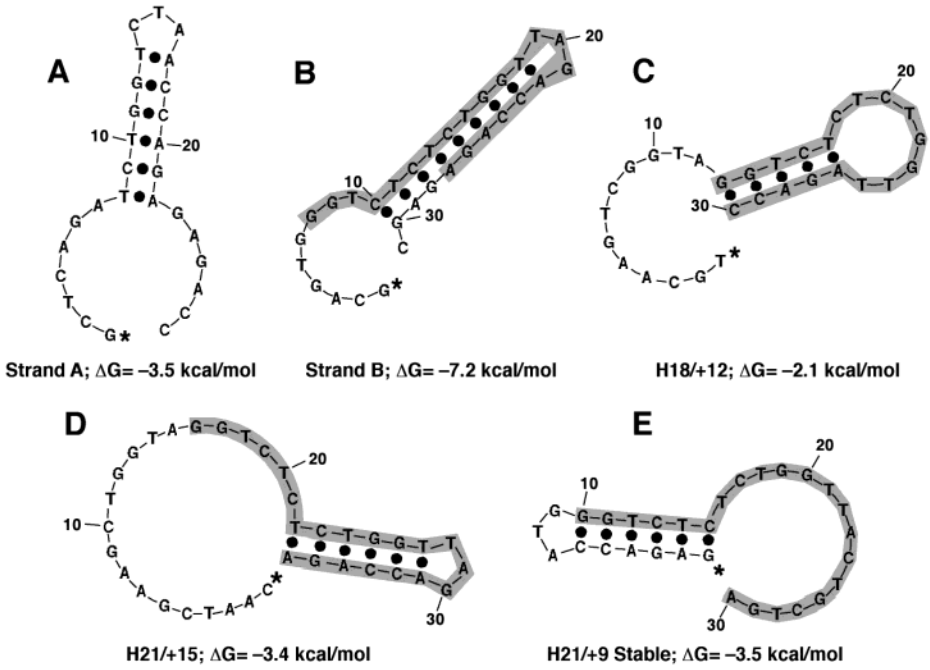

FIGURE 2: Most stable conformations of the DNA oligonucleotide primers and acceptors: (A) strand A, (B) strand B, (C) H18/+12, (D) H21/+15, and (E) H21/+9 stable oligonucleotides. A star indicates the 5′ end of each oligonucleotide. Formation of base pairs is indicated by a dot between the two bases. DNA secondary structure predictions were performed using mfold version 3.0 software (see Materials and Methods). In panels B−E, nucleotides that can hybridize to the strand A primer are shaded in gray.

+*Y*" DNA oligonucleotide had *X* bases complementary to the 3′ end of one of the two DNA primers and a *Y*-base extension at the 5′ end. To avoid any possible effect on polymerization kinetics due to pause sites in the template, we used acceptor strands with short 5′ extensions (less than 15 bases) that would not form secondary structures after they had annealed to the primer strand. Acceptor strands that had up to 15 bases of complementarity to the primer strand were not able to form secondary structures before annealing to the primer strand (Table 1). Acceptor strands with longer regions of complementarity with the primer strand could form secondary structures before annealing to the primer strand (except the H21/+10 unstable oligonucleotide) (Table 1, Figure 2). We also designed an oligonucleotide with 21 bases of complementarity with the strand A short modified oligonucleotide that could not form secondary structures before annealing to the primer strand (H21/+10 unstable oligonucleotide; Figure 1, Table 1).

Competition for Annealing of Strand A to Two Different Acceptors. ^{32}P-5′-end-labeled strand A oligonucleotide was incubated with one of the DNA oligonucleotide acceptors (Figure 3A). No strand transfer product was observed with the H3/+2 and the H5/+5 acceptor strands (data not shown). Transfer to a DNA oligonucleotide acceptor with less than 10 bases of complementarity with the primer (H7/+8 and H8/+8 oligonucleotides) was very inefficient (lanes 2 and 3). When the acceptor strand had at least 10 bases of complementarity, complete strand transfer occurred during the time of the incubation (lanes 5−9). For some of the oligonucleotides, two strand transfer products were present (lanes 4 and 7). The upper band was the result of nontemplated nucleotide addition to the 3′ end of the full-length strand transfer product (lower band). Both bands were used to measure the efficiency of annealing. Because the nontemplated nucleotide is added only after the acceptor strand has been fully copied, the addition of a nontemplated nucleotide does not affect the rate of annealing.

If two different oligonucleotide acceptors (acceptor 1 and acceptor 2) are both present in an annealing reaction, the relative amounts of the two strand transfer products (products of the transfer of the primer to acceptor 1 and acceptor 2) depend on the relative rates of annealing of the primer to acceptor 1 and acceptor 2. Competition experiments were used to measure the transfer of strand A oligonucleotide to two different acceptor oligonucleotides (Figure 3B). As we showed previously (Figure 3A), transfer of the strand A oligonucleotide to the H9/+4 oligonucleotide was very inefficient in comparison to the transfer to the H10/+12 oligonucleotide (lane 2); the only products seen were from the transfer to the H10/+12 oligonucleotide even when the concentration of the H9/+4 oligonucleotide (400 nM) was higher than the concentration of the H10/+12 oligonucleotide (100 nM). Transfer of the strand A oligonucleotide to the H13/+15 oligonucleotide was faster than transfer to the H10/+12 oligonucleotide (lanes 3 and 6). Transfer to the H15/+2 oligonucleotide was faster than transfers to the H13/+15 (lane 9), H18/+12 (lanes 12 and 15), and H21/+15 (lane 18) oligonucleotides, and transfer to H18/+12 was faster than the transfer to H21/+15 in the absence of NC. In experiments with acceptor oligonucleotides that had less than 15 bases of complementarity with the strand A oligonucleotide (lanes 2, 3, 6, and 9), the rate of strand transfer increased with the size of the complementary region. With acceptor oligonucleotides with complementary regions longer than 15 nucleotides, the rate was slower (lanes 12, 15, 18, and 21). These results show that there is no simple correlation between the size of the complementarity between the primer and the acceptor strands and the rate of annealing.

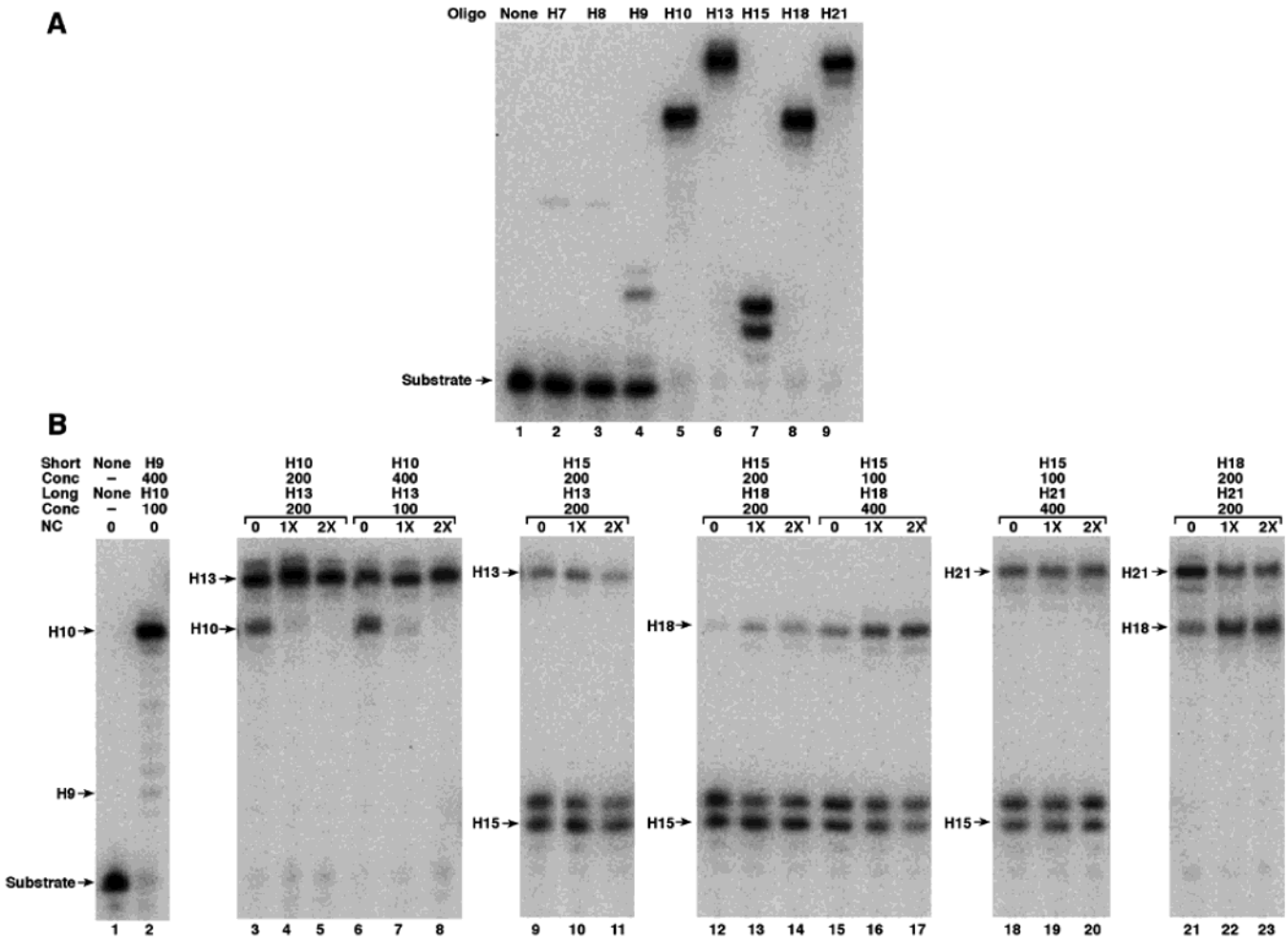

FIGURE 3: Competition for annealing of the strand A oligonucleotide to two DNA oligonucleotide acceptors with different lengths of complementarity with the strand A oligonucleotide. (A) The strand A oligonucleotide was incubated for 15 min with a DNA oligonucleotide acceptor (specified at the top of the gel), and the reactions were carried out as described in Materials and Methods. Reaction products were analyzed on a 12% denaturing polyacrylamide gel. (B) For each reaction, the strand A oligonucleotide was incubated with two DNA oligonucleotide acceptors (names and concentrations are specified at the top of the gel) with different lengths of complementarity with the strand A oligonucleotide for 15 min in the absence or in the presence of HIV-1 NC (lanes designated 1× or 2×). For the reactions presented in the lanes labeled 1× and 2×, HIV-1 NC was added in 1- or 2-fold excess by comparison to the total DNA concentration, respectively. The oligonucleotide acceptor with the shorter 5′-end template extension is called short; the other oligonucleotide is called long. Concentrations of the acceptor strands are expressed in nanomolar and are specified at the top of the gel. H7 is the H7/+8 oligonucleotide, H10 is the H10/+12 oligonucleotide, H13 is the H13/+15 oligonucleotide, H15 is the H15/+2 oligonucleotide, H18 is the H18/+12 oligonucleotide, and H21 is the H21/+15 oligonucleotide. The positions on the gel of the various strand transfer products are indicated by arrows. The name of the acceptor strand oligonucleotide used to form each of these products is specified near each arrow.

We did similar competition reactions in the presence of a 1-fold or a 2-fold excess of HIV-1 NC. The excess of HIV-1 NC was calculated on the assumption that each NC molecule covers seven bases (*17*). The effects of HIV-1 NC on the distribution of the strand transfer products were clearly different for different pairs of oligonucleotide acceptors. For reactions done in the presence of the H10/+12 and H13/+15 oligonucleotides (200 nM each, lanes 3−5; 400 and 100 nM, respectively, lanes 6−8), the addition of NC increased the difference in the rate of annealing between the faster and the slower reactions as measured by the amount of the two different strand transfer products. When a 1-fold excess of NC was present, a small amount of transfer to the H10/+12 acceptor was seen. When a 2-fold excess of HIV-1 NC was added, essentially all of the transfer was to H13/+15 (compare lanes 5 and 8 with lanes 4 and 7, respectively). The distribution of the strand transfer products was not affected by the addition of NC for reactions performed with the H13/+15 and H15/+2 oligonucleotides (200 nM each, lanes 9−11) and the H15/+2 and H21/+15 oligonucleotides (100 and 400 nM, respectively, lanes 18−20). For the reactions performed with the H15/+2 and H18/+12 oligonucleotides (200 nM each, lanes 12−14; 100 and 400 nM, respectively, lanes 15−17) and the H18/+12 and H21/+15 oligonucleotides (200 nM each, lanes 21−23), the addition of NC decreased the difference in the relative amounts of the strand transfer products. We believe that the explanation for these results lies in the effects of NC on the annealing of oligonucleotides that do (or do not) have secondary structure (see Discussion).

Kinetics of Annealing of the Strand A Oligonucleotide. To try to understand the results of the competition experiments, we performed kinetic analysis of the annealing of the strand A oligonucleotide to several acceptor oligonucleotides. The strand A oligonucleotide was incubated with dNTPs and HIV-1 RT in RT binding buffer (Materials and Methods). The reaction was started by addition of a mixture composed of the H10/+12 oligonucleotide present in large excess and

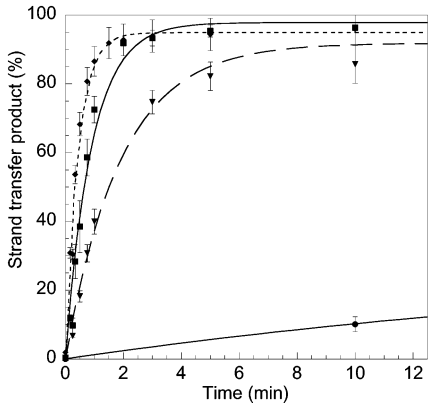

FIGURE 4: If the DNA oligonucleotide acceptor has no secondary structure, the rate of annealing increases with the length of complementarity between the DNA primer and the DNA acceptor strands. The strand A oligonucleotide (20 nM) was incubated with either the H9/+4 (●), the H10/+12 (▼), the H13/+15 (■), or the H15/+2 (◆) oligonucleotide (200 nM), and the reactions were carried out as described in Materials and Methods. The products were analyzed on a 12% denaturing polyacrylamide gel. Curves were fit using the KaleidaGraph software using a single-exponential equation. Each curve is the average of at least four independent reactions.

$MgCl_2$. The annealing reaction (as measured by the formation of the strand transfer product) could be fitted by a single-exponential equation with a k_0 of 0.49 min^{-1} (Figure 4). Because the observed rate of formation of the strand transfer product is much slower than templated polymerization [typically around 10 s^{-1} (*16*)], the observed rate of the reaction is equivalent to the rate of the annealing step. Doubling the concentration of the RT or the dNTPs had no effect on the reaction (data not shown); previous analysis showed that RT binding and the initiation of reverse transcription are very rapid under our conditions (*12*). Because the concentration of the acceptor was much larger than the concentration of the primer, the concentration of the acceptor strand could be considered to be constant during the reaction. Consequently, the annealing step was a pseudo-first-order reaction with a k_0 equal to the product of k_{ann}, the rate of the annealing step, and the concentration of the acceptor strand (200 nM in this specific reaction). For this specific reaction, we found a k_{ann} of 2.4×10^6 min^{-1} M^{-1} (Table 2).

Similar experiments were performed with the H7/+8 (10 μM), H9/+4 (200 nM), H13/+15 (200 nM), and H15/+2 (75 nM) oligonucleotides (Figure 4, Table 3). Except for the reaction performed with the H7/+8 oligonucleotide, the reactions went to completion in less than 1 h, and k_{ann} could be calculated using an exponential equation (Table 2). In the case of the H7/+8 oligonucleotide, the reaction did not go to completion in less than 1 h, and the linear part of the data was fitted using a linear equation with a slope equal to $[H7/+8]k_{ann}$ (Table 2). Similar experiments were performed with the H18/+12 (200 nM) and the H21/+15 (75 nM) oligonucleotides. With these two acceptor strands, a two-exponential fit was required $[A_0(1 + 1/(k_1 - k_0))$-

Table 2: Rate of Annealing of DNA Oligonucleotide Acceptors to the Strand A Oligonucleotide[a]

oligonucleotide acceptor[b]	concn (nM)[c]	k_0 without NC (min^{-1})	k_{obs} without NC (min^{-1} M^{-1})[d]	k_0 with 2× NC (min^{-1})	k_{obs} with 2× NC (min^{-1} M^{-1})[d]
H7/+8	10000	0.12 ± 0.02	$(1.2 \pm 0.2) \times 10^4$		
H9/+4	200	$(6.9 \pm 1.3) \times 10^{-3}$	$(3.4 \pm 0.6) \times 10^4$	$(1.7 \pm 0.3) \times 10^{-3}$	$(8.5 \pm 1.5) \times 10^3$
H10/+12	200	0.49 ± 0.07	$(2.4 \pm 0.3) \times 10^6$	0.021 ± 0.06	$(1.0 \pm 0.3) \times 10^5$
H13/+15	200	1.14 ± 0.14	$(5.7 \pm 0.7) \times 10^6$	1.06 ± 0.18	$(5.3 \pm 0.9) \times 10^6$
H15/+2	75	0.75 ± 0.17	$(1.0 \pm 0.2) \times 10^7$	0.85 ± 0.17	$(1.1 \pm 0.2) \times 10^7$
H18/+12	200	0.31 ± 0.02	$(1.6 \pm 0.1) \times 10^6$	1.76 ± 0.3	$(8.8 \pm 1.5) \times 10^6$
H21/+15	75	0.20 ± 0.03	$(2.7 \pm 0.4) \times 10^6$	0.30 ± 0.05	$(4.0 \pm 0.7) \times 10^6$
strand B	300	0.21 ± 0.03	$(7.0 \pm 1.0) \times 10^5$	0.77 ± 0.12	$(2.6 \pm 0.4) \times 10^6$

[a] The rate of annealing of the strand A oligonucleotide to different acceptors was measured in the presence of 10 μM dNTPs, 6 mM $MgCl_2$, and 200 nM HIV-1 RT in RT binding buffer at 37 °C in the presence or absence of HIV-1 NC as described in Materials and Methods. Aliquots were quenched after different incubation times, and products were analyzed on a 12% denaturing polyacrylamide gel. Curves were fitted using the KaleidaGraph software. When the reaction did go to completion in less than 1 h, data were fit using a single- or a double-exponential equation (see Results). When the reaction did not go to completion in less than 1 h, the linear part of the curve was fit using a linear equation (percentage of product = k_0t). Rates are the average of at least four independent reactions. [b] Name of the oligonucleotide used as acceptor in the annealing reaction with the strand A oligonucleotide. [c] Concentration of the oligonucleotide used as the acceptor. [d] k_{obs} is equal to k_0 divided by the concentration of the acceptor oligonucleotide.

Table 3: Comparison of the Rates of Annealing of Some DNA Oligonucleotide Acceptors to the Strand A or the Strand A Short Modified Oligonucleotides[a]

oligonucleotide primer[b]	oligonucleotide acceptor[c]	concn (nM)[d]	k_0 without NC (min^{-1})	k_{obs} without NC (min^{-1} M^{-1})[e]	k_0 with 2× NC (min^{-1})	k_{obs} with 2× NC (min^{-1} M^{-1})[e]
strand A	H13/+15	200	1.14 ± 0.14	$(5.7 \pm 0.7) \times 10^6$	1.06 ± 0.18	$(5.3 \pm 0.9) \times 10^6$
strand A short modified	H13/+15	40	1.48 ± 0.24	$(3.7 \pm 0.6) \times 10^7$	1.0 ± 0.1	$(2.5 \pm 0.3) \times 10^7$
strand A	H21/+15	75	0.20 ± 0.03	$(2.7 \pm 0.4) \times 10^6$	1.76 ± 0.3	$(8.8 \pm 1.5) \times 10^6$
strand A short modified	H21/+10 unstable	3	2.70 ± 0.37	$(9.0 \pm 1.2) \times 10^8$	1.97 ± 0.20	$(6.6 \pm 0.6) \times 10^8$
strand A short modified	H21/+9 stable	40	1.05 ± 0.2	$(2.6 \pm 0.5) \times 10^7$	1.07 ± 0.21	$(2.7 \pm 0.5) \times 10^7$

[a] The rate of annealing of the primers to the acceptors was measured in the presence or absence of HIV-1 NC as described in Materials and Methods. When the reaction went to completion in less than 1 h, data were fitted using a single-exponential equation. When the reaction did not go to completion in less than 1 h, the linear part of the curve was fit using a linear equation (percentage of product = k_0t). Rates are the average of at least four independent reactions. [b] Name of the oligonucleotide used as the primer in the annealing reaction. [c] Name of the oligonucleotide used as acceptor. [d] Concentration of the oligonucleotide used as acceptor. [e] k_{obs} is equal to k_0 divided by the concentration of the acceptor oligonucleotide.

($k_0 \exp(-k_1 t) - k_1 \exp(-k_0 t)$)]. This type of equation describes a reaction with two successive steps. In both cases, k_1 was much larger than k_0 (6.9 ± 1.00 and 0.31 ± 0.02 min^{-1} with the H18/+12 oligonucleotide and 7.0 ± 1.3 and 0.20 ± 0.03 min^{-1} with the H21/+15, respectively). When the concentration of the H21/+15 oligonucleotide was tripled, k_1 was not affected, but k_0 increased (data not shown). Thus, k_1 cannot be related to the annealing step because it does not depend on the concentration of the acceptor strand. Because k_0 does, it is related to the annealing reaction; k_1 represents some step that occurs prior to annealing. k_1 might represent a transition step between two conformations of the oligonucleotide before annealing. Similar observations were made with the strand B oligonucleotide (Table 2). If an acceptor strand can form a secondary structure (like H18/+12, H21/+15, and strand B), only the portion of the acceptor that is not folded can participate in the annealing reaction. This slows the annealing reaction relative to transfers to acceptors that have no secondary structure. If the acceptor can form a secondary structure, the acceptor strand (Acc) is in rapid equilibrium between two conformations (the folded and the unfolded conformations) with an equilibrium constant, K_e. Only the unfolded acceptor strand (Acc_{unfold}) can anneal to the primer. Because the acceptor strand is in large excess, $k_0 = k_{ann}[Acc_{unfold}] = k_{ann}K_e[Acc]$. To be able to compare annealing reactions with different acceptor strands, we used the constant $k_{obs} = k_{ann}K_e$ (Table 2). When the acceptor strand cannot form a secondary structure, $K_e = 1$ and $k_{obs} = k_{ann}$. Under our reaction conditions, 10 bases of complementarity were necessary for efficient transfer of the strand A oligonucleotide. The k_{obs} increased with the length of the complementarity between the strand A oligonucleotide and the acceptor strand (up to 15 bases); however, with longer regions of complementarity, it decreased, probably due to the formation of the secondary structure in the acceptor strand.

Effect of HIV-1 NC on the Annealing of the Strand A Oligonucleotide to a DNA Oligonucleotide Acceptor. Similar experiments were performed in the presence of HIV-1 NC. Each strand was incubated separately with HIV-1 NC for 5 min at 37 °C, before the annealing reaction was started (see Materials and Methods). Experimental conditions were identical to those previously used in the reactions performed in the absence of HIV-1 NC. When the H10/+12 oligonucleotide was used as the acceptor, the addition of HIV-1 NC reduced the rate of annealing (k_{obs}) (Figure 5A, Table 2). The observed rate of the annealing reaction decreased as the concentration of HIV-1 NC increased (Figure 5A, 3- and 24-fold decrease when a 0.2- and 2-fold excess of HIV-1 NC was added). Similar results were obtained with the H9/+4 oligonucleotide (Table 2). The addition of HIV-1 NC had no measurable effect on the observed rate of annealing of the strand A oligonucleotide to the H15/+2 (Figure 5B, Table 2) and H13/+15 oligonucleotides (Table 2). For annealing reactions involving the H18/+12 and the strand B oligonucleotides, the addition of HIV-1 NC increased the observed rate of annealing (5- and 4-fold, respectively; Figure 5C, Table 2); with the H21/+15 oligonucleotide, there was a more modest increase in the observed rate (1.5-fold; Figure 5D, Table 2).

Effects of the Formation of Secondary Structures in the DNA Acceptors or Primers. As has already been mentioned,

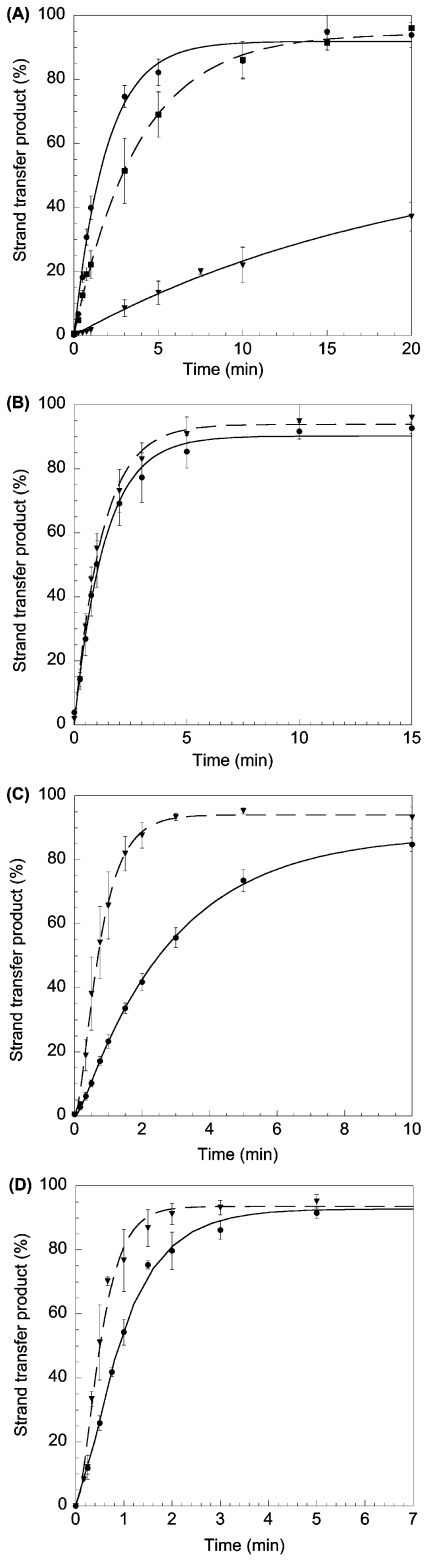

FIGURE 5: Effects of HIV-1 NC on the rate of formation of the strand transfer product are functions of the size of the complementarity region between the primer and the acceptor strands, the presence or the absence of secondary structures, and their positions. Transfer of the strand A oligonucleotide (20 nM) to the H10/+12 (200 nM) (panel A), the H15/+2 (75 nM) (panel B), the H18/+12 (200 nM) (panel C), or the H21/+15 oligonucleotide (75 nM) (panel D) in the absence of the HIV-1 NC (●) or in the presence of a 0.2-fold excess (■) or 2-fold excess (▼) of HIV-1 NC was measured as described in Materials and Methods. Curves were fit using the KaleidaGraph software using a single- or a double-exponential equation. Each curve is the average of at least four independent reactions.

the strand A oligonucleotide could form a secondary structure (Table 1, Figure 2). We modified the sequence of the strand A oligonucleotide to create a DNA primer that was not able to form any stable secondary structures (strand A short modified oligonucleotide; Table 1, Figure 1). The results were similar to those obtained with the strand A oligonucleotide; there was no detectable transfer of the strand A short modified oligonucleotide to an oligonucleotide acceptor with less than seven bases of complementarity (data not shown). The H13/+15 oligonucleotide acceptor is not able to form a stable secondary structure (Table 1). The annealing of the strand A short modified oligonucleotide to the H13/+15 oligonucleotide was 6 times faster than the annealing of the strand A oligonucleotide to the same acceptor (Table 3), which suggests that secondary structure in the primer strand slows the rate of annealing. The addition of HIV-1 NC to the annealing reaction slightly reduced the observed rate of a reaction with the strand A short modified oligonucleotide as primer and did not significantly affect the rate of an annealing reaction with the strand A oligonucleotide as the primer (Table 3).

As shown in Table 2, the observed rate of annealing of the strand A oligonucleotide to the H18/+12 oligonucleotide was about 6 times slower than the observed rate of annealing to the H15/+2 oligonucleotide (Table 2). To see if this decrease could be attributed to the formation of the secondary structure in the H18/+12 oligonucleotide (Figure 2), we designed two DNA oligonucleotide acceptors with 21 bases of complementarity to the strand A short modified oligonucleotide. The H21/+10 unstable oligonucleotide was not able to form a stable secondary structure; the H21/+9 stable oligonucleotide was able to form a stable secondary structure (Table 1, Figure 2). The annealing of the strand A short modified oligonucleotide to the H21/+10 unstable oligonucleotide was more than 30 times faster than to the H21/+9 stable oligonucleotide (Table 3). The addition of HIV-1 NC caused a decrease in the observed rate of annealing of strand A short modified oligonucleotide to the H21/+10 unstable oligonucleotide as previously observed for the transfer to the H13/+15 oligonucleotide; however, it did not affect the rate of annealing to the H21/+9 stable oligonucleotide (Table 3).

DISCUSSION

Two key steps of retroviral reverse transcription are the two strand transfers, the first between the growing DNA strand and the 3′ end of the RNA (minus-strand transfer) and the second involving DNA templates (plus-strand transfer) (*3*). HIV-1 NC protein facilitates a variety of annealing and strand exchange processes, including the annealing of DNA and RNA oligonucleotides (*18*−*26*), tRNA primer unwinding (*27*, *28*) and the annealing of a tRNA primer to the PBS (*29*−*37*), retroviral RNA dimerization (*38*, *39*), and ribozyme catalysis (*20*, *40*−*43*). NC is able to produce these effects because of its nucleic chaperone activity (*40*, *44*); i.e., it catalyzes conformational rearrangements of nucleic acids to produce thermodynamically more stable structures (*18*, *20*, *43*, *45*). NC clearly promotes minus-strand transfer (*19*, *21*−*23*, *26*, *34*, *46*−*53*) and plus-strand transfer (*24*, *26*, *54*−*56*) during retroviral DNA synthesis. In addition to the strand transfer reaction, NC might affect other steps in reverse transcription including initiation (*47*, *57*), rate of polymerization (*53*, *58*−*60*), pausing during polymerization (*47*, *58*−*60*), ability to polymerize through secondary structures (*61*, *62*), abortion of synthesis by strand separation (*58*), self-priming of −sssDNA (*9*−*11*, *48*, *63*), reassociation of RT with the nucleic acid during polymerization (*47*), RNase H activity of RT (*51*, *53*), removal of the tRNA Lys3 primer from the 5′ end of minus-strand DNA (*24*, *26*), and the rate of internal recombination (*58*, *64*, *65*). Our goal was to develop a simple in vitro system to measure the annealing rates of two DNA oligonucleotides and to monitor the different parameters (size of the region of complementarity between the primer and the acceptor strands, presence or absence of secondary structures in either the primer or the acceptor strand, presence or absence of NC) that might affect this rate. Our approach was to use a DNA oligonucleotide primer that can be extended by only few nucleotides after it has annealed to a DNA oligonucleotide acceptor. Because the formation of the nucleic acid−RT−dNTP ternary complex and the extension reaction are significantly faster than the annealing reaction, measuring the rate of formation of the extended primer provides a measure of the rate of the annealing reaction.

We found that the size of the complementarity between the primer and the acceptor strands, the presence of secondary structure in the primer and/or the acceptor strand, and the presence or the absence of the HIV-1 NC protein affected the rate of annealing of two DNA oligonucleotides. With our in vitro system, when the size of the region of complementarity between the primer and the acceptor strands was short (less than 13 bases), transfer was possible if the complementarity was at least nine nucleotides, but was inefficient. This agrees with what was previously observed in vitro (*10*, *11*, *66*) and in vivo (*67*−*70*). This is not surprising because the typical melting temperature of a DNA duplex of 12 base pairs is around 37 °C, the temperature used in our assays. Efficient and specific in vivo strand transfer was observed only when the complementarity between the primer and the acceptor strands was at least 12−14 bases long (*67*, *69*−*71*). It is likely that the minimal size of the complementarity for an efficient strand transfer will depend on the conditions of the reaction, on the G·C composition in the region of complementarity, and on the nature of the duplex (DNA·DNA vs DNA·RNA).

Competition reactions involving the strand A oligonucleotide and the H9/+4 and the H10/+12 or the H10/+12 and the H13/+15 oligonucleotides (Figure 3) showed that the rate of annealing increased with the size of the complementarity between the primer and the acceptor strands when a stable secondary structure cannot be formed in the acceptor strand. This result was confirmed by kinetic analysis (Table 2). The increase in the rate of the strand transfer with the size of the complementarity can be explained as an increase in the k_{on} of the annealing reaction. Strand transfer is clearly based on a region of complementary sequence (*72*) rather than on the presence of specific sequences (*46*, *66*). The longer the region of complementarity between the two strands, the faster the annealing of the strands. Thus, the strand transfer reaction is intrinsically selective; specific annealing is favored in comparison to the annealing of segments with short complementary segments, as has been observed in vivo (*68*).

Under our conditions, HIV-1 NC reduced the rate of annealing between oligonucleotides with short regions of complementarity (less than 12 bases). NC has a moderate negative effect on annealing when the region of complementarity between the primer and acceptor was longer than 12 nucleotides if the acceptor strand cannot form a stable secondary structure (transfer of the strand A oligonucleotide to the H13/+12 or the H15/+2 oligonucleotides, for example), probably because NC binds to the nucleic acid. These results are similar to those observed in reactions involving ribozymes (*43*). NC inhibited the reaction of the least stable ribozyme−substrate complexes (less than 12 bases) while cleavage of complexes in which the complementary segment was 13−14 base pairs was not affected. NC also enhanced the rate of dissociation of the products when the double-stranded portion of both cleavage products was less than seven base pairs.

The presence of a secondary structure in the primer acceptor strand slows the rate of annealing (compare the rate of annealing of strand A to the annealing of the strand A short modified oligonucleotide to the H13/+15 oligonucleotide; Table 3). The formation of a secondary structure in the acceptor strand decreased the observed rate of annealing (compare the rate of annealing of the strand A short modified oligonucleotide to the H21/+9 stable and H21/+10 unstable oligonucleotides; Table 3). The position of the secondary structure in the acceptor strand also affected the observed rate of annealing. If the secondary structure extended to the 5′ end of the region of complementarity in the acceptor strand (for example, for the H18/+12 oligonucleotide; Table 1), the decrease of the observed rate of annealing was larger than when a structure of similar free energy did not extend all the way to the 5′ end of the region of complementarity in the acceptor strand (H21/+15 oligonucleotide; Table 1). For secondary structures at similar positions in the acceptor oligonucleotide there was a direct correlation between the stability of the secondary structure in the acceptor strand and the observed rate of annealing (compare the annealing of the strand A oligonucleotide to the H21/+15 and to the strand B oligonucleotide; Table 2). If there was secondary structure in the acceptor strand, the secondary structure competed with the annealing of the acceptor strand to the primer strand, which slowed the observed rate of strand transfer.

HIV-1 NC increased the rate of annealing when secondary structures could be formed in the primer and/or acceptor strands (H18/+12, H21/+15, H21/+9 stable, strand B, strand A oligonucleotides, for example). NC is able to destabilize secondary structures (*43*, *56*) by lowering the cooperativity of the helix−coil transition and the kinetic barrier so that the equilibrium between the double-stranded and the single-stranded conformations is rapidly reached and the maximum number of complementary base pairs is formed (*19*, *20*, *73*−*76*). We propose that NC promotes the annealing of single-stranded nucleic acids that have secondary structure by increasing K_e, the equilibrium constant between the folded and the unfolded forms. NC shifts the equilibrium toward unfolded conformations, increasing the concentration of primer and acceptor strands in the appropriate conformation for annealing. The effect of NC depends on the free energy of the secondary structure in the acceptor strand (compare the transfer of the strand A oligonucleotide to the H18+12 and to the strand B oligonucleotides). If the secondary structure is relatively stable, then the difference in the free energy between the two conformations is large. If there is extensive secondary structure, the binding of NC may not be sufficient to significantly increase the concentration of the unfolded strands. As a consequence, NC will have only a moderate effect on the rate of annealing. If the secondary structure is relatively unstable, NC will have a significant effect on the amount of unfolded nucleic acid and on the rate of annealing.

Secondary structures are present at the 3′ end of HIV-1, HIV-2, HTLV-1 RNA, and −sssDNA (*10*). The 3′ end of genomic RNA from both Moloney murine leukemia virus (MoMLV) and Rous sarcoma virus (RSV) can also form stable secondary structures (data not shown). The presence of secondary structures in the U_3 and R segments of MoMLV and RSV would explain the role NC plays in facilitating minus-strand transfer during the replication of these viruses. According to our model, the effect of NC on these secondary structures, together with its effects on RNase H activity, accounts for its effects on the rate of the strand transfer reaction (*19*, *21*−*24*, *26*, *34*, *46*−*56*). A previous study measuring copy choice strand transfer showed that certain sequences are hot spots for strand transfer and that NC modifies the pattern of the hot spots (*77*). The presence of secondary structures in the primer and/or the acceptor strands reduces the rate of strand transfer, which could explain hot spots for strand transfer (more efficient transfer occurs when no secondary structure can be formed). The effects of NC on the rate of strand transfer in the presence or absence of secondary structures (positive effect when secondary structure can be formed, slightly negative effect in absence of secondary structure) could explain some of the differences in the pattern of hot spots for transfer seen in the presence and absence of NC.

Strand transfers involving low complementarity between the primer and the acceptor strands are inefficient, and NC reduces the efficiency of these low specificity strand transfers (this report; *11*). NC does not generally promote strand transfer; rather it promotes specific transfers between strands that have secondary structures. The reverse transcription of the genome of retroviruses involves strand transfer in which the 3′ ends of the primer and/or acceptor strands have significant secondary structures. NC helps the process of reverse transcription (polymerization through secondary structures, strand transfer between ends with secondary structures), but it also clearly increases the accuracy of recombination during reverse transcription by reducing nonspecific strand transfers that might occur internally at breaks in the RNA.

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