A Complementary Single-Stranded Docking Site Is Required for Enhancement of Strand Exchange by Human Immunodeficiency Virus Nucleocapsid Protein on Substrates That Model Viral Recombination†

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Received October 15, 2004; Revised Manuscript Received December 3, 2004

ABSTRACT: Enhancement of strand exchange by nucleocapsid protein (NC) is proposed to occur during retroviral recombination. The mechanism was examined using an RNA (donor)−DNA hybrid that mimicked a retrovirus replication intermediate. This consisted of a 25 base pair hybrid region flanked on each side by single-stranded RNA or DNA. A second set of acceptor RNAs that could bind to the 25-base hybrid region and to various lengths of additional bases on the DNA was used to displace the donor by hybridizing with the DNA. Displacement required a complementary single-stranded DNA region outside the donor−DNA 25-nucleotide hybrid region. NC enhanced displacement slightly when the acceptor could bind 10 nucleotides and significantly when binding 22 or more nucleotides in the single-stranded region. Two mutated acceptors that bound over 47 total nucleotides on the DNA (22 in the single-stranded region plus 25 in the hybrid region) were constructed. One had three mismatches in the hybrid region; the other, three in the single-stranded region and one in the hybrid region. Each acceptor bound the DNA with approximately equal thermodynamic stability, yet NC stimulated exchange with the former and actually inhibited with the latter. This emphasized the importance of the single-stranded region in NC stimulation. The results support a mechanism where NC enhances the docking of the acceptor to the single-stranded region and then the acceptor "zippers" through the hybrid and displaces the donor. Results with the mutated acceptors indicate that NC may actually inhibit strand exchange between genomes in nonhomologous regions.

Recombination is crucial for generating diversity among retroviruses. Most recombination occurs during minus strand DNA synthesis when DNA being synthesized on one of the two RNA genomes in the virion switches to the second genome where synthesis continues. Using reconstituted in vitro assays several groups have shown that nucleocapsid protein (NC)[1] can enhance recombination (*1*−*8*). During reverse transcription by HIV reverse transcriptase (HIV-RT), the RNase H activity of RT degrades the RNA strand behind the 3′ terminus of the nascent DNA. Therefore, at any given time, the putative replication intermediate would consist of a relatively small hybrid region [perhaps 15−30 bases (*9*)] between the genomic RNA and DNA and large regions of single-stranded RNA and DNA downstream and upstream, respectively, of the 3′ terminus. The "dock and lock" model proposes that NC accelerates strand exchange by promoting binding of the second RNA (acceptor) to the single-stranded region of the nascent DNA. The acceptor then "zippers" down the DNA through the RNA (donor)−DNA hybrid region and displaces the donor (*4*, *10*). Variations of this mechanism involving pause sites, structural motifs on the acceptor, and genome regions with little secondary structure have been studied (*1*, *4*, *11*). Overall, the results point to the importance of the single-stranded DNA region as a docking site for the acceptor template, with NC playing a pivotal role in enhancing this process. However, relatively little is known concerning the extent of complementarity and the length of the single-stranded region that is required for NC stimulation. These factors are especially important for recombination between diverse genomes such as those from different viral subtypes.

Human immunodeficiency virus NC is a highly basic 55 amino acid protein that contains two CCHC-type zinc fingers (*12*). The protein possesses nucleic acid chaperone activity that can enhance the rearrangement of nucleic acids to encourage formation of the most stable hybrid (*13*−*18*). In the virion NC coats and protects the viral RNAs and has been shown to participate in several steps of the viral life cycle. These include annealing of tRNA to the viral primer binding site (pbs) (as part of the Gag precursor protein) (*19*−*24*), augmenting reverse transcription by destabilizing some of the secondary structures in the viral genome and enhancing RNase H activity (*7*, *25*−*32*), enhancing transfer of the minus and plus strand strong-stop DNAs [(−)ssDNA and (+)ssDNA, respectively] (*26*, *33*−*40*), helping to package the genomes (as part of the Gag precursor protein) (*41*−*46*), aiding in proper folding and dimerization of the

† This work was supported by National Institute of General Medicine Grant GM051140-10A2.

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[1] Abbreviations: NC, nucleocapsid protein; HIV, human immunodeficiency virus; RNase H, ribonuclease H; RT, reverse transcriptase; pbs, primer binding site; (−)ssDNA and (+)ssDNA, minus and plus sense strong-stop DNA.

10.1021/bi0477945 CCC: $30.25
Published on Web 02/12/2005

Table 1: PCR Primers Used To Synthesize DNAs Used as Templates for Donor and Acceptor RNA Synthesis

Acceptor[a]	Primer 1[b] 5' to 3'	Primer 2[c] 5' to 3'
25acc	TTGTAATACGACTCACTATA	TAACCCTCACTAAAGGGAAC
35acc	TTGTAATACGACTCACTATA	AGCTCGAAATTAACCCTCACT
47acc	TTGTAATACGACTCACTATA	ATGATTACGCCAAGCTCGAA
63acc	TTGTAATACGACTCACTATA	GGAAACAGCTATGACCATGA
80acc	TTGTAATACGACTCACTATA	GTGTGGAATTGTGAGCGGAT

[a] Two versions of the acceptors were used. Long and short versions were produced using the same PCR primers but different plasmid DNA for amplification. The long versions in the paper are denoted as *XX*acc with *XX* = the number of bases on the DNA that the acceptor can bind. The short versions can bind the same number of DNA bases but have 50 bases removed from the 5′ end of the acceptor and are denoted *XX*shortacc in the text. Refer to Material and Methods for details of the PCR reactions. [b] This primer was used to create the end of the PCR DNA near the T7 start site. The same primer was used for each acceptor because all acceptors have the same 5′ end which starts at the T7 site. [c] This primer was used to create the end of the PCR DNA that produced the 3′ end of the RNA transcript. The length of the acceptor 3′ end increased with the higher numbered acceptors, increasing the region of the acceptor that would anneal to the single-stranded region of the DNA. Refer to Figure 1 for a schematic diagram of the acceptors.

two genomes (*5*, *14*, *15*, *18*, *47*), and potentially serving a role as a cofactor in integration (*48*−*50*).

Results suggest that NC's chaperone activity is driven mostly by a helix-destabilizing activity that unwinds secondary structures and destabilizes weak hybrids (*2*, *4*, *13*, *17*, *24*, *26*, *35*, *38*, *51*−*57*). A second component that can directly enhance annealing even in the absence of structure may also contribute (*53*).

In this report strand exchange assays were performed using a system that mimicked strand transfer occurring during retrovirus recombination. The system consisted of acceptor RNAs that could hybridize to DNA that was part of a DNA−donor RNA complex. This complex was designed to mimic the replication intermediate occurring during minus strand DNA synthesis. It therefore consisted of an RNA−DNA hybrid region (25 base pairs) flanked by single-stranded regions of RNA and DNA (described above). The ability of various acceptors to bind the DNA and displace the donor RNA was monitored. The results indicated that enhancement of strand exchange by HIV-1 NC occurs when the complementary region of the acceptor extends past the donor−DNA hybrid region for approximately 10 or more nucleotides into the single-stranded DNA region. They also indicated that for NC to accelerate strand exchange it is vital for the single-stranded region of the DNA, and the portion of the acceptor that binds this region, to have a high degree of complementarity. This point is especially relevant to strand exchanges occurring in nature between different HIV subtypes. No evidence for direct invasion of the duplex by the acceptor in the presence or absence of NC was observed. Strand exchange was enhanced by NC only if binding of the acceptor to the single-stranded DNA region could occur. This was the case even if the acceptor−DNA product had greater thermodynamic stability than the donor−DNA. Overall, the results support a mechanism where NC stimulates binding of the acceptor to the upstream single-stranded region of DNA and then facilitates migration of the acceptor through the donor−DNA duplex region. This results in donor displacement and strand exchange. The results are discussed with respect to the potential implications on recombination between genomes with different sequences.

MATERIALS AND METHODS

Materials. Primers used in PCR reactions and to synthesize mutant acceptors were purchased from Integrated DNA Technologies. Taq polymerase and T4 polynucleotide kinase were purchased from New England Biolabs, and T7 RNA polymerase and ribonucleotide triphosphates were purchased from Roche. Proteinase K was purchased from Kodak. RNasin was purchased from Promega. NC was prepared as described previously (*58*). Aliquots of HIV-1 NC were prepared and stored in 50 mM Tris-HCl, pH 7.5, 10% glycerol, and 5 mM 2-mercaptoethanol at 80 °C. Radiolabeled nucleotides were from Amersham. All other chemicals or enzymes used were from Fisher Scientific or Sigma.

Preparation of RNAs. RNAs were prepared by runoff transcription completed on double-stranded DNA products synthesized by PCR (see below). In strand exchange experiments PCR was completed using pBSM13+ or pBSM13Δ for the acceptors and donor, respectively. The pBSM13Δ plasmid was created from pBSM13+ by removing 50 base pairs with *Eco*RI and *Hin*dIII restriction enzymes as described previously (*59*). These extra base pairs gave the acceptors 50 additional nucleotides which allowed for a gel shift when the DNA was hybridized to the acceptors as opposed to the donors. In donor displacement experiments pBSM13Δ was used to synthesize both the donor and acceptors. A list of the primers used in the PCR reactions is shown in Table 1. The various types of acceptors used are described and illustrated more specifically in the Results section and Figure 1.

PCR reactions were run to amplify the desired DNA fragments. The PCR reaction included 0.1 μg of template DNA, 50 pmol of each primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, and 1.5 units of Taq DNA polymerase (New England Biolabs). The denaturing temperature used was 94 °C, with an annealing temperature of 48 °C and an extension temperature of 72 °C. Each temperature was held for 1 min, and 35 cycles were conducted. Completed reactions were run on 8% nondenaturing polyacrylamide gels (*60*), and the DNA bands were located by ultraviolet shadowing, then excised, and eluted overnight in TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). DNA was filtered and precipitated as described (*61*). After the DNA was resuspended in water, it was used as a template for runoff transcription as described below.

For mutated acceptors, DNA primers were purchased that contained the mutations. Fifty-eight nucleotide DNAs that were complementary to the 58 bases at the 3′ end of a 77-base DNA were used to make the three mutated acceptors

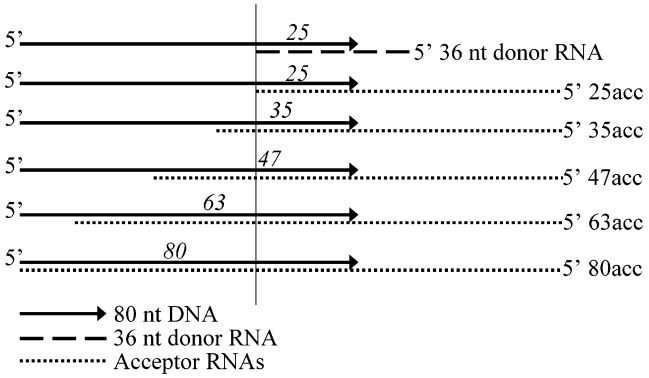

FIGURE 1: Schematic of hybrid regions between donor and DNA and acceptors and DNA. The hybrid regions between the donor RNA (dashed line) and DNA (solid line with arrowhead at 3′ end) are shown at the top of the figure. The donor−DNA hybrid (referred to as 25hyb) was preformed and isolated as described in the text. The hybrid regions with each acceptor (dotted lines) are shown below the donor−DNA hybrid. From top to bottom the DNA is shown bound to 25acc, 35acc, 47acc, 63acc, and 80acc. The vertical line illustrates the point on the DNA where the 3′ end of the donor is bound. This is used to highlight the 3′ end of the acceptor relative to the 3′ end of the donor. The number of base pairs each donor or acceptor forms with the DNA is marked above the DNA in italics. Acceptors shown are those used in the strand exchange experiments which had an additional 50 nucleotides (relative to the donor) at the 5′ end. These were denoted *XX*acc in the text with *XX* = number of nucleotides on the DNA that the acceptor can bind. For donor displacement experiments 50 nucleotides at the 5′ end were eliminated, and these acceptors are denoted *XX*shortacc in the text.

(47shortacc6mut, 47shortacc4mut, and 47shortacc3mut). Each set of primers was hybridized in hybridization buffer (50 mM Tris-HCl, pH 8, 80 mM KCl) by heating 50 pmol of each primer to 65 °C and slowly cooling to below 37 °C. The 5′ end was then filled in using 10 units of Klenow polymerase and 200 μM dNTPs for 15 min at 37 °C. The filled-in bases corresponded to the T7 RNA polymerase promoter. The hybrids were phenol−chloroform extracted and precipitated. The pellets were resuspended in water, and the DNA was used as template for transcription reactions.

Transcription reactions were prepared using 1 μg of template DNA and 40 units of T7 RNA polymerase and run for 2 h at 37 °C. The final concentration of components in the reaction was as follows: 0.8 units/μL RNase inhibitor, 40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine, and 500 μM rNTPs. Fifteen units of DNase 1 was added for 15 min to digest template DNA after transcription. Transcription reactions were phenol−chloroform extracted and precipitated. RNA was run on 8% or 10% denaturing polyacrylamide gels (*60*). RNA was excised, and the crushed gel was suspended in RNA elution buffer [80% formamide, 400 mM NaCl, 1 mM EDTA, 40 mM Tris-HCl (pH 7.0)]. After overnight elution RNA was precipitated as described previously. RNA was quantified by optical absorbance using a GeneQuant spectrophotometer (Pharmacia).

Preparation of 80-mer DNA for Strand Transfer Experiments. Fifty picomoles of DNA was 5′-^{32}P end labeled using T4 polynucleotide kinase (PNK). The labeling reaction was done at 37 °C for 30 min. The T4 PNK was heat inactivated by incubating the reaction at 65 °C for 15 min. The DNA was centrifuged on a Sephadex G-25 column to remove excess radiolabeled nucleotide. The DNA sequence was 5′-GATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAG-3′.

Preparation of Hybrid for Strand Transfer Experiments. Fifty picomoles of 5′ end labeled DNA was resuspended with 75−100 pmol of 36-nucleotide donor RNA in hybridization buffer (50 mM Tris-HCl, pH 8, 80 mM KCl). The solution was heated to 65 °C for 5 min and then slowly cooled to below 37 °C. The hybrid was run on a 10% native polyacrylamide gel (*60*) along with a sample of 5′ end labeled DNA. This allowed for accurate retrieval of the hybrid. The hybrid band was located by autoradiography, excised, and eluted for 5−6 h in the hybridization buffer. The eluted hybrid was filtered and quantified. The amount of hybrid retrieved was determined using a scintillation counter to measure a known quantity of the labeled DNA and the unknown quantity of the hybrid. The counts per minute per picomole was determined using the known quantity of DNA. This could then be used to determine how many picomoles of hybrid was recovered. Alternatively, hybrids were eluted for a half hour in 1× TBE (89 mM Tris-HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA). In this case the gel slice containing the hybrid was placed in dialysis tubing in 1 mL of 1× TBE. The tubing was placed in 1× TBE, and current was applied to draw the hybrid out of the gel into the buffer in the tubing. Hybrids obtained from both elution methods were analyzed on native gels to determine what proportion of the DNA remained hybridized to the donor. Only preparations with less than 10% dissociated DNA were used for assays.

Strand Exchange Reactions. Strand exchange reactions were performed at 37 °C. Four solutions of equal volume were made. Two solutions contained the hybrid while the others contained the acceptor. Six and a half microliters of NC or control buffer (50 mM Tris-HCl, pH 8, 2 mM DTT, 0.1 mM EDTA) was added to 58.5 μL of the hybrid or acceptor solutions. The solutions were preincubated with NC or buffer for 2 min. After 2 min 7.5 μL of the acceptor reaction with NC was added to 7.5 μL of the hybrid reaction with NC in proteinase K (PK) stop solution (2 mg/mL PK, 10 mM Tris-HCl, pH 8, 1.25% SDS, 15 mM EDTA) for time zero. The same was done for the reactions without NC. To start the reactions, 57 μL of the acceptor solution with NC was added to 57 μL of the hybrid solution containing NC. The same was done for the reactions without NC. The final concentrations were 2 nM hybrid, 4 nM acceptor RNA, 1 μM NC (or 0 μM NC for reactions without NC), 50 mM Tris-HCl, pH 8, 0.1 mM EDTA, 2 mM DTT, 0.1 μg/μL bovine serum albumin (BSA), 25 μM $ZnCl_2$, 6 mM $MgCl_2$, and 80 mM KCl. At specific time points (1, 2, 4, 8, 16, and 32 min) 15 μL of each reaction was removed and added to 15 μL of PK solution. Each reaction was incubated in PK for 4 min to digest NC protein, and then 6 μL of 6× native dye (40% sucrose, 0.17% xylene cyanol, 0.17% bromophenol blue) was added. Reactions were placed on ice immediately. Control reactions were performed by adding 6.75 μL of hybrid reaction to 6.75 μL of buffer without acceptor and 1.5 μL of NC or control buffer. Controls were incubated at 37 °C for 32 min, and then PK solution was added. After 4 min 6 μL of 6× native dye was added, and control reactions were placed on ice. Reactions were then run on 10% native polyacrylamide gels (*60*). Gels were dried, and autoradiographs were obtained. Gels were quantified using a Bio-Rad G-525 phosphorimager. The percent transfer was obtained

by dividing the amount of DNA hybridized to the acceptor (*T*) by the amount of DNA hybridized to the donor (*H*) and that transferred to the acceptor (*T*) and multiplying by 100 [% transferred = $(T/(H + T)) \times 100$].

Preparation of RNA for Donor Displacement Experiments. The 36-nucleotide donor RNA was internally labeled for donor displacement experiments. This was done by synthesizing the RNA in runoff transcription reactions which included [α-^{32}P]UTP. Reactions included 1 μg of template DNA, 40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 10 mM DTT, 2 mM sperimidine, 10 nmol of cold ATP, CTP, and GTP, 2 nmol of cold UTP, 100 pmol of radiolabeled UTP (800 Ci/mmol), 20 units of T7 RNA polymerase, and 20 units of RNase inhibitor in a 20 μL reaction. Transcription reactions were run for 1 h at 37 °C. Fifteen units of DNase 1 was added, and the reaction was incubated for an additional 15 min. One volume of 2× formamide dye [90% formamide (w/v), 10 mM EDTA, pH 8, 0.1% (w/v) each bromophenol blue and xylene cyanol] was added, and the RNA was purified on a 10% polyacrylamide denaturing gel (*60*). The RNA was excised and eluted in RNA elution buffer as previously described. The eluted RNA was removed from the gel and filtered. Two volumes of ethanol was added, and the RNA was precipitated as described above. RNA was quantified using a scintillation counter, and the amount of RNA was determined by the specific activity.

Preparation of Hybrid for Donor Displacement Experiments. Hybrids for donor displacement experiments were prepared as described above for strand transfer experiments.

Donor Displacement Experiments. Donor displacement experiments were completed essentially as described above for the strand transfer experiments. The percent donor displacement was obtained by dividing the amount of single-stranded RNA (*S*) by the amount of RNA hybridized to the 80-mer (*H*) added to the amount of single-stranded RNA and multiplying by 100 [% donor displaced = $(S/(H + S)) \times 100$]. The percent RNA displaced at time zero was subtracted from each time point. This was done because during hybrid purification a small amount of the hybrid (less than 10%) would fall apart. The donor displaced after hybrid purification was subtracted so that it would not be considered displaced due to exchange of the DNA to the acceptor.

RESULTS

Strand Exchange to Acceptors Forming Hybrid Regions with 10 or More Additional Base Pairs than the DNA−Donor Hybrid Is Enhanced by NC. The first experiments completed were strand exchange experiments with a labeled DNA. An 80-mer DNA was 5′ end labeled with ^{32}P and hybridized to 36-mer RNA (referred to as donor RNA). This hybrid, referred to as 25hyb, contained 25 base pairs formed between the 3′ ends of the nucleic acids. The other 55 and 11 nucleotides of the DNA and RNA, respectively, were single stranded. The hybrid is shown in Figure 1 along with the different acceptors used in the experiments. After purification the hybrid was used to examine transfer of the labeled DNA to acceptors. The acceptors used are denoted 25acc, 35acc, 47acc, 63acc, and 80acc. These can hybridize to an additional 0, 10, 22, 38, and 55 bases, respectively, outside the donor−DNA hybrid region. The number preceding each acceptor indicates the total length of the hybrid it can form with the DNA. For example, 47acc forms a 47 base pair hybrid with the 80-mer DNA. The hybrid includes the 25 bases that are part of the donor−DNA hybrid plus an additional 22 bases outside the hybrid region as depicted in Figure 1. Note that the system is designed to mimic strand exchange occurring during HIV recombination. The donor−DNA hybrid represents a putative "replication intermediate" consisting of nascent DNA bound to RNA template by a relatively small hybrid zone between the two. The portion of the RNA originally used as a template for the upstream DNA would be digested away by RT RNase H during or immediately after synthesis (see the introduction).

To test strand exchange to different acceptors, the hybrid was incubated with each acceptor in the presence or absence of NC over 32 min. The experiments were run on native polyacrylamide gels, and autoradiograms were obtained. Figure 2A shows transfer of DNA from the 36-mer RNA to the 47acc acceptor. The gel show that as the DNA transfers from the donor RNA to the acceptor, it shifts up. This allowed for quantification of the transferred DNA. The upshift is due to the presence of 50 additional bases (when compared to the donor) at the 5′ end of the acceptor that makes the acceptor−DNA hybrid substantially larger than the donor−DNA hybrid. Note also that the acceptor−DNA hybrid does not run as a distinct band but rather a major band with a smear above. This did not result from the presence of NC in the reactions as the same pattern was observed with or without NC. All acceptors with the additional 50 nucleotides at the 5′ end showed this pattern, and it probably results from more than one possible folding conformation in this 50-nucleotide region or from intermolecular interactions between the regions.

As shown in Figure 2A, strand exchange with 47acc was significantly stimulated by NC. Graphs compiled from several experiments for transfer with 25acc and 35acc (Figure 2B), 47acc (Figure 2C), and 63acc and 80acc (Figure 2D) are shown. No significant transfer of the DNA was observed with 25acc either with or without NC. This acceptor has the same hybrid region as the donor. All of the other acceptors showed some transfer in the absence of NC, and transfer was enhanced when NC was present. In the case of 35acc, transfer was very low compared to the others (note the different *y*-axis on graphs). The 47acc, 63acc, and 80acc acceptors all transferred reasonably well without NC but were strongly enhanced by NC. The results show that NC enhances strand exchange provided that a longer hybrid can form between the DNA and acceptor as compared to the DNA and donor. The extent of enhancement is dependent on the number of additional bases in the acceptor−DNA hybrid, with a large jump observed between 10 (35acc) and 22 (47acc). Experiments performed using single-stranded 80-mer DNA and each acceptor RNA showed that NC accelerated hybridization in all cases (data not shown). This included 25acc for which no strand exchange was observed using 25hyb.

Donor Displacement Experiments Support Results from the Strand Exchange Assays. The strand exchange experiments presented above showed that the length of the complementary region between the DNA and acceptor is important in NC-enhanced strand exchange. If acceptor binding to the DNA is a precursor to strand exchange, then the donor template should be displaced from the DNA only

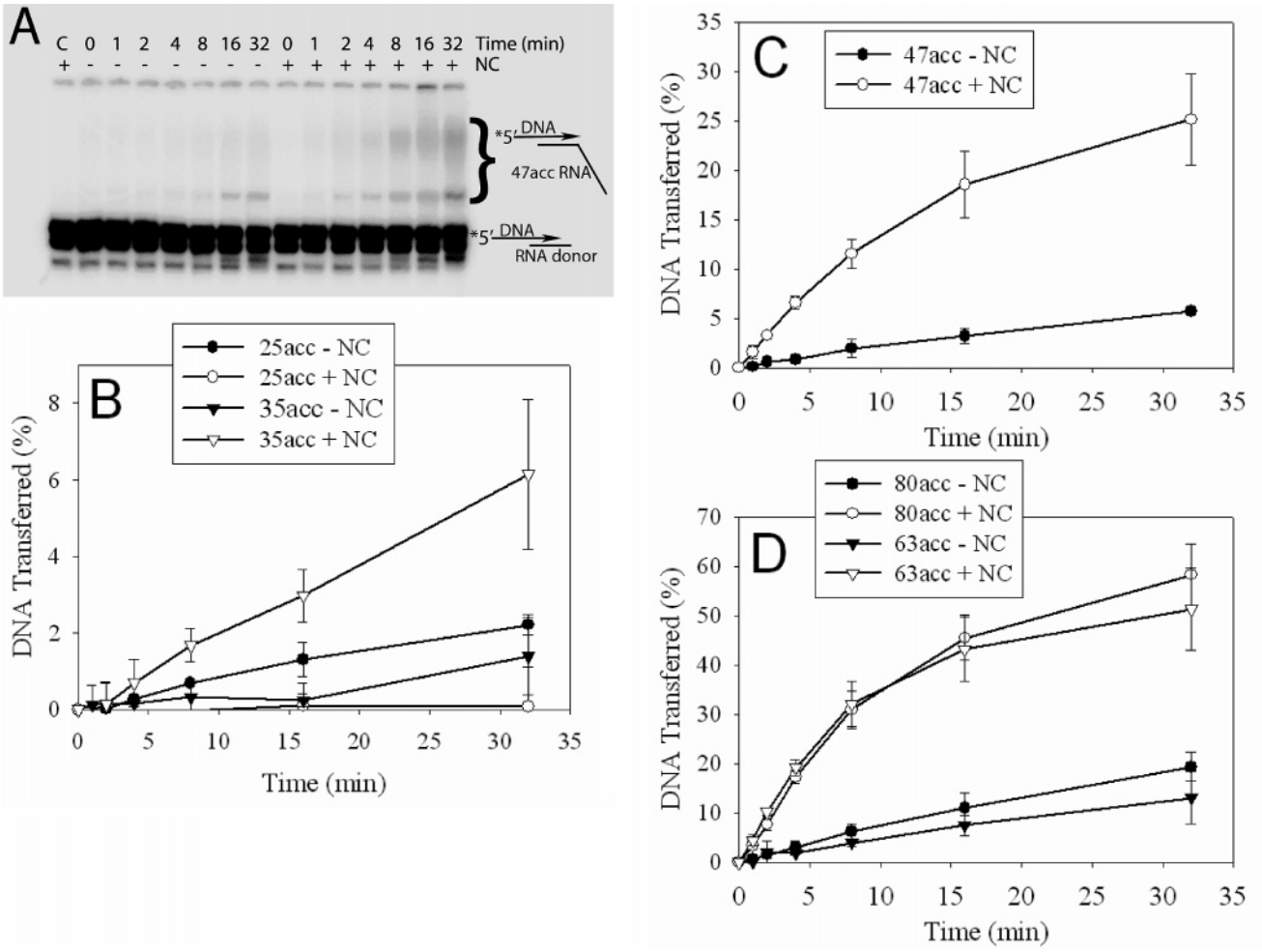

FIGURE 2: Autoradiogram and graphs showing DNA strand exchange from donor RNA to long acceptors. (A) A representative autoradiogram showing the binding of 5′-^{32}P-labeled DNA to the 47acc acceptor is shown. The experiments were performed by mixing 2 nM 80-nucleotide DNA hybridized to the 36-nucleotide donor RNA (referred to as 25hyb because of the 25 base pair hybrid between the DNA and donor) with 4 nM acceptor RNAs and removing aliquots at the time points (min) indicated above each lane as described under Materials and Methods. Samples were run on a 10% nondenaturing polyacrylamide gel. Positions where the acceptor−DNA hybrid and donor−DNA hybrid (starting material) run are marked by schematic drawings. NC was included in reactions with a (+) symbol. The control lane (C) had NC but no acceptor. Control reactions were completed over 32 min. Graphs showing the percent of DNA transferred from the donor RNA to acceptor RNA in the presence or absence of NC are shown in panel B for 25acc (circles) and 35acc (triangles), panel C for 47acc, and panel D for 63acc (triangles) and 80acc (circles). Open symbols are with NC and closed symbols without. Note the difference in the *Y*-axis values in each graph. The average of three experiments is shown, and the error bars represent the standard deviation.

by those acceptors for which strand exchange was observed, and displacement should not be observed in the absence of acceptor. We therefore employed a different method to directly test acceptor-mediated donor displacement. In the donor displacement experiments the 36-nucleotide donor RNA used above was internally labeled with ^{32}P, and the 80-mer DNA was not labeled. The hybrid (25hyb) was formed and isolated as for the strand exchange experiments above. It was then incubated with different acceptors (see below) in the presence and absence of NC over a 32 min time course. The experiments were run on nondenaturing polyacrylamide gels, and autoradiograms were obtained. In these experiments the amount of the donor that was displaced from the hybrid was monitored.

Using the donor displacement method was beneficial for a number of reasons. First, we could see what types of acceptors enhanced the removal of the donor from the DNA. This allowed us to differentiate between acceptor-mediated displacement and dissociation of the donor resulting from the prolonged incubation period or the presence of NC. Second, this technique allowed the removal of a variable that could potentially have an affect on the strand exchange experiments. For the strand exchange experiments it was necessary to have an additional 50 nucleotides on the 5′ end of the acceptors so that the DNA would adequately shift up in the gel once bound to the acceptor. However, in the donor displacement experiments the 50 nucleotides could be removed because the monitored change was the donor being displaced from the hybrid. This is beneficial because, in the strand exchange assays, the extra 50 nucleotides would change the secondary structure of the acceptor RNA, and therefore the structure of the acceptor and donor RNAs would differ. Because RNA secondary structure could play a role in strand exchange, and the experiments were not intended to test differences in secondary structure, it was desirable to remove these 50 nucleotides. Because of these advantages the donor displacement method was used to further examine the effects of NC on strand transfer.

A typical autoradiogram for a donor displacement experiment using 63shortacc (these acceptors are designated as

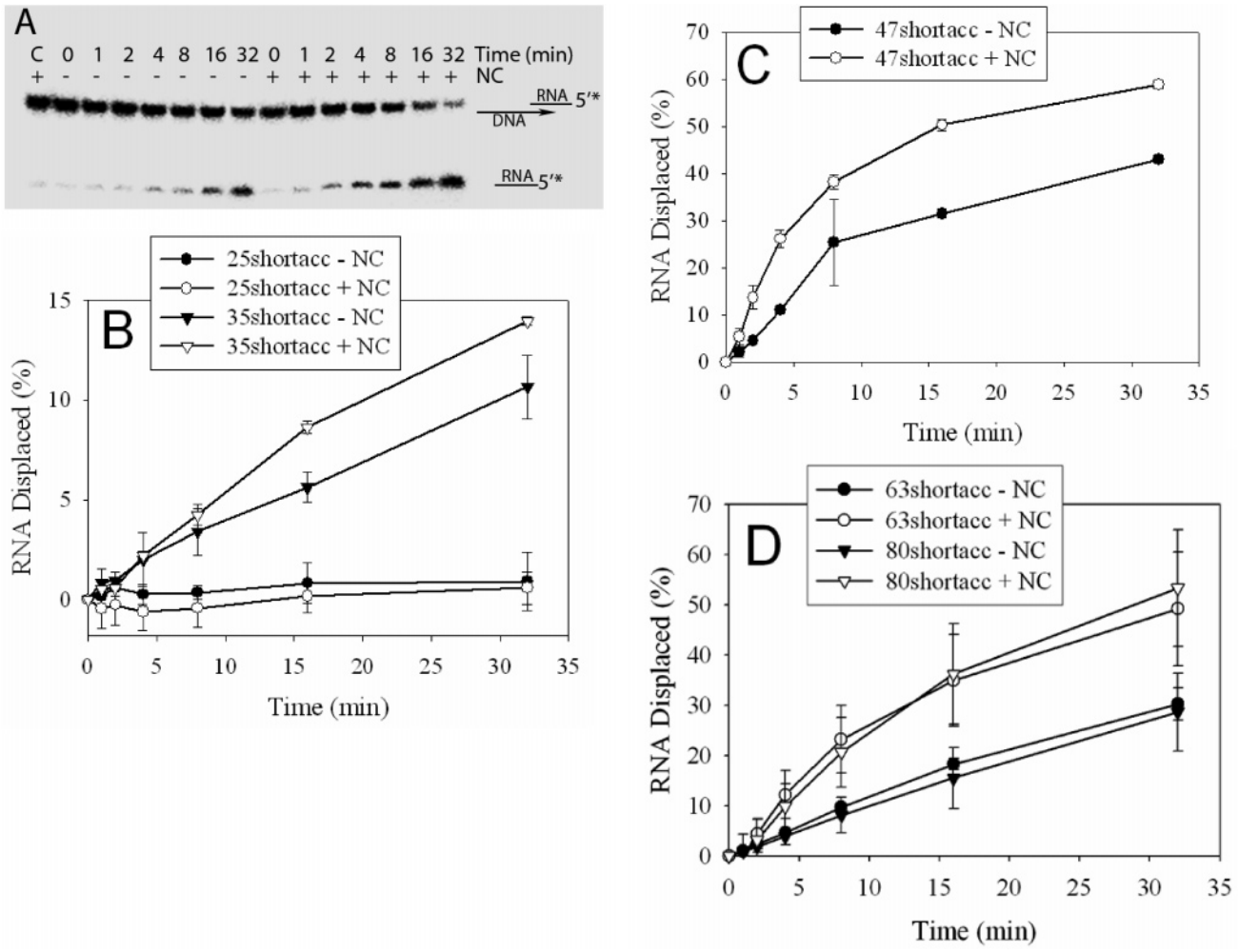

FIGURE 3: Autoradiogram and graphs of donor displacement experiments. (A) A representative autoradiogram showing the displacement of the radiolabeled donor RNA in the presence of 63shortacc is shown. The experiment was performed by mixing 2 nM 80-nucleotide DNA hybridized to the ^{32}P internally labeled 36-nucleotide donor RNA (referred to as 25hyb because of the 25 base pair hybrid between the DNA and donor) with 4 nM acceptor RNAs and removing aliquots at the time points (min) indicated above each lane as described under Materials and Methods. Samples were run on a 10% nondenaturing polyacrylamide gel. Positions where the donor−DNA hybrid (starting material) and displaced donor RNA run are marked with schematic drawings. NC was included in the reactions with a plus (+) symbol. The control lane (C) had NC but no acceptor. Control reactions were completed over 32 min. Graphs showing the percent donor RNA displaced by the acceptor are shown in panel B for 25shortacc (circles) and 35shortacc (triangles), panel C for 47acc, and panel D for 63shortacc (circles) and 80shortacc (triangles). Open symbols are with NC and closed symbols without. Note the difference in the *Y*-axis values in the graph shown in panel B. The average of three experiments is shown, and the error bars represent the standard deviation.

"shortacc" to distinguish them from the longer versions used in strand exchange reactions which had 50 additional bases at the 5′ end) is shown in Figure 3A. The control reaction ("C") shows the substrate incubated with NC but no acceptor for 32 min. A very small amount of displaced single-stranded labeled donor RNA is observed in the reaction. This was also observed in time 0 reactions with or without NC. Therefore, it results from a small amount of unhybridized donor RNA in the isolated starting substrate rather than NC-induced dissociation. When acceptor was included in the reaction, displacement was observed and increased substantially in the presence of NC. Graphical results for 25shortacc and 35shortacc (Figure 3B), 47shortacc (Figure 3C), and 63shortacc and 80shortacc (Figure 3D) are presented. The graphs support the strand exchange experiments discussed previously. Again, there was not a substantial amount of displacement until 47shortacc was used; however, a small enhancement with NC was observed with 35shortacc. The donor is not displaced when 25shortacc is used (note that in this case 25shortacc is identical to the 36-base donor RNA). This confirms the strand exchange result that indicated NC cannot exchange between hybrids of equal length. In this case even an RNA that was identical to the donor RNA could not displace the donor, at least not at the 2:1 acceptor:donor ratio used in these experiments.

To further confirm the results with 25acc and 25shortacc, a second hybrid using radiolabeled 35shortacc as the donor and the 80-mer DNA was prepared (referred to as 35hyb). This hybrid showed no strand displacement when incubated with unlabeled 35shortacc, while displacement was observed with 47shortacc (data not shown). The result confirms the finding that NC cannot catalyze exchange of hybrids that are equal.

Displacement Experiments with Mutated Acceptors Show That Complementary Bases in the Acceptor and Single-Stranded DNA Region Are Pivotal in Promoting NC Enhancement of Strand Exchange. The results obtained from displacement experiments indicated that the region outside of the donor−DNA hybrid is vital to the enhancement of strand exchange with NC. For considerable stimulation an

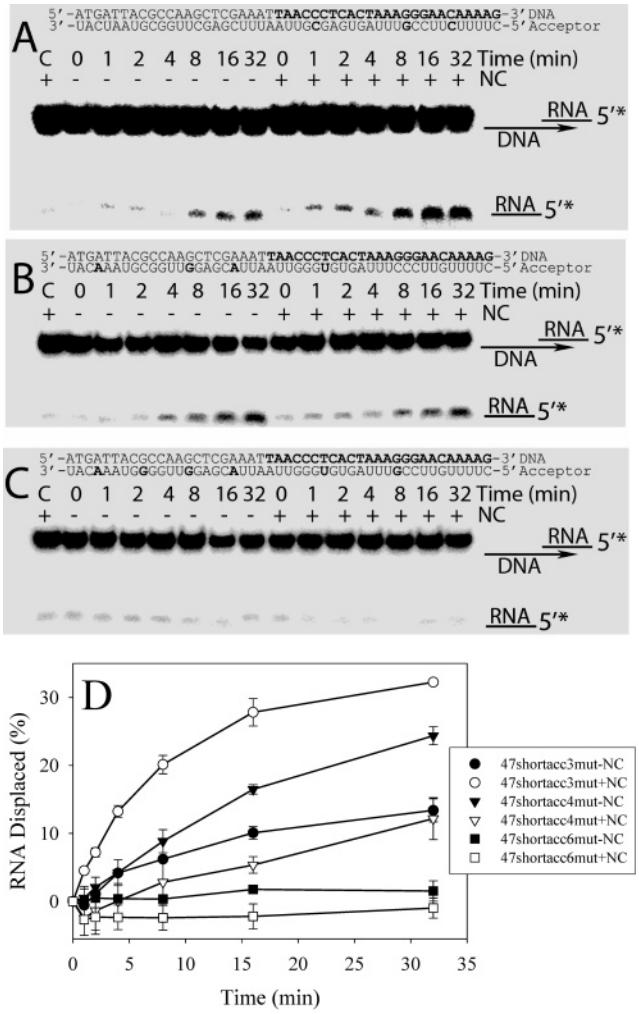

FIGURE 4: Autoradiograms and graph of donor displacement assays with mutant acceptors. Autoradiograms showing the displacement of radiolabeled donor RNA in the presence of 47shortacc3mut (A), 47shortacc4mut (B), and 47shortacc6mut (C) are shown. For each autoradiogram, the sequence of the mutated acceptor RNA is shown just below the DNA sequence to which the acceptor can bind. The bold nucleotides in the acceptor are the point mutations that produce mismatches. The bold portion of the DNA is the 25 nucleotides originally bound to the donor RNA. Experiments were performed as described in Figure 2. Samples were run on a 10% nondenaturing polyacrylamide gel. Positions where the donor−DNA hybrid (starting material) and displaced donor RNA run are marked with schematic drawings. NC was included in the reactions with a plus (+) symbol. The control lane (C) had NC but no acceptor. Control reactions were completed over 32 min. A graph showing the percent donor RNA displaced by the acceptor is shown in panel D with 47shortacc3mut (circles), 47shortacc4mut (triangles), and 47shortacc6mut (squares). Open symbols are with NC and closed symbols without. The average of three experiments is shown, and the error bars represent the standard deviation.

additional 22 nucleotides in the acceptor−DNA hybrid were necessary. Therefore, we wanted to determine the importance of complete complementarity in this region. Three mutated acceptors derived from 47shortacc were designed. These acceptors had different point mutations. The sequences of the hybrid region between the mutant acceptors and the DNA are shown above the autoradiograms in Figure 4. The first acceptor, 47shortacc3mut (Figure 4A), had three mutations in the region of the donor−DNA hybrid. This acceptor was completely complementary in the 22-nucleotide portion that binds to the single-stranded region of DNA. The second acceptor, 47shortacc4mut (Figure 4B), had four mutations. Three mutations were in the single-stranded region, and one was in the hybrid region. The last mutant acceptor, 47shortacc6mut (Figure 4C), had six mutations which were in the region that would anneal to the single-stranded DNA as well as the hybrid region between the donor and the DNA. The predicted change in thermodynamic stability between the donor−DNA and acceptor−DNA using these acceptors and others is shown in Table 2. It is important to note that though 47shortacc3mut and 47shortacc4mut have mutations in different regions, the predicted change in stability between the donor−DNA and acceptor−DNA is similar (−5.19 and −8.83 kcal/mol for 47shortacc3mut and 47shortacc4mut, respectively, with the acceptor−DNA being more stable). This is also similar to the change in stability predicted with 35shortacc (−8.57 kcal/mol). Therefore, these experiments test transfer to acceptors with little difference in overall thermodynamic stability but different complementarity.

Table 2: Increase in Stability When DNA Transfers from Donor RNA to Acceptor RNA

acceptor	change in thermodynamic stability[a]
25shortacc	0
35shortacc	−8.57
47shortacc	−20.34
47shortacc3mut	−5.19
47shortacc4mut	−8.83
47shortacc6mut	−3.01
63shortacc	> −34.42
80shortacc	> −34.42

[a] Thermodynamic stability of hybrids predicted using the MELTING program with 4 nM nucleic acid (strand in excess) and 80 mM NaCl (*69*). The free energy change (ΔG in kcal/mol at 37 °C) of the acceptor−DNA hybrid minus the free energy change of the donor−DNA hybrid (−18.96 kcal/mol) is shown. RNA−DNA hybrids greater than 60 base pairs cannot be computed by the program so values for 63shortacc and 80shortacc are listed as greater than the value for binding of the first 60 bases.

Displacement experiments with 25hyb and the mutant acceptors are shown in Figure 4A−C and displayed graphically in Figure 4D. When 47shortacc6mut was used, no displacement was observed with or without NC (Figure 4C). This result indicates that extensively disturbing the complementarity between the acceptor and the DNA is detrimental to transfer. However, we wanted to pinpoint what region of complementarity is important for enhanced transfer with NC. Therefore, experiments were completed with 47shortacc3mut and 47shortacc4mut. As was stated earlier, 47shortacc3mut has mutations in the region of the donor−DNA hybrid, while 47shortacc4mut has one mutation in that region and 3 mutations in the single-stranded region of the DNA. The results with 47shortacc3mut showed a similar, though somewhat decreased, displacement profile compared to 47shortacc (Figure 3C). With 47shortacc3mut there is a substantial amount of displacement without NC, but the rate of displacement is considerably increased with NC (Figure 4A). Therefore, when the mutations are in the donor−DNA hybrid region, there is not a great effect on NC enhancement of exchange. The results with 47shortacc4mut are drastically different. With this acceptor displacement was observed in the absence of NC. However, when the reaction was completed in the presence of NC, donor displacement was considerably inhibited (Figure 4B). This result clearly shows

the importance of complementarity between the single-stranded region of DNA and the acceptor. When this region was completely complementary (47shortacc and 47shortacc3mut), NC enhanced transfer. However, when 3 of 22 nucleotides in the region were not complementary, transfer was actually inhibited with NC.

DISCUSSION

Nucleocapsid protein promotes annealing of complementary nucleic acids and can enhance strand exchange leading to the formation of a stronger hybrid at the expense of a weaker one (*17*, *54*). The substrates used in this report were designed to model strand exchanges that would occur during retroviral recombination. As such, the donor−DNA hybrid had a duplex region and flanking single-stranded RNA (donor) or DNA regions. The RNA acceptor was complementary to the DNA. We showed that NC enhances the rate of strand exchange by stimulating the binding of the acceptor template to a single-stranded region of the DNA outside the duplex region. The thermodynamic stability of the acceptor−single-stranded DNA region, presumably an intermediate in strand exchange, is important in allowing the acceptor to "dock" with the region. No evidence for NC enhancement was observed if a stable hybrid could not form between the acceptor and single-stranded DNA region. This was true even in cases where the overall thermodynamic stability of the acceptor−DNA hybrid was greater than the donor−DNA hybrid (47shortacc4mut, for example).

Conclusions in the experiments were based on the assumption that the rate of the reactions was determined by the actual strand exchange event and not unwinding of the acceptor or single-stranded region of the DNA. Using a reconstituted in vitro system designed to mimic (−)ssDNA transfer, unfolding of the (−)ssDNA and RNA acceptor (R region of the genome) was shown to be the rate-limiting step in association between the DNA and RNA (*36*). It is possible that unfolding could have played a role in determining the reaction rate when the longer acceptors (80acc and 80shortacc or 63acc and 63shortacc) were used. However, 47acc underwent strand exchange much more rapidly than 35acc (Figure 2) despite the latter folding less stably [predicted ΔG's of −25.0 and −30.7 kcal/mol for 35 and 47acc, respectively, using M-fold and default conditions (*62*)]. Therefore, the strand exchange rate in reactions with 35acc is limited by the association of the acceptor with the DNA, in particular, with the single-stranded DNA region. In this region 47acc can form 12 additional base pairs. This was also the case for the donor displacement reactions using 35shortacc and 47shortacc (predicted ΔG's of −11.2 and −14 kcal/mol for 35 and 47shortacc, respectively) (Figure 3).

Experiments with the mutated acceptor templates clearly demonstrated the importance of the single-stranded DNA region as a docking site for strand exchange. Acceptor 47shortacc which hybridizes to the DNA over a 47-base stretch (the 25 bases in the donor−DNA hybrid region plus 22 additional bases in the single-stranded DNA region) was used as a basis for these experiments. When the acceptor had mutations in the donor−DNA hybrid region and the single-stranded DNA region (47shortacc6mut), no displacement was observed either with or without NC (Figure 4C,D). When the acceptor was used that had mutations in the donor−DNA hybrid region but not the region exterior to that (47shortacc3mut), NC clearly enhanced strand exchange, shown by a large stimulation of donor displacement (Figure 4A,D). The third mutated acceptor had three mutations in the single-stranded binding region and one in the hybrid region (47shortacc4mut). The hybrid between the DNA and this acceptor had a ΔG value similar to that of 47shortacc3mut bound to the DNA. Therefore, overall these acceptors are approximately thermodynamically equivalent (Table 2). Both acceptors displaced the donor to a similar extent in the absence of NC, but displacement with 47shortacc4mut was actually inhibited by NC (Figure 4B,D). The lack of correlation between product thermodynamics and NC enhancement is further illustrated by comparing displacement with acceptors 35shortacc and 47shortacc3mut. Both had similar gains in thermodynamic stability for the DNA−acceptor compared to the DNA−donor (see Table 2). However, NC had only a very small effect on the displacement with 35acc (Figure 3B). Taken together, these results show that high complementarity between the acceptor and single-stranded DNA region is what allows NC to enhance strand exchange. Mismatched nucleotides in the donor−DNA hybrid region have a diminished effect when compared to mutations in the single-stranded DNA region. The results point toward a mechanism where NC initiates strand exchange by accelerating (or stabilizing) the formation of a trimeric intermediate consisting of the acceptor bound to the free single-stranded DNA region of the donor−DNA hybrid (see below). The properties of NC are consistent with the enhancement resulting from the disruption of intramolecular hydrogen bonding (secondary structure) in the complementary regions of the single-stranded DNA and acceptor RNA (see the introduction).

Inhibition of hybrid formation by NC as was found with 47shortacc4mut has been observed by others when relatively weak hybrids are used (*63*). Although NC is generally regarded as stimulating the formation of hybrids, it is actually a helix-destabilizing protein that weakens hybrid stability and melts secondary structure (*9*, *52*, *54*). Since NC accomplishes this through binding to the nucleic acid strand, it probably needs to be displaced by strong base complementarity to allow hybrid formation. On 47shortacc4mut, the three mismatches over the 22-base region that binds to the single-stranded DNA likely resulted in a "weak" hybrid capable of forming in the absence but not presence of NC. The complete 22 base pair hybrid formed with 47shortacc3mut over this same region was apparently strong enough to displace NC.

Overall, our results support a model where NC enhances the binding of acceptor to the single-stranded region upstream of the donor−DNA hybrid. Enhancement can occur with as few as 10 bases, but strong enhancement requires more. After binding the single-stranded region, the acceptor "zippers" through the donor−DNA hybrid displacing the donor. The model is in agreement with a recent model proposed to explain NC stimulation of tRNA binding to the primer binding site (pbs) (*24*). In this case a short 4−5-nucleotide stretch on the tRNA was proposed to melt and associate with the pbs in a relatively slow reaction that is stimulated by NC. This was followed by a rapid zipping reaction that unwinds the tRNA and allows the complete 18 base pair tRNA−pbs hybrid to form. The substrates used in our

experiments had much longer single-stranded docking sites as would be expected during recombination, and when the docking site was only 10 bases (35acc and 35shortacc), NC stimulation was very small. It is possible that structural intricacies and unique interactions between the pbs, tRNA, and NC allow a very short 4−5-base docking site to be used. In further support of this model, other reports have also shown that the rate of NC-catalyzed strand exchange using small (approximately 30 base pair) substrates is enhanced when the hybrid region is flanked by single-stranded bases complementary to the acceptor (*17*).

These results highlight that during NC-stimulated strand exchange there is a necessity for a short stretch of complementary sequences between the acceptor and the DNA that can anneal outside the donor−DNA hybrid region. This could be a particularly important factor for recombination between different HIV subtypes or clades where significant differences in genome nucleotide sequence are observed. Recombination between different clades is particularly important since it can lead to new intersubtype viruses that present a major challenge to the development of vaccines and drug therapies (*64*−*68*). Strand transfer in these cases may be relatively inefficient, especially in regions that are more divergent. In fact, the results with 47shortacc4mut suggest that NC may even inhibit recombination between genome regions with sequence variations. During intersubtype recombination, this inhibition coupled with the high homology in conserved genome regions could serve to strongly focus recombination to these conserved regions. Consistent with this, an analysis of 34 HIV-1 intersubtype recombinants, for which the full genome sequence is known, indicated that most recombination breakpoints were near conserved genome regions (*64*). Also, assays in which different virus subtypes were used to co-infect cells showed that most crossover sites in the *env* gene focused to the conserved regions (*68*). These studies analyzed viable recombinants that were potentially subjected to selection. Therefore, they cannot be used to directly determine the proportion of crossovers that occur at various genome sites. It would be interesting to see if results using single cycle infection assays that can map crossovers in the absence of selection also show these tendencies.

ACKNOWLEDGMENT

We thank Dr. Charles McHenry from the University of Colorado for the plasmid clone for wild-type NC overexpression.

REFERENCES

1. Moumen, A., Polomack, L., Unge, T., Veron, M., Buc, H., and Negroni, M. (2003) Evidence for a mechanism of recombination during reverse transcription dependent on the structure of the acceptor RNA, *J. Biol. Chem. 278*, 15973−15982.
2. DeStefano, J. J. (1995) Human immunodeficiency virus nucleocapsid protein stimulates strand transfer from internal regions of heteropolymeric RNA templates, *Arch. Virol. 140*, 1775−1789.
3. Negroni, M., and Buc, H. (2001) Mechanisms of retroviral recombination, *Annu. Rev. Genet. 35*, 275−302.
4. Roda, R. H., Balakrishnan, M., Hanson, M. N., Wohrl, B. M., Le Grice, S. F., Roques, B. P., Gorelick, R. J., and Bambara, R. A. (2003) Role of the Reverse transcriptase, nucleocapsid protein, and template structure in the two-step transfer mechanism in retroviral recombination, *J. Biol. Chem. 278*, 31536−31546.
5. Balakrishnan, M., Roques, B. P., Fay, P. J., and Bambara, R. A. (2003) Template dimerization promotes an acceptor invasion-induced transfer mechanism during human immunodeficiency virus type 1 minus-strand synthesis, *J. Virol. 77*, 4710−4721.
6. Derebail, S. S., Heath, M. J., and DeStefano, J. J. (2003) Evidence for the differential effects of nucleocapsid protein on strand transfer in various regions of the HIV genome, *J. Biol. Chem. 278*, 15702−15712.
7. Rodriguez-Rodriguez, L., Tsuchihashi, Z., Fuentes, G. M., Bambara, R. A., and Fay, P. J. (1995) Influence of human immunodeficiency virus nucleocapsid protein on synthesis and strand transfer by the reverse transcriptase in vitro, *J. Biol. Chem. 270*, 15005−15011.
8. Negroni, M., and Buc, H. (2000) Copy-choice recombination by reverse transcriptases: reshuffling of genetic markers mediated by RNA chaperones, *Proc. Natl. Acad. Sci. U.S.A. 97*, 6385−6390.
9. DeStefano, J. J. (1996) Interaction of human immunodeficiency virus nucleocapsid protein with a structure mimicking a replication intermediate. Effects on stability, reverse transcriptase binding, and strand transfer, *J. Biol. Chem. 271*, 16350−16356.
10. Chen, Y., Balakrishnan, M., Roques, B. P., and Bambara, R. A. (2003) Steps of the acceptor invasion mechanism for HIV-1 minus strand strong stop transfer, *J. Biol. Chem. 278*, 38368−38375.
11. Derebail, S. S., and Destefano, J. J. (2004) Mechanistic analysis of pause site-dependent and -independent recombinogenic strand transfer from structurally diverse regions of the HIV genome, *J. Biol. Chem. 279*, 47446−47454.
12. Henderson, L. E., Bowers, M. A., Sowder, R. C., II, Serabyn, S. A., Johnson, D. G., Bess, J. W., Jr., Arthur, L. O., Bryant, D. K., and Fenselau, C. (1992) Gag proteins of the highly replicative MN strain of human immunodeficiency virus type 1: post translational modifications, *J. Virol. 4*, 1856−1865.
13. Azoulay, J., Clamme, J. P., Darlix, J. L., Roques, B. P., and Mely, Y. (2003) Destabilization of the HIV-1 complementary sequence of TAR by the nucleocapsid protein through activation of conformational fluctuations, *J. Mol. Biol. 326*, 691−700.
14. Baba, S., Takahashi, K., Koyanagi, Y., Yamamoto, N., Takaku, H., Gorelick, R. J., and Kawai, G. (2003) Role of the zinc fingers of HIV-1 nucleocapsid protein in maturation of genomic RNA, *J. Biochem. (Tokyo) 134*, 637−639.
15. Bampi, C., Jacquenet, S., Lener, D., Decimo, D., and Darlix, J. L. (2004) The chaperoning and assistance roles of the HIV-1 nucleocapsid protein in proviral DNA synthesis and maintenance, *Int. J. Biochem. Cell Biol. 36*, 1668−1686.
16. Takahashi, K., Baba, S., Koyanagi, Y., Yamamoto, N., Takaku, H., and Kawai, G. (2001) Two basic regions of NCp7 are sufficient for conformational conversion of HIV-1 dimerization initiation site from kissing-loop dimer to extended-duplex dimer, *J. Biol. Chem. 276*, 31274−31278.
17. Urbaneja, M. A., Wu, M., Casas-Finet, J. R., and Karpel, R. L. (2002) HIV-1 nucleocapsid protein as a nucleic acid chaperone: spectroscopic study of its helix-destabilizing properties, structural binding specificity, and annealing activity, *J. Mol. Biol. 318*, 749−764.
18. Feng, Y. X., Copeland, T. D., Henderson, L. E., Gorelick, R. J., Bosche, W. J., Levin, J. G., and Rein, A. (1996) HIV-1 nucleocapsid protein induces "maturation" of dimeric retroviral RNA in vitro, *Proc. Natl. Acad. Sci. U.S.A. 93*, 7577−7581.
19. Li, X., Quan, Y., Arts, E. J., Li, Z., Preston, B. D., de Rocquigny, H., Roques, B. P., Darlix, J. L., Kleiman, L., Parniak, M. A., and Wainberg, M. A. (1996) Human immunodeficiency virus Type 1 nucleocapsid protein (NCp7) directs specific initiation of minus-strand DNA synthesis primed by human tRNA(Lys3) in vitro: studies of viral RNA molecules mutated in regions that flank the primer binding site, *J. Virol. 70*, 4996−5004.
20. Huang, Y., Khorchid, A., Wang, J., Parniak, M. A., Darlix, J. L., Wainberg, M. A., and Kleiman, L. (1997) Effect of mutations in the nucleocapsid protein (NCp7) upon Pr160(gag-pol) and tRNA-(Lys) incorporation into human immunodeficiency virus type 1, *J. Virol. 71*, 4378−4384.
21. Barat, C., Schatz, O., Le Grice, S., and Darlix, J. L. (1993) Analysis of the interactions of HIV1 replication primer tRNA(Lys,3) with nucleocapsid protein and reverse transcriptase, *J. Mol. Biol. 231*, 185−190.
22. Brule, F., Marquet, R., Rong, L., Wainberg, M. A., Roques, B. P., Le Grice, S. F., Ehresmann, B., and Ehresmann, C. (2002) Structural and functional properties of the HIV-1 RNA-tRNA-(Lys)3 primer complex annealed by the nucleocapsid protein: comparison with the heat-annealed complex, *RNA 8*, 8−15.

23. Hargittai, M. R., Mangla, A. T., Gorelick, R. J., and Musier-Forsyth, K. (2001) HIV-1 nucleocapsid protein zinc finger structures induce tRNA(Lys,3) structural changes but are not critical for primer/template annealing, *J. Mol. Biol. 312*, 985−997.
24. Hargittai, M. R., Gorelick, R. J., Rouzina, I., and Musier-Forsyth, K. (2004) Mechanistic insights into the kinetics of HIV-1 nucleocapsid protein-facilitated tRNA annealing to the primer binding site, *J. Mol. Biol. 337*, 951−968.
25. Cameron, C. E., Ghosh, M., Le Grice, S. F., and Benkovic, S. J. (1997) Mutations in HIV reverse transcriptase which alter RNase H activity and decrease strand transfer efficiency are suppressed by HIV nucleocapsid protein, *Proc. Natl. Acad. Sci. U.S.A. 94*, 6700−6705.
26. Peliska, J. A., Balasubramanian, S., Giedroc, D. P., and Benkovic, S. J. (1994) Recombinant HIV-1 nucleocapsid protein accelerates HIV-1 reverse transcriptase catalyzed DNA strand transfer reactions and modulates RNase H activity, *Biochemistry 33*, 13817−13823.
27. Ji, X., Klarmann, G. J., and Preston, B. D. (1996) Effect of human immunodeficiency virus type 1 (HIV-1) nucleocapsid protein on HIV-1 reverse transcriptase activity in vitro, *Biochemistry 35*, 132−143.
28. Wu, W., Henderson, L. E., Copeland, T. D., Gorelick, R. J., Bosche, W. J., Rein, A., and Levin, J. G. (1996) Human immunodeficiency virus type 1 nucleocapsid protein reduces reverse transcriptase pausing at a secondary structure near the murine leukemia virus polypurine tract, *J. Virol. 70*, 7132−7142.
29. Drummond, J. E., Mounts, P., Gorelick, R. J., Casas-Finet, J. R., Bosche, W. J., Henderson, L. E., Waters, D. J., and Arthur, L. O. (1997) Wild-type and mutant HIV type 1 nucleocapsid proteins increase the proportion of long cDNA transcripts by viral reverse transcriptase, *AIDS Res. Hum. Retroviruses 13*, 533−543.
30. Rong, L., Liang, C., Hsu, M., Kleiman, L., Petitjean, P., de Rocquigny, H., Roques, B. P., and Wainberg, M. A. (1998) Roles of the human immunodeficiency virus type 1 nucleocapsid protein in annealing and initiation versus elongation in reverse transcription of viral negative-strand strong-stop DNA, *J. Virol. 72*, 9353−9358.
31. Iwatani, Y., Rosen, A. E., Guo, J., Musier-Forsyth, K., and Levin, J. G. (2003) Efficient initiation of HIV-1 reverse transcription in vitro. Requirement for RNA sequences downstream of the primer binding site abrogated by nucleocapsid protein-dependent primer-template interactions, *J. Biol. Chem. 278*, 14185−14195.
32. Wisniewski, M., Chen, Y., Balakrishnan, M., Palaniappan, C., Roques, B. P., Fay, P. J., and Bambara, R. A. (2002) Substrate requirements for secondary cleavage by HIV-1 reverse transcriptase RNase H, *J. Biol. Chem. 277*, 28400−28410.
33. Heilman-Miller, S. L., Wu, T., and Levin, J. G. (2004) Alteration of nucleic acid structure and stability modulates the efficiency of minus-strand transfer mediated by the HIV-1 nucleocapsid protein, *J. Biol. Chem. 279*, 44154−44165.
34. Hsu, M., Rong, L., de Rocquigny, H., Roques, B. P., and Wainberg, M. A. (2000) The effect of mutations in the HIV-1 nucleocapsid protein on strand transfer in cell-free reverse transcription reactions, *Nucleic Acids Res. 28*, 1724−1729.
35. Hong, M. K., Harbron, E. J., O'Connor, D. B., Guo, J., Barbara, P. F., Levin, J. G., and Musier-Forsyth, K. (2003) Nucleic acid conformational changes essential for HIV-1 nucleocapsid protein-mediated inhibition of self-priming in minus-strand transfer, *J. Mol. Biol. 325*, 1−10.
36. You, J. C., and McHenry, C. S. (1994) Human immunodeficiency virus nucleocapsid protein accelerates strand transfer of the terminally redundant sequences involved in reverse transcription, *J. Biol. Chem. 269*, 31491−31495.
37. Guo, J., Wu, T., Anderson, J., Kane, B. F., Johnson, D. G., Gorelick, R. J., Henderson, L. E., and Levin, J. G. (2000) Zinc finger structures in the human immunodeficiency virus type 1 nucleocapsid protein facilitate efficient minus- and plus-strand transfer, *J. Virol. 74*, 8980−8988.
38. Guo, J., Henderson, L. E., Bess, J., Kane, B., and Levin, J. G. (1997) Human immunodeficiency virus type 1 nucleocapsid protein promotes efficient strand transfer and specific viral DNA synthesis by inhibiting TAR-dependent self-priming from minus-strand strong-stop DNA, *J. Virol. 71*, 5178−5188.
39. Darlix, J. L., Vincent, A., Gabus, C., de Rocquigny, H., and Roques, B. (1993) Trans-activation of the 5′ to 3′ viral DNA strand transfer by nucleocapsid protein during reverse transcription of HIV1 RNA, *C. R. Acad. Sci., Ser. III 316*, 763−771.
40. Allain, B., Lapadat-Tapolsky, M., Berlioz, C., and Darlix, J. L. (1994) Transactivation of the minus-strand DNA transfer by nucleocapsid protein during reverse transcription of the retroviral genome, *EMBO J. 13*, 973−981.
41. Gorelick, R. J., Gagliardi, T. D., Bosche, W. J., Wiltrout, T. A., Coren, L. V., Chabot, D. J., Lifson, J. D., Henderson, L. E., and Arthur, L. O. (1999) Strict conservation of the retroviral nucleocapsid protein zinc finger is strongly influenced by its role in viral infection processes: characterization of HIV-1 particles containing mutant nucleocapsid zinc-coordinating sequences, *Virology 256*, 92−104.
42. Housset, V., De Rocquigny, H., Roques, B. P., and Darlix, J. L. (1993) Basic amino acids flanking the zinc finger of Moloney murine leukemia virus nucleocapsid protein NCp10 are critical for virus infectivity, *J. Virol. 67*, 2537−2545.
43. Rein, A., Harvin, D. P., Mirro, J., Ernst, S. M., and Gorelick, R. J. (1994) Evidence that a central domain of nucleocapsid protein is required for RNA packaging in murine leukemia virus, *J. Virol. 68*, 6124−6129.
44. Gorelick, R. J., Chabot, D. J., Rein, A., Henderson, L. E., and Arthur, L. O. (1993) The two zinc fingers in the human immunodeficiency virus type 1 nucleocapsid protein are not functionally equivalent, *J. Virol. 67*, 4027−4036.
45. Kim, M. Y., and Jeong, S. (2004) Inhibition of the functions of the nucleocapsid protein of human immunodeficiency virus-1 by an RNA aptamer, *Biochem. Biophys. Res. Commun. 320*, 1181−1186.
46. Amarasinghe, G. K., De Guzman, R. N., Turner, R. B., Chancellor, K. J., Wu, Z. R., and Summers, M. F. (2000) NMR structure of the HIV-1 nucleocapsid protein bound to stem-loop SL2 of the psi-RNA packaging signal. Implications for genome recognition, *J. Mol. Biol. 301*, 491−511.
47. Muriaux, D., De Rocquigny, H., Roques, B. P., and Paoletti, J. (1996) NCp7 activates HIV-1Lai RNA dimerization by converting a transient loop-loop complex into a stable dimer, *J. Biol. Chem. 271*, 33686−33692.
48. Carteau, S., Gorelick, R. J., and Bushman, F. D. (1999) Coupled integration of human immunodeficiency virus type 1 cDNA ends by purified integrase in vitro: stimulation by the viral nucleocapsid protein, *J. Virol. 73*, 6670−6679.
49. Poljak, L., Batson, S. M., Ficheux, D., Roques, B. P., Darlix, J.-L., and Kas, E. (2003) Analysis of NCp7-dependent activation of HIV-1 cDNA Integration and its conservation among retroviral nucleocapsid proteins, *J. Mol. Biol. 329*, 411−421.
50. Carteau, S., Batson, S. C., Poljak, L., Mouscadet, J. F., de Rocquigny, H., Darlix, J. L., Roques, B. P., Kas, E., and Auclair, C. (1997) Human immunodeficiency virus type 1 nucleocapsid protein specifically stimulates Mg^{2+}-dependent DNA integration in vitro, *J. Virol. 71*, 6225−6229.
51. Bernacchi, S., Stoylov, S., Piemont, E., Ficheux, D., Roques, B. P., Darlix, J. L., and Mely, Y. (2002) HIV-1 nucleocapsid protein activates transient melting of least stable parts of the secondary structure of TAR and its complementary sequence, *J. Mol. Biol. 317*, 385−399.
52. Williams, M. C., Rouzina, I., Wenner, J. R., Gorelick, R. J., Musier-Forsyth, K., and Bloomfield, V. A. (2001) Mechanism for nucleic acid chaperone activity of HIV-1 nucleocapsid protein revealed by single molecule stretching, *Proc. Natl. Acad. Sci. U.S.A. 98*, 6121−6126.
53. Heath, M. J., Derebail, S. S., Gorelick, R. J., and DeStefano, J. J. (2003) Differing roles of the N-terminal and C-terminal zinc fingers in HIV-1 nucleocapsid protein enhanced nucleic acid annealing, *J. Biol. Chem. 278*, 30755−30763.
54. Tsuchihashi, Z., and Brown, P. O. (1994) DNA strand exchange and selective DNA annealing promoted by the human immunodeficiency virus type 1 nucleocapsid protein, *J. Virol. 68*, 5863−5870.
55. De Rocquigny, H., Gabus, C., Vincent, A., Fournie-Zaluski, M. C., Roques, B., and Darlix, J. L. (1992) Viral RNA annealing activities of human immunodeficiency virus type 1 nucleocapsid protein require only peptide domains outside the zinc fingers, *Proc. Natl. Acad. Sci. U.S.A. 89*, 6472−6476.
56. Beltz, H., Piemont, E., Schaub, E., Ficheux, D., Roques, B., Darlix, J. L., and Mely, Y. (2004) Role of the structure of the top half of HIV-1 cTAR DNA on the nucleic acid destabilizing activity of the nucleocapsid protein NCp7, *J. Mol. Biol. 338*, 711−723.

57. Khan, R., and Giedroc, D. P. (1992) Recombinant human immunodeficiency virus type 1 nucleocapsid (NCp7) protein unwinds tRNA, *J. Biol. Chem. 267*, 6689−6695.
58. You, J. C., and McHenry, C. S. (1993) HIV nucleocapsid protein. Expression in *Escherichia coli*, purification, and characterization, *J. Biol. Chem. 268*, 16519−16527.
59. DeStefano, J. J., Mallaber, L. M., Rodriguez-Rodriguez, L., Fay, P. J., and Bambara, R. A. (1992) Requirements for strand transfer between internal regions of heteropolymer templates by human immunodeficiency virus reverse transcriptase, *J. Virol. 66*, 6370−6378.
60. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
61. Raja, A., and DeStefano, J. J. (2003) Interaction of HIV reverse transcriptase with structures mimicking recombination intermediates, *J. Biol. Chem. 278*, 10102−10111.
62. Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction, *Nucleic Acids Res. 31*, 3406−3415.
63. Golinelli, M. P., and Hughes, S. H. (2003) Secondary structure in the nucleic acid affects the rate of HIV-1 nucleocapsid-mediated strand annealing, *Biochemistry 42*, 8153−8162.
64. Magiorkinis, G., Paraskevis, D., Vandamme, A. M., Magiorkinis, E., Sypsa, V., and Hatzakis, A. (2003) In vivo characteristics of human immunodeficiency virus type 1 intersubtype recombination: determination of hot spots and correlation with sequence similarity, *J. Gen. Virol. 84*, 2715−2722.
65. Fang, G., Weiser, B., Kuiken, C., Philpott, S. M., Rowland-Jones, S., Plummer, F., Kimani, J., Shi, B., Kaul, R., Bwayo, J., Anzala, O., and Burger, H. (2004) Recombination following superinfection by HIV-1, *AIDS 18*, 153−159.
66. Kijak, G. H., Rubio, A. E., Quarleri, J. F., and Salomon, H. (2001) HIV type 1 genetic diversity is a major obstacle for antiretroviral drug resistance hybridization-based assays, *AIDS Res. Hum. Retroviruses 17*, 1415−1421.
67. Motomura, K., Kusagawa, S., Kato, K., Nohtomi, K., Lwin, H. H., Tun, K. M., Thwe, M., Oo, K. Y., Lwin, S., Kyaw, O., Zaw, M., Nagai, Y., and Takebe, Y. (2000) Emergence of new forms of human immunodeficiency virus type 1 intersubtype recombinants in central Myanmar, *AIDS Res. Hum. Retroviruses 16*, 1831−1843.
68. Quinones-Mateu, M. E., Gao, Y., Ball, S. C., Marozsan, A. J., Abraha, A., and Arts, E. J. (2002) In vitro intersubtype recombinants of human immunodeficiency virus type 1: comparison to recent and circulating in vivo recombinant forms, *J. Virol. 76*, 9600−9613.
69. Le Novere, N. (2001) MELTING, computing the melting temperature of nucleic acid duplex, *Bioinformatics 17*, 1226−1227.

BI0477945