Nucleotide Excision Repair and Template-independent Addition by HIV-1 Reverse Transcriptase in the Presence of Nucleocapsid Protein*

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During HIV replication, reverse transcriptase (RT), assisted by the nucleocapsid protein (NC), converts the genomic RNA into proviral DNA. This process appears to be the major source of genetic variability, as RT can misincorporate nucleotides during minus and plus strand DNA synthesis. To investigate nucleotide addition or substitution by RT, we set up in vitro models containing HIV-1 RNA, cDNA, NC, and various RTs. We used the wild type RT and azidothymidine- and didanosine-resistant RTs, because they represent the major forms of resistant RTs selected in patients undergoing therapies. Results show that all RTs can add nucleotides in a non-template fashion on the cDNA 3’-end, a reaction stimulated by NC. Nucleotide substitutions were examined using in vitro systems where 3’-mutated cDNAs were extended by RT on an HIV-1 RNA template. With NC, RT extension of the mutated cDNAs was efficient, and surprisingly, mutations were frequently corrected. These results suggest for the first time that RT has excision-repair activity that is triggered by NC. Chaperoning of RT by NC might be explained by the fact that NC stabilizes an RT-DNA binary complex. In conclusion, RT-NC interactions appear to play critical roles in HIV-1 variability.

Human immunodeficiency virus type 1 (HIV-1)2 replication is characterized by a high level of genetic variability, which leads to the appearance of quasispecies within a single individual (1). The emergence of quasispecies poses serious issues for AIDS treatment and vaccine development. Indeed, during most antiretroviral therapies, drug-resistant variants and viruses escaping the immune surveillance quickly appear (2, 3). Understanding the molecular determinants of HIV-1 variability is of critical importance for the design of new highly active antiretroviral drugs.

Several mechanisms contribute to HIV-1 hypermutability. Reverse transcription, which comprises the early stage of the viral replication cycle, is the major source of retrovirus variability. Reverse transcriptase (RT) catalyzes the conversion of the single-stranded genomic RNA into a double-stranded viral DNA that is subsequently imported into the nucleus and integrated into the host cell genome by integrase. RT is error-prone and has the ability to misincorporate nucleotides during polymerization and to extend the mispaired nucleotides (4, 5). This is thought to generate one or several mutations per round of virus replication. In addition to the obligatory first and second DNA strand transfers, RT mediates random strand transfers that generate recombinant viruses (6). These transfer events can be triggered by pauses during reverse transcription because of secondary structures in the viral RNA, mispaired nucleotides, or nicks (7–9). Previous results indicate that 30% of the transfers are coupled with point mutations at the site of transfer (10). Moreover, RT is able to add one or more non-template nucleotides on the cDNA 3’-end while copying RNA ends (11–13). As the genomic RNA is not fully protected against cellular nuclease degradation, RT is thought to encounter multiple ends during reverse transcription (5, 14). cDNAs bearing the extra nucleotides are likely transferred onto the second RNA molecule to allow polymerization rescue. Data show that these transfers often result in substitutions (9). During polymerization, RT interacts with and is assisted by the viral nucleocapsid protein NC (5, 10, 15). NC is a well conserved small basic protein with two zinc finger motifs flanked by regions rich in basic residues. NC molecules coat the dimeric genome, forming the nucleocapsid structure within the virion, and are implicated in the early phases (proviral DNA synthesis) and late phases (genomic RNA dimerization and packaging) of the viral replication cycle. NC promotes reverse transcription by directing the annealing of primer tRNA18S-3 to the primer binding site (15). In addition, NC stimulates minus and plus strand DNA transfers by promoting hybridization of the complementary repeat (R) sequences from the cDNA and the RNA template and by annealing of the tRNA and primer binding site sequences, respectively (10, 16–18). NC also promotes specific proviral DNA synthesis by inhibiting self-primed reverse transcription (19–21). Recently, NC was found to play a role in the maintenance of the newly made HIV-1 DNA by protecting the long terminal repeat sequences from nuclease attack and was also found to assist integrase (22–24).

Because NC plays key roles in the early steps of reverse transcription and in the DNA strand transfers, we wanted to investigate its possible function in viral variability. To investigate the influence of NC on nucleotide addition or substitution by RT, we established in vitro model systems containing HIV-1 RNA and cDNA and NC and RT proteins. In

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‡ The abbreviations used are: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; NC, nucleocapsid protein; AZT, 3’-azido-3’-deoxythymidine; AZTTP, 3’-azido-3’-deoxythymidine 5’-triphosphate; wt, wild type; ddI, didanosine; ddT, ddT-resistant.
addition to the wild type (wt) RT, we used AZT- and ddI-resistant RTs, because they represent the major forms of resistant RTs selected in patients under highly active antiretroviral therapy. Our results show that all RTs can add nucleotides in a non-template fashion at the cDNA 3’-end. Purines were preferentially added, and this was independent of the ultimate residue. NC was found to stimulate this non-template addition of nucleotides by RTs. Nucleotide substitutions by RT were examined using the same in vitro systems, where 3’-mutated cDNAs were extended. In the presence of NC, RT extension of the mutated cDNAs was efficient, and more importantly in most cases, mutations were corrected.

To further investigate how the presence of NC affects the interaction between RT and its substrate and thus its activity, we examined the influence of NC on the dissociation rate of RT on a duplex DNA. Our data indicate that NC significantly stabilizes the binary complex between HIV-1 RT and duplex DNA and therefore should be viewed as a critical RT co-factor.

EXPERIMENTAL PROCEDURES

RNA and DNA—RNAs were either HIV-1 RNA-generated in vitro by T7 phage RNA polymerase (10) or synthetic oligoribonucleotides. DNA and RNA oligonucleotides were supplied by Invitrogen or Integrated DNA Technologies. All of the other reagents were purchased from Sigma and were of the highest purity available. Radiolabeled oligonucleotides were prepared using phage T4 polynucleotide kinase (Invitrogen) and [γ-32P]ATP. DNAs were purified by 8% PAGE in 7 M urea. Sequences of nucleic acids used in this study are presented in Table 1.

Protein Expression and Purification—Recombinant HIV-1 reverse transcriptase (RT p66/p51) was purified according to the above protocol.

Highly pure NC-(1–72), -(1–55), and -(12–53) were prepared by pentafluorophenyl ester chemical synthesis by D. Ficheux (Institut de Biologie et Chimie des Protéines, Lyon, France) as done previously (26). To test fluorescence, RTs were purified according to the above protocol.

To further investigate how the presence of NC affects the interaction between RT and its substrate and thus its activity, we examined the influence of NC on the dissociation rate of RT on a duplex DNA. Our data indicate that NC significantly stabilizes the binary complex between HIV-1 RT and duplex DNA and therefore should be viewed as a critical RT co-factor.

Formation of Nucleoprotein Complexes—RNA (0.5 pmol), 5’-end 32P-labeled DNA oligonucleotides (0.5 pmol), and NC at protein to nucleotide molar ratios of either 0 or 1.6 were incubated for 10 min at 37 °C in 10 μl of 20 mM Tris-HCl, pH 7.5, 30 mM NaCl, 0.2 mM MgCl2, 5 mM dithiothreitol, 0.01% (v/v) Triton X-100. When included in the mixture, the concentration was 1.2 μM for NC-(1–71) and up to 2.4 μM for NC-(1–55)rec.

To assess the concentration-dependent effect of NC on single nucleotide extension, the reaction was modified as follows: a mixture containing 25 nM template-primer and 25 nM HIV-1 RT was preincubated with varying concentrations of NC (0.1–1.2 μM NC-(1–71) or 0.2–3.2 μM NC-(1–55)rec in 12.5 mM Tris-HCl, pH 7.8, 40 mM NaCl, 9 mM MgCl2, 5 mM dithiothreitol, and 0.01% (v/v) Triton X-100. After 3 min at 37 °C, heparin was added to sequester dissociated RT and ensure single round nucleotide incorporation. For reactions lacking NC, the minimal heparin concentration (10 μg/ml) that inhibits rebinding of RT to the primer-template was used. For reactions containing NC, the heparin concentration was increased to 2 mg/ml to account for a potential decrease in effective heparin concentration because of binding with the basic NC. Incubation was continued for times ranging from 9 to 360 s before dATP was added to a final concentration of 25 μM to initiate single nucleotide incorporation. The sample was then placed on ice and the reaction stopped by mixing with an equal volume of 89 mM Tris borate (pH 8.3), 2 mM EDTA, and 95% (v/v) formamide containing 0.1% (v/v) bromphenol blue and xylene cyanol. Reaction products were resolved by high voltage-denaturing 10% PAGE and visualized by phosphorimaging. The fraction of enzyme bound to the template was plotted as a function of time and the dissociation rate constant koff estimated by fitting the data with KaleidaGraph to the single exponential function γ = Aexp(−kofft), where A is amplitude, koff is dissociation rate, and t is incubation time with the trap. Note that initiating the primer extension with dATP at varying times after the addition of heparin reveals the fraction of RT still bound to the template at this time point.

To assess the concentration-dependent effect of NC on single nucleotide extension, the reaction was modified as follows: a mixture containing 25 nM template-primer and 25 nM HIV-1 RT was preincubated with varying concentrations of NC (0.1–1.2 μM NC-(1–71) or 0.2–3.2 μM NC-(1–55)rec in 12.5 mM Tris-HCl, pH 7.8, 40 mM NaCl, 9 mM MgCl2, 5 mM dithiothreitol, and 0.01% (v/v) Triton X-100. After 3 min, heparin was added to a final concentration of 2 mg/ml, and the mixture was incubated for 50 s at 37 °C. Primer extension was initiated by the addition of dATP to a final concentration of 25 μM. In a second set of experiments, reaction mixtures contained, in addition to the varying amounts of NC-(1–71) (0.1–1.2 μM), a constant and excess amount of 1.6 μM NC-(1–55)rec. Products were resolved and analyzed as described above.

DNA Sequencing—cDNAs synthesized by RT extension of wild type or mutated DNA primers with or without NC-(1–72) were purified by phenol-chloroform extraction and ethanol-precipitated. cDNAs were PCR-amplified using a high fidelity Taq polymerase (Promega) and specific primers bearing restriction sites TARamp (–) (‘5’-GGGATCC-CGGTACCCGAGGAGCTCCGCGG-3’) and TARamp (+) (‘5’-GGAAATTCGGTCTTAAAGTTAGTACC-3’). Amplified DNAs were then digested with EcoRI and BamHI and cloned into pSP64 vector digested with the same enzymes. Ligations were performed using Invitrogen T4 DNA ligase transformed and amplified in an E. coli recA- strain.
Plasmids were sequenced and sequences analyzed using the Edit View software.

**RESULTS**

**Influence of NC on Non-template Nucleotide Addition by RT**—Non-template addition of nucleotides by RT is thought to occur at the blunt end of the newly made duplex template. During HIV-1 reverse transcription, such additions may take place during the two obligatory strand transfers and also at non-obligatory strand transfers caused by nicks in the genomic RNA (11–13).

To study non-template nucleotide additions at the cDNA 3'-end by HIV-1 RT, we derived an in vitro system formed of an RNA template, a primer DNA corresponding to the newly synthesized cDNA, NC, HIV-1 RT, and dNTPs (Fig. 1). Four types of template-primer were used with either one of the four possible cDNA 3'-ends. Sequences used were derived from the HIV-1 genome or from non-viral sequences (Table 1). The addition of NC to RNA and 5'-end 32P-labeled primer DNA caused the concomitant formation of nucleoprotein complexes (20, 27) and hybridization of the primer DNA and template RNA. In the absence of NC, annealing was carried out by heating (see “Experimental Procedures”). Next, either one or a mixture of the four dNTPs and RT were added. Reactions were performed at 37 °C for 30 min and [32P]cDNAs were performed for 30 min at 37 °C. cDNAs were visualized by autoradiography.

Non-template nucleotide addition activity was assayed with wild type RT, NC-(1–72) and several different classes of template-primer complexes with each cDNA 3'-end as indicated in Fig. 2. Results show that RT, in the absence of NC, can efficiently add one nucleotide at the cDNA 3'-end, preferentially A or G when the 3'-end is T, G, or C (Fig. 2, B–D) and not when it is A (Fig. 2A). These results are in good agreement with previous data from several groups (9, 11–13). In the presence of NC, RT was able to add A, G, or T at the cDNA 3'-A (Fig. 2A). Moreover, the addition of 4–6 consecutive T or G bases was also observed on template-primer complexes 3 and 7 (Fig. 2, B and D). However, NC did not change the pattern of the RT-directed addition, which remained A,G,T>G>C (Fig. 2). The non-template addition of nucleotides by RT requires a hybrid, because when DNA was not hybridized to the RNA template, no addition was observed (data not shown).

To further investigate the role of NC in the RT-directed non-template addition of nucleotides, we used NC-(1–55), because it is the ultimately processed form of HIV-1 NC and also the NC-(12–53) mutant corresponding to the central globular domain of the viral protein (see supplemental data). NC-(1–55) was found to be clearly less active than NC-(1–72) (Fig. 3, compare lanes 6 and 7), whereas the NC-(12–53) deletion mutant was very poorly active (Fig. 3, lane 8). These results suggest that the basic rich regions of NC are important for enhancing the non-template addition of nucleotides by HIV-1 RT.

**Non-template Addition of Nucleotides by RT Mutants Resistant to Antiviral Drugs**—Non-template addition of nucleotides by RT was analyzed with the AZT (T215Y)- and ddI (L74V)-resistant RTs, NC-(1–72), and four different classes of template-primer complexes, with each cDNA 3'-end as reported in Fig. 4. Results show that the AZT- and ddI-resistant RTs retained their non-template nucleotide addition activity (Fig. 4A), which was also enhanced by NC with cDNA 3'-A (P/T #1) and 3'C (P/T #7) (Fig. 4A, lanes 3, 4, 7, 11, and 12). However, in contrast to wt RT, these mutants were unable to catalyze multiple nucleotide additions (Fig. 4A, compare P/T panel #7, lanes 4, 8, and 12).

**TABLE 1**

**Nucleic acids used in this study**

P/T stands for primer/template. Underlined sequences indicate mutated nucleotide(s) at the primer 3'-end used in the assays.

| System | Sequences | Origin |
|--------|-----------|--------|
| P/T 01 | 5'TTCCAGC/CGATAATG/CGCCATAC3' | HIV-1 |
| P/T 02 | 5'TTCCAGCAGCTATGTCGGGAA3' | HIV-1 |
| P/T 03 | 5'TTCCAGCAGCTATGTCGGGAA3' | HIV-1 |
| P/T 04 | 5'AAATTCGGCTCTTCCATAT3' | HIV-1 |
| P/T 05 | 5'AAATTCGGCTCTTCCATAT3' | HIV-1 |
| P/T 06 | 5'AAATTCGGCTCTTCCATAT3' | HIV-1 |
| P/T 07 | 5'AAATTCGGCTCTTCCATAT3' | HIV-1 |
| P/T 08 | 5'AAATTCGGCTCTTCCATAT3' | HIV-1 |

**FIGURE 1.** Formation of HIV-1 nucleoprotein complexes in vitro to study template-independent addition and excision of nucleotides by RT. HIV-1 blunt end nucleoprotein complexes were formed by incubating an RNA representing part of the HIV-1 genome and a 5'-end 32P-labeled DNA corresponding to the viral cDNA without or with NC for 10 min at 37 °C. HIV-1 RT and each dNTP alone or equimolar amounts of the four dNTPs or AZTTP were then added, and reactions were performed for 30 min at 37 °C. cDNAs were fractionated on a 12% denaturing polyacrylamide gel and visualized by autoradiography.
Evidence That RT Has a DNA Repair Activity—As already pointed out, a major source of HIV-1 variability is the fact that RT can introduce point mutations into the cDNA chain during the polymerization reaction (4). Previous experiments from this laboratory showed that HIV-1 RT was able to extend 3’-mutated cDNAs, although copying a HIV-1 RNA template in the presence of NC-(1–72) (20). Under these conditions, cDNA extension efficiencies were ~40%, 23 and 5% for one, three, and five mutations at the cDNA 3’-end, respectively, compared with the non-mutated cDNA. Given the propensity of HIV-1 RT to add nucleotides in a non-template fashion and incorporate mutations in growing cDNA chains, we re-investigated the RT extension of cDNA with 1–5 mutations at the 3’-end, respectively, compared with the 5’-end, whereas the 5’-end, respectively, compared with the

FIGURE 2. Template-independent nucleotide addition by wild type HIV-1 RT at the cDNA 3’-end. Nucleoprotein complexes were formed as described under “Experimental Procedures.” Four types of RNA-DNA hybrids were used: 3’A DNA (P/T#1–2) (A), 3’T DNA (P/T#3–4) (B), 3’ G DNA (P/T#5–6) (C), and 3’ C DNA (P/T#7–8) (D). Nucleic acid sequences are presented in Table 1. NC-(1–72) to nucleotide molar ratios used were 0 (lanes 1, 3, 5, 7, 9, and 11) and 1:6 (lanes 2, 4, 6, 8, 10, and 12). After nucleoprotein complex formation, blunt end RNA-DNA hybrids were incubated with 0.25 µM wt HIV-1 RT and either no dNTP (lanes 1 and 2), all four dNTPs (lanes 3 and 4), or one dNTP (lanes 5–12). Arrows indicate [32P]DNA primer, and +1 shows DNA with one nucleotide added. The dark vertical line corresponds to cDNAs with several nucleotides added.

FIGURE 3. Influence of NC variants on the template-independent addition of nucleotide by RT. Nucleoprotein complexes were formed as described under “Experimental Procedures.” Two types of RNA-DNA hybrids were used: 3’A (P/T#1) and 3’C (P/T#7) DNAs (Table 1). NCs used were NC-(1–72) (lanes 2 and 6), NC-(1–55) (lanes 3 and 7), and mutant NC-(12–53) (lanes 4 and 8). The NC to nucleotide molar ratios used were 0 (lanes 1 and 5) and 1:6 (lanes 2–4 and 6–8). After formation, blunt end RNA-DNA hybrids were incubated with WT HIV-1 RT at 0.25 µM and either no dNTP (lanes 1–4) or all four dNTPs (lanes 5–8). Arrows indicate [32P]DNA primer, +1 shows DNA with one nucleotide added, and black vertical line corresponds to cDNAs with multiple nucleotides added.

A terminal addition pattern similar to wild type RT was observed for the mutant enzymes, namely A,G>T>C (data not shown). Moreover, stimulation of the non-template addition of mutant RTs by NC was dependent on the basic regions of NC (Fig. 4B).
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TABLE 2

Ability of HIV-1 RT to correct mispaired nucleotides at the primer 3' end

Percentages were determined as the number of each type of sequences relative to the total numbers of DNA sequenced (40–92 sequences/type). DNA primers are shown hybridized to the RNA template. Underlined sequences indicate mutations at the primer cDNA 3' end. Observed WT sequences are those where the original mutations have been corrected. Not corrected sequences correspond to cDNAs where initial mutations were retained. Partially corrected indicates cDNAs where 1, 2, 3, or 4 mutations were corrected. Corrected with additional mutations are sequences where original mutations were corrected, with additional mutations elsewhere.

| DNA primer           | NC-(1–72) | Observed WT | Not corrected | Partially corrected | Corrected with additional mutations |
|----------------------|-----------|-------------|---------------|---------------------|-------------------------------------|
| 3' WT AGAGACC→3'UCUCUGG | +         | 100         | 0             | 0                   | 0                                   |
| 3' mut1 AGAGACA→3'UCUCUGG | +         | 86          | 14            | 0                   | 0                                   |
| 3' mut3 AGAGCGA→3'UCUCUGG | +         | 67          | 3             | 10                  | 20                                  |
| 3' mut5 AGAGCGA→3'UCUCUGG | +         | 83          | 0             | 1                   | 16                                  |
| 3' mut1 AGAGACA→3'UCUCUGG | −         | 68          | 32            | 0                   | 0                                   |
| 3' mut3 AGAGCGA→3'UCUCUGG | −         | 19          | 3             | 10                  | 68                                  |

the nucleoprotein complex. Thereafter, HIV-1 RT and dNTPs were added to allow cDNA synthesis. The levels of cDNA synthesis were higher with NC than without (data not shown) and were similar to those already published (20). Full-length cDNAs were PCR-amplified, cloned, and sequenced. A high fidelity Taq polymerase was used to prevent introducing mutations during the amplification step (see “Experimental Procedures”).

Sequencing results summarized in Table 2 were unexpected, because they show that the original mutations were rarely found when cDNA synthesis was performed in the presence of NC-(1–72). For example, a single mutation was corrected >80% of the time. Similarly, high correction values were obtained with 3 and 5 consecutive mutations. It was interesting to note that a single mutation was retained ~14% of the time, whereas partially corrected sequences were found in 10% of the cDNAs sequenced (Tables 1 and 2, 3' mut3). These results indicate that RT in the presence of NC-(1–72) can correct mismatches at the cDNA polymerization site in vitro, although not completely. In the absence of NC, RT was still capable of partially correcting a single mutation at the cDNA polymerization site, but this was decreased by at least 3-fold when three consecutive mutations were present (Table 2, 3' mut1 and 3' mut3 without NC). In addition, the absence of NC during reverse transcription was associated with an increased number of mutations elsewhere in the 370-nucleotide-sequenced cDNAs (Table 2). Taken together, these data show that HIV-1 RT has an excision-repair activity in vitro and that NC stimulates this new activity.

HIV-1 RT-mediated Nucleotide Excision—To understand the repair reaction carried out by RT during elongation of cDNAs originally containing 1–5 mutations at their 3'-end (Table 2), we reasoned that the RT enzyme could excise the mismatched residues at the polymerization site, thus allowing cDNA synthesis to occur. To investigate this possibility, we used the template–primer hybrid as before to form a nucleoprotein complex upon NC addition. Thereafter, we added RT and the nucleotide analogue AZT in its triphosphorylated form, AZTTP, to prevent elongation after a putative nucleotide excision (Fig. 1).

In the presence of AZTTP, cDNA that was one nucleotide shorter than the original DNA was observed (Fig. 5). Nucleotide removal occurred with wt (Fig. 5A, lanes 1–4), AZTTP (T215Y) (lanes 5–8), and ddI (L74V) (lanes 9–12) RTs. Also, NC-(1–72) was found to increase, by ~10-fold, the efficiency of nucleotide excision by WT, AZT, and ddI RT (Fig. 5A, lanes 4, 8, and 12). First of all, we excluded the possibility that nucleotide excision resulted from a DNase activity of RT (data not shown). To further examine the influence of NC on nucleotide...
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removal by RT, we again used NC-(1–55) and NC-(12–53) corresponding to the central zinc fingers (see "Experimental Procedures"). The NC-(12–53) deletion mutant, missing the basic regions, was unable to stimulate nucleotide excision (Fig. 5B, lane 8). These data indicate that the NC basic domains are required for an efficient nucleotide excision by RT. The NC-(1–55) variant also did not promote nucleotide removal by RT in the presence of AZTTP (Fig. 5B, lane 7). To examine whether AZTTP has a direct effect on the RT excision activity, we used other chain terminators, such as ddATP, ddTTP, ddGTP, and ddCTP, in the presence or absence of NC. However, no excision was observed under these conditions (data not shown). Because ATP was described as a catalyst of AZT removal by AZT-resistant RT (28), we analyzed its effect on nucleotide removal by HIV-1 RT. In the presence of NC, AZTTP, and ATP, nucleotide excision efficiency by wt RT was lower than in assays performed without ribose ATP (data not shown). This suggests that ATP negatively influences nucleotide excision by RT. In addition, when inorganic pyrophosphatase was added to a reaction with AZTTP, nucleotide excision was partially hampered, indicating that pyrophosphate is involved in nucleotide removal by HIV-1 RT (data not shown).

Influence of NC on RT-DNA Complex Stability—Taken together, the above results show that NC can stimulate non-template additions and excision-repair of nucleotides by RT at the cDNA 3′-end. One plausible mechanism underlying this stimulation could be that NC has a stabilizing effect on the RT-substrate complex, prolonging the time RT remains productively bound. As a more quantitative measurement of interactions between RT and its substrate and the influence of NC, dissociation rate constants were evaluated by single nucleotide incorporation. The presence of heparin in the reaction mixture ensured single RT binding events, such that the relative amount of primer extension product at a given time reflects the fraction of enzyme in complex with the substrate. Fig. 6 illustrates the formation of the primer extension product (p + 1) in the absence (A) or presence (B) of NC-(1–71), when single nucleotide extension was initiated at varying time points after the addition of heparin. Fitting the data with the single exponential equation $y = A \exp(-k_{off}t)$, where $A$ is amplitude and $t$ is incubation time, yields the dissociation rate $k_{off}$ for RT on the substrate. For the reaction without NC, the dissociation rate $k_{off}$ was determined as $0.059 \pm 0.0014$ s$^{-1}$, whereas in the presence of NC-(1–71), $k_{off}$ was $0.0036 \pm 0.00028$ s$^{-1}$ (Fig. 6C). This 10-fold decrease in dissociation rate constant suggests that NC significantly stabilizes the complex between HIV-1 RT and template-primer. In contrast, the ultimate processed form of NC, namely NC-(1–55), had no effect (data not shown). Fig. 7 displays the concentration-dependent effect of NC-(1–71) on the formation of the primer extension product by RT at a given time point. The solid curve represents reactions performed with increasing concentrations of NC. The dashed curve represents a second set of experiments, where all reactions contained, in addition to the varying amounts of NC-(1–71), a constant and excess amount of 1.6 μM NC-(1–55)mm.

DISCUSSION

In the present study, we have used in vitro systems mimicking the in vivo HIV-1 nucleoprotein complex called the viral nucleocapsid in which reverse transcription occurs. We have revealed new roles for NC in the synthesis of HIV-1 proviral DNA. NC-(1–72) stimulates the template-independent nucleotide addition mediated by wild type and drug-resistant RTs. Using four different blunt end template-primer hybrids, we observed variations in the NC stimulation levels, suggesting that NC influence on the non-template addition of nucleotides by RT depends on the local sequence or secondary structure of the blunt end hybrid. In addition, NC-(1–72) was found to stimulate multiple additions of dTMP and dGMP, which might be correlated with RT-NC interactions (30) (see also below) and the higher affinity of NC for T and G residues (31).

In another series of experiments, we observed that HIV-RT chaper-
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oned by NC-(1–72) was able to efficiently correct one, three, and five consecutive mismatches at the polymerization site in vitro (Table 2). These findings indicate, for the first time, that HIV-1 RT could have an excision-repair activity in vitro. It has been assumed for a long time that retroviral RTs lack a proofreading activity (32, 33), because they can efficiently misincorporate nucleotides and subsequently extend mismatches (4, 34–38). This lack of 3′-5′-exonuclease activity was proposed to account for the high error rate of retroviral RT. However, in the majority of mispair extension assays, cDNAs extended were not sequenced and thus the presence of the initial mutation was not confirmed. In the present system, data on several hundred sequences confirm extension of one (A:C) and three consecutive mismatches (A:C, G:G, C:U) (Table 2). More interestingly, we observed that mispair correction occurred more frequently than mispair extension, especially in the presence of NC-(1–72). In agreement with this, HIV-1 RT has the ability to excise a nucleotide from the cDNA 3′-end using two different mechanisms, namely by pyrophosphorolysis corresponding to the reverse reaction of polymerization and by ATP-dependent hydrolysis. Also, RT was found to efficiently remove AZT from AZT-blocked DNA (39, 40). Excision of AZT by HIV-1 WT and AZT-resistant RT has been proposed to explain resistance to AZT inhibition. Furthermore, RT was recently shown to selectively remove mispairs (41) and to excise an incorporated dUMP in the presence of uracil-DNA glycosylase type 2 (42). Excision of dGMP with the insertion of the correct dAMP opposite incorporated dUMP in the presence of uracil-DNA glycosylase type 2 (39, 40). Excision of AZT by HIV-1 WT and AZT-resistant RT has been proposed to explain resistance to AZT inhibition. Similarly, Lener et al. (30) have reported that NC-(1–72) functions more efficiently than NC-(1–55) in forming replicative nucleoprotein complexes in vitro (15). Thus, one can imagine that these two NC variants, which coexist in the viral particle, act differently in the course of cDNA synthesis by RT (30) and in proviral DNA integration (22, 50).

Taken together, our findings highlight the dual role played by both NC and RT in viral replication. On one hand, RT and NC ensure virus viability by allowing a specific conversion of the RNA genome into a complete double-stranded DNA flanked by long terminal repeats in a process that necessitates two obligatory strand transfers. In addition and when required, forced interstrand transfers can occur at nicks in the RNA genome to allow continuation of cDNA synthesis by the replicative complex. On the other hand, RT chaperoned by NC appears to induce a balanced variability in the newly synthesized cDNA by virtue of non-template nucleotide addition or nucleotide excision at mispaired sites. This balanced mechanism of virus variability and viability would thus ensure both efficient virus replication and enough diversity to escape drug treatments and immune responses.

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