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Human Immunodeficiency Virus Type 1 Nucleocapsid Protein Reduces Reverse Transcriptase Pausing at a Secondary Structure near the Murine Leukemia Virus Polypurine Tract

WEIXING WU,[1] LOUIS E. HENDERSON,[2] TERRY D. COPELAND,[3] ROBERT J. GORELICK,[2] WILLIAM J. BOSCHE,[2] ALAN REIN,[4] AND JUDITH G. LEVIN[1]*

Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, Bethesda, Maryland 20892,[1] and AIDS Vaccine Program, SAIC-Frederick,[2] and Special Program in Protein Chemistry[3] and Retroviral Genetics Section,[4] ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702

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In an earlier study on minus-strand DNA synthesis catalyzed by murine leukemia virus reverse transcriptase, we described a prominent pause site near the polypurine tract (J. Guo, W. Wu, Z. Y. Yuan, K. Post, R. J. Crouch, and J. G. Levin, Biochemistry 34:5018–5029, 1995). We now report that pausing at this site is due to a stem-loop structure in the RNA template, formed by interaction of a number of bases in the polypurine tract, including the six G's, and a 3′ sequence which includes four C's. Addition of human immunodeficiency virus type 1 (HIV-1) nucleocapsid (NC) protein to reverse transcriptase reactions reduces pausing by ~8- to 10-fold and stimulates synthesis of full-length DNA. Thus, NC functions as an accessory protein during elongation of minus-strand DNA and increases the efficiency of DNA synthesis, in this case, by apparently destabilizing a region of secondary structure in the template. Since NC is associated with genomic RNA in the viral core and is likely to be part of a viral replication complex, these results suggest that NC may also promote efficient DNA synthesis during virus replication. Mutational analysis indicates that the features of HIV-1 NC which are important for reduction of pausing include the basic amino acids flanking the first zinc finger, the zinc fingers, and the cysteine and aromatic amino acids within the fingers. These findings suggest that reverse transcription might be targeted by drugs which inactivate the zinc fingers of HIV-1 NC.

Replication of all retroviruses is dependent on conversion of the single-stranded RNA genome to linear double-stranded DNA. This process is catalyzed by the virion-associated reverse transcriptase (RT) in a complex series of reactions involving the RNA- and DNA-dependent DNA polymerase activities, as well as the RNase H activity, of the enzyme (45, 76).

In earlier studies on in vitro minus-strand DNA synthesis with purified murine leukemia virus (MuLV) RTs, we (35) and others (73) showed that the RNase H domain facilitates processive DNA synthesis by stabilizing the interaction between RT and primer-template. However, even the wild-type enzyme, which has the requisite RNase H domain, is not completely processive and many small intermediate products were found to accumulate in the RT reactions (35, 73). Indeed, in our system, we detected a major pause product (35) whose 3′ terminus is at a pair of U's, 14 nucleotides (nt) downstream of the polypurine tract (PPT), a short sequence serving as the primer for plus-strand DNA synthesis (reviewed in reference 9). If such pausing occurred during virus replication, RT would have difficulty extending minus-strand DNA beyond the PPT and both minus and plus-strand DNA synthesis would be impaired. (A DNA copy of the PPT must be made to provide an RNA-DNA substrate for the RNase H cleavage event which generates the free 3′-OH used for initiation of plus-strand DNA synthesis [9].)

Since the efficiency of viral DNA synthesis has a direct effect on virus replication, it is likely that in vivo, accessory proteins (either cellular or viral) are recruited to increase the rate and extent of reverse transcription during virus infection. One candidate for this function is the viral nucleocapsid protein (NC), which is associated in a complex with genomic RNA, tRNA primer, RT, and integrase in the retrovirus core (11, 16, 26, 50). A review of the recent literature on NC is given in reference 17.

The NC protein is derived from the C-terminal region of the Gag precursor protein (36, 37). It is a small, basic protein which binds single-stranded nucleic acids (44) and catalyzes conformational changes which result in more thermodynamically stable structures (7, 21, 25, 41, 59, 74, 75, 79; reviewed in reference 40). On the basis of these properties, NC has been classified as a nucleic acid chaperone (40).

All retroviral NC proteins (except those of the spumaretrovirus group [53]) contain either one or two copies of an invariant motif, $C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_4\text{-}C$, where X denotes variable amino acids which are found in different retroviruses; the motif has been termed a zinc finger, cysteine array, or Cys-His box (4, 14, 38). Zinc is present in retrovirus particles (8) and is bound to the NC cysteine arrays by coordination with the cysteine and histidine residues (10, 54, 68–70).

Mutations in the zinc fingers are associated with loss of infectivity and a dramatic reduction in genomic RNA packaging (1, 23, 33, 34, 55–57). In the case of viruses containing two zinc fingers, such as human immunodeficiency virus type 1 (HIV-1) (32 [and references therein], 36), both fingers are required for infectivity and encapsidation of viral RNA (22, 32, 34, 56); in addition, the positions of the two finger sequences in the HIV-1 protein cannot be exchanged (22, 32). Recent studies with virions containing heterologous NC proteins suggest that specific recognition of the viral RNA packaging signal is

* Corresponding author. Mailing address: Laboratory of Molecular Genetics, NICHD, Building 6B, Room 216, NIH, Bethesda, MD 20892. Phone: (301) 496-1970. Fax: (301) 496-0243. Electronic mail address: jlevin@lmgvax.nichd.nih.gov.

mediated by NC in an interaction which may involve the zinc finger(s) (6, 24, 80). However, the mechanism is not understood.

Several investigators have observed that virions with certain mutations in NC are noninfectious but still encapsidate significant levels of genomic RNA (31–33, 55–57, 80). These findings led to the conclusion that NC has other essential functions in addition to RNA packaging, including roles in virus maturation (25, 27, 28), reverse transcription (2, 16, 19, 43, 50, 60, 66, 71, 72, 77–79), and possibly integration (50).

In this report, we describe the role of NC as an accessory protein during viral DNA synthesis. We examined the effect of HIV-1 NC on pausing near the MuLV PPT during elongation of minus-strand DNA by MuLV RT. In addition, we prepared mutant NC proteins to identify elements within NC which are important for activity in this system. Our results show that pausing is due to a stem-loop structure in the RNA template formed by sequences within and downstream of the PPT. NC, acting as an RNA chaperone, presumably destabilizes this structure: thus, NC dramatically reduces pausing and stimulates synthesis of full-length DNA, resulting in an increase in the efficiency of viral DNA synthesis. Mutational analysis of NC function indicated that the conserved features of the NC protein, including the basic amino acids flanking the first zinc finger, as well as the zinc finger structures and certain residues therein, all contribute to the ability of NC to reduce pausing.

MATERIALS AND METHODS

Materials. Restriction enzymes, T4 ligase, and *Taq* polymerase were purchased from Boehringer Mannheim. T4 polynucleotide kinase was obtained from Promega Biotech. A MEGAscript kit, purchased from Ambion, was used to make T7 transcripts. [γ-^{32}P]ATP (3,000 Ci/mmol) and [α-^{35}S]dATPαS were purchased from Amersham Life Science Inc. The deoxynucleoside triphosphates were obtained from Pharmacia LKB Biotechnology Inc. DNA oligonucleotides were synthesized on a Biosearch Synthesizer model 8750 (New Brunswick Scientific Co.) or were purchased from Lofstrand. DNA sequencing was performed with a Sequenase kit obtained from U.S. Biochemical Corp.

Construction of plasmids. Clone pPPT-A (35), which contains the AKR MuLV PPT and surrounding 5′ and 3′ sequences (nt 7602 to 8033 [39]) in the pGEM3Z vector, was used to generate three new clones with mutations in the PPT and/or surrounding sequences. PCRs were carried out with pPPT-A as the template and a 17-nt universal sequencing primer, 5′-GTTTTCCCAGTCAC GAC (U.S. Biochemical Corp.), as the forward primer in all cases. The reverse primers were as follows. (i) The primer used to change the four C's downstream of the PPT (nt 7834 to 7837 [39]) to 5′-TGTA (clone pWW4C) was 5′-AAAA CTGCAGTTAGCTGGCTAAGCCTTATGAATACATCTTTCATTCCCCCC (complementary to nt 7819 to 7865 [39]). (ii) The primer used to change the 6 G's in the PPT (nt 7819 to 7824 [39]) to 5′-TCATAC (clone pWW6G) was 5′-AA AACTGCAGTTAGCTGGCTAAGCCTTATGAAGGGGTCTTTCATTGTA TGATCTTTCTGTAA (complementary to nt 7808 to 7865 [39]). (iii) The primer used to exchange the positions of the four C's and the six G's (clone pWWsw) was 5′-AAAACTGCAGTTAGCTGGCTAAGCCTTATGAACCCCCCTCTTT CATTGGGGTCTTTCTGTAA (complementary to nt 7808 to 7865 [39]). The PCR products were digested with *Eco*RI and *Pst*I, which removed the four A's at the 5′ ends of the reverse primers and also provided the ends for subsequent ligation. To replace the wild-type sequence with mutant sequences, each of the PCR fragments was ligated together with *Eco*RI-*Sal*I (~3.2 kb) and 180-bp *Pst*I-*Sal*I fragments from pPPT-A. The entire viral insert, including the region containing the mutation, was sequenced in each of the clones.

Preparation of recombinant or chemically synthesized NC proteins. Fusion protein vectors for bacterial expression of recombinant NC were constructed as follows. DNA fragments containing NC coding sequences for wild-type and zinc finger "switch" mutants (1:1, 2:2, and 2:1) were generated by PCR from proviral clones described previously (32, 34 [and references therein]). The PCR primer used to amplify the 5′ end of the NC gene was 5′-GAGCTCGGTACCCGGCC GGGG; the primer used for amplification of the 3′ end was 5′-GTACGTGTC GACTTAATTAGCCTGTCTCTCAGTACAATTTTTGGCTATGTGCCC. The PCR fragments were purified, digested with *Kpn*I and *Sal*I, and cloned into the corresponding sites in pMALc (Pharmacia Biotech, Piscataway, N.J.). DNA sequencing was used to verify the sequence of each construct.

Bacterial clones containing the wild-type NC and zinc finger switch mutant sequences were grown in Luria-Bertani broth at 37°C to an A_{600} of 0.5. Isopropyl β-D-thiogalactopyranoside was then added to a final concentration of 0.3 mM, and fusion proteins were induced for 3.5 h. The bacteria were pelleted and resuspended in buffer containing 10 mM Tris–0.25% (vol/vol) Tween 20–10 mM β-mercaptoethanol, pH 7.2. The suspension was sonicated, and NaCl was added to a final concentration of 0.5 M. Cellular debris was removed by centrifugation at 10,000 × *g* for 20 min. The NaCl concentration was raised to 1 M, and the solution was extracted three times with acetonitrile to remove the detergent. The suspension was centrifuged at 2,000 × *g*, and the resulting pellet was washed twice with 50% (vol/vol) acetonitrile in 20 mM Tris-HCl–0.5 M NaCl, pH 8.0, and pelleted as before. The pellet was resuspended in 30 ml of factor Xa buffer as specified by the supplier (New England Biolabs, Inc.). The pH was raised and lowered three times to 12.0 (by using 3 M NaOH) and 4.5 (by using 20% [vol/vol] trifluoroacetic acid in H_2O), respectively). The suspension was pelleted as before and resuspended in 50 ml of factor Xa buffer. The pH was adjusted to 12.0, and the solution was left for 3 min at room temperature; the pH was then lowered to 9.0. Fifty microliters of factor Xa (New England Biolabs, Inc.) was added to the solution, which was then incubated at room temperature for 10 min. The pH was lowered to 8.0, and the solution was incubated for an additional 16 h at 20°C. The NC proteins were purified by high-performance liquid chromatography (HPLC) as described by Henderson et al. (38).

To prepare NC modified with 3 equivalents of *N*-ethylmaleimide (NEM) (3NEM-NC), NC protein (with two bound zinc ions) was reacted at room temperature for 30 min with a threefold molar excess of NEM at pH 8 in 0.1 M Tris-HCl buffer. The reaction products and various modified proteins were separated by HPLC. Separate peaks were obtained for each possible modified protein product (i.e., proteins with zero to six modified cysteine residues). However, under these conditions, the major product of the reaction is an NC protein with cysteine residues modified by NEM alkylation at positions 36, 39, and 49 (data not shown). NC with all six cysteine residues alkylated with NEM (6NEM-NC) was prepared by the same method, except that the protein was reacted with a 20-fold excess of NEM reagent. Both the 3NEM-NC and 6NEM-NC proteins were characterized by complete amino acid sequence analysis to identify the locations of modified cysteine residues (data not shown).

Chemically synthesized wild-type and mutant NC proteins were constructed by solid-phase synthesis in an Applied Biosystems 430A peptide synthesizer. Proteins were deprotected and then purified by HPLC with an increasing gradient of acetonitrile on a Waters C18 column. Purified proteins gave correct amino acid compositions. The recombinant and synthetic wild-type NC proteins had equivalent activities.

The NC preparations contained 2 molar equivalents of $ZnCl_2$; mutants containing only one zinc finger contained 1 molar equivalent of $ZnCl_2$. All NC proteins were reconstituted in 10 mM Tris, pH 8.0, divided into aliquots, and stored at −80°C; a fresh aliquot was used for each experiment.

Primer extension assay. Reaction conditions and preparation of the 467-nt AKR MuLV RNA template and the ^{32}P 5′-end-labeled primer have been described by Guo et al. (35). Under the conditions of the assay, multiple initiation events from extended or nonextended primer molecules are possible. AKR MuLV RT was expressed and purified as described by Post et al. (62). The ratio of RT to primer-template was 10:1. Where indicated, NC was added together with the four deoxynucleoside triphosphates and $MgCl_2$. Control experiments showed that with low concentrations of NC, the same results were obtained when NC was added to primer-template prior to incubation with RT or together with RT; however, if NC was added before RT in reactions containing NC at concentrations of 1 μM or higher, primer extension was inhibited in a dose-dependent manner (data not shown). This inhibition presumably reflects inability of RT to bind to primer-template because at high concentrations of NC, all of the available template sites were covered and/or the nucleic acid was precipitated (35a, 74). Incubation was performed for 10 min at 37°C, unless indicated otherwise. Reactions were terminated by addition of 2 μg of proteinase K and incubation for 30 min at 65°C, followed by extraction with 1 volume of phenol-chloroform. The aqueous layer was added to 4 μl of STOP solution from the Sequenase kit, and the mixture was then heated at 90°C for 5 min; a 2-μl aliquot was loaded onto a 6% sequencing gel. Radioactivity in DNA products was quantified by using a PhosphorImager (Molecular Dynamics) and ImageQuant software. The data were plotted by using the Excel program.

RESULTS

RT pausing on an MuLV RNA template near the PPT. To measure minus-strand DNA synthesis in our primer extension assay, we incubated AKR MuLV RT with a preformed primer-template complex consisting of a short ^{32}P-labeled DNA oligonucleotide hybridized to a 467-nt AKR MuLV RNA template containing the PPT in its central portion (Fig. 1A) (35). As shown in earlier work (35), the extension products included the full-length 321-nt DNA, as well as a number of shorter pause products (Fig. 1A and C). The predominant pause product was a 75-nt DNA (Fig. 1A and C) (35), which terminates at a pair of U's immediately downstream from a run of four C's (nt 7834 to 7837 [39]) in the vicinity of the PPT (Fig. 1B). The 75-nt DNA was the focus of this study. Our interest in inves-

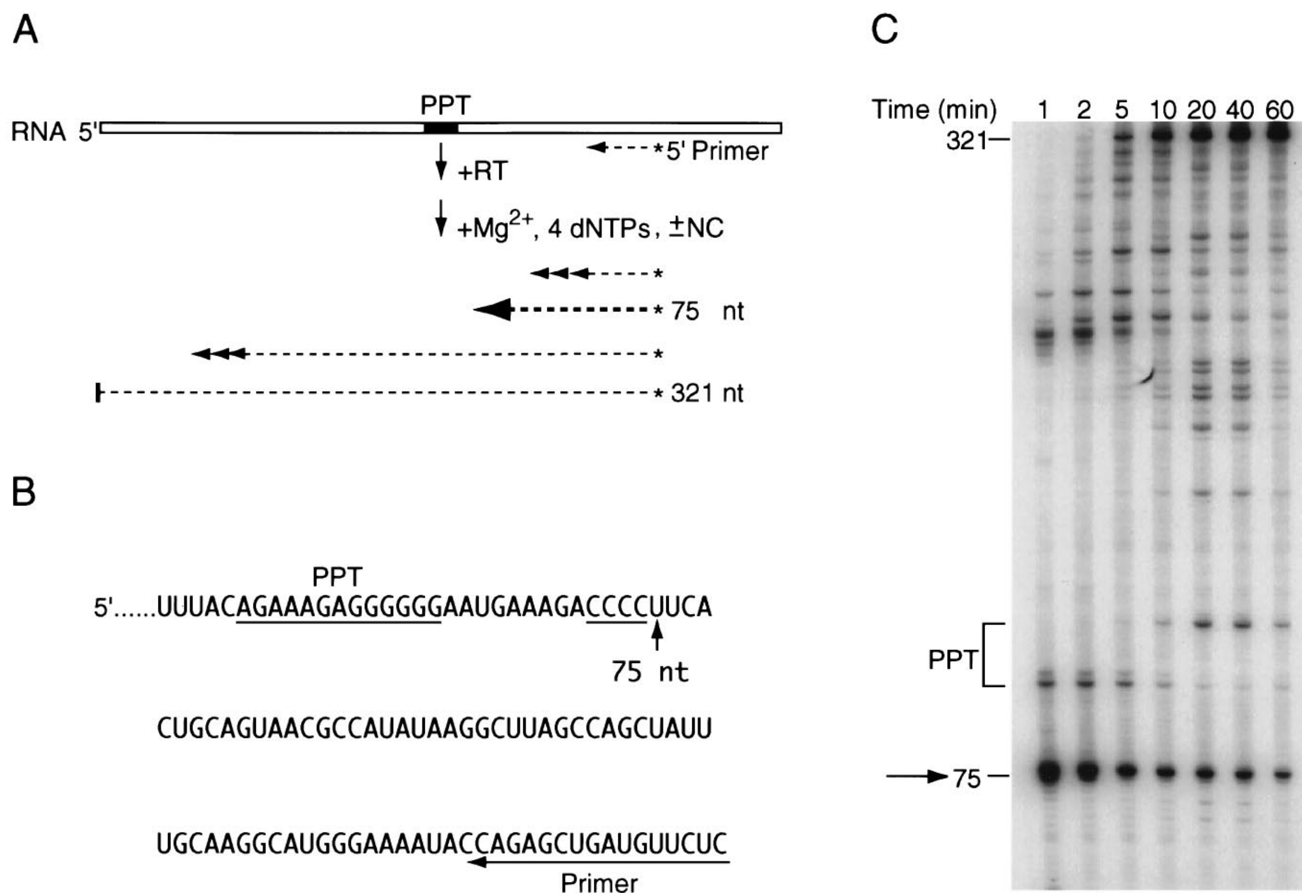


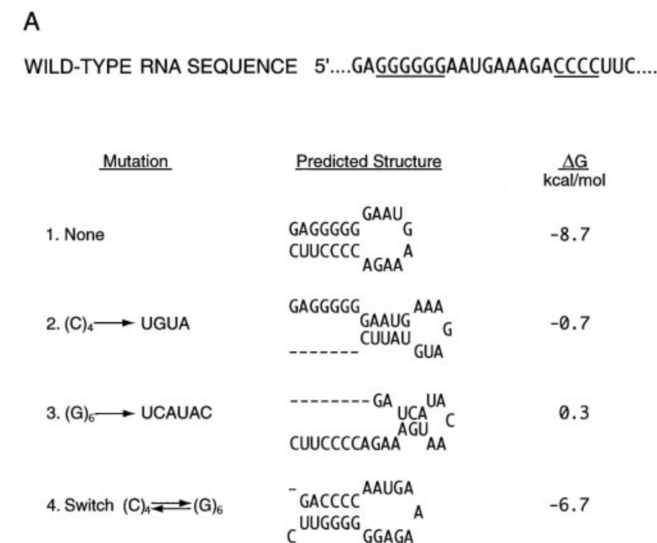
FIG. 1. Schematic diagram of primer extension assay and time course of DNA synthesis with a PPT-containing AKR MuLV RNA template. (A) Primer extension assay. The RNA template (open rectangle) containing the PPT sequence (solid area within open rectangle) is shown annealed to a short, 5′ ^{32}P-labeled DNA primer (thin, dashed line with arrow; the star denotes the ^{32}P label). The RNA-DNA hybrid is incubated with MuLV RT and then further incubated with Mg^{2+} and the four deoxynucleoside triphosphates in the presence or absence of NC protein as indicated in Materials and Methods. The labeled products include the full-length 321-nt DNA (dashed line with a solid bar at the end) and shorter DNAs (dashed lines with multiple arrowheads); the major 75-nt DNA product is highlighted by a thick, dashed line and a large arrowhead. The diagram is not drawn to scale. (B) Partial sequence of the template. The PPT, four downstream C's, and the sequence in the template which is complementary to the primer are underlined; an arrow points to the U which directs incorporation of the last residue in the 75-nt DNA pause product. (C) Time course of DNA synthesis. The reaction conditions used are described in Materials and Methods. Five-microliter aliquots were removed at each of the time points and prepared for gel electrophoresis as described in Materials and Methods. The positions of the 321-nt full-length product, the PPT, and the 75-nt pause product (arrow) are indicated on the left.

tigating factors affecting formation of the 75-nt DNA was motivated by the following consideration: if significant pausing occurred near the PPT, there would be detrimental effects on both elongation of minus-strand DNA and initiation of plus-strand DNA synthesis; this, in turn, would reduce the overall efficiency of virus replication.

The time course of DNA synthesis over a 60-min period is shown in Fig. 1C. With increasing times of incubation, the amount of 75-nt DNA was reduced, whereas the amount of full-length product was dramatically increased. These observations are consistent with a precursor-product relationship between the two DNAs.

Effect of secondary structure in the RNA template on RT pausing near the PPT. Since RT can pause at sites of secondary structure in the viral RNA template (3, 12, 20, 47, 58), a region near the PPT (nt 7817 to 7840 [39]) was analyzed with the FoldRNA program. The program predicts that a stable, 24-nt stem-loop structure can form by interaction of sequences within the PPT, including the six G's, and sequences downstream of the PPT, including the four C's (Fig. 2A). To determine whether this structure is involved in the formation of the 75-nt pause product, we made mutant RNA templates in which (i) the four C's were changed to a random sequence (UGUA), (ii) the six G's in the PPT were changed to a random sequence (UCAUAC), and (iii) the positions of the four C's and six G's were switched (Fig. 2A). The mutations which change either the four C's or six G's destroy the predicted secondary structure (Fig. 2A) and would be expected to reduce or eliminate pausing near the PPT. In contrast, the compensatory mutation in which the four C's and six G's are exchanged is predicted to form an alternate stem-loop structure (Fig. 2A); in this case, pausing would be expected to occur at the position of the six G's.

To test these predictions, mutant and wild-type RNA templates were incubated in primer extension reaction mixtures (Fig. 1A). The portion of the gel containing DNA products terminating near the PPT is shown alongside a sequencing ladder corresponding to the template used in the reaction (Fig. 2B). As already shown (Fig. 1C) (35), the pause product obtained with the wild-type template was the 75-nt DNA (note the migration of this product to the position immediately downstream of the first C in the run of four C's). By contrast,



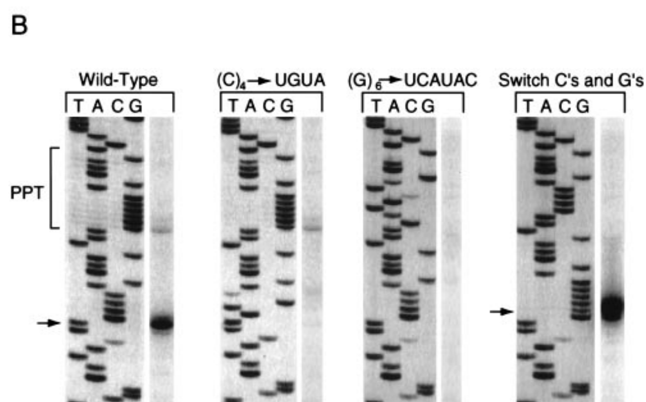


FIG. 2. Effect of secondary structure in the PPT region of the AKR MuLV template RNA on formation of the 75-nt pause product. (A) Computer predictions of secondary structure. The FoldRNA (Genetics Computer Group) program was used to predict RNA secondary structure. The wild-type RNA sequence in the vicinity of the PPT is indicated at the top; the six G's in the PPT and the four downstream C's are underlined. The 24-nt stable stem-loop which can be formed by the wild-type sequence in this region (nt 7817 to 7840 [39]) and the free-energy value of the putative structure are shown. The predictions for RNAs with mutations which change the four C's or six G's to a random sequence or which exchange the positions of the G's and C's are also shown. (B) Gel analysis of reactions with wild-type or mutant RNA templates. Reactions were carried out and analyzed as described in Materials and Methods. DNA products synthesized from RNA sequences in the vicinity of the PPT and sequencing ladders for each template are shown. The arrows beside the gels point to the positions of the pause products. The position of the PPT in the wild-type template is indicated on the left.

pausing was essentially eliminated in reaction mixtures containing templates with the substitution mutations in either the four C's or six G's. However, with the compensatory switch mutant, significant pausing occurred at the site of the six G's. These findings are in complete agreement with the predicted results and indicate that secondary structure in the RNA template near the PPT is responsible for the formation of the 75-nt pause product.

HIV-1 NC protein reduces formation of the 75-nt pause product. One way in which the virus could overcome the potential problem caused by RT pausing near the PPT would be to exploit the ability of the NC protein to destabilize RNA secondary structure (40, 46, 79); in other words, NC would

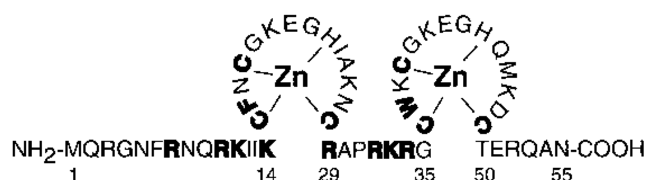


FIG. 3. Amino acid sequence of HIV-1 NC. The total number of residues is 55. There are two zinc fingers (residues 15 to 28 and 36 to 49), each having the highly conserved Cys-His motif C-X_2-C-X_4-H-X_4-C (32 [and references therein], 36). Residues in bold type were changed to alanine (see Table 1).

function as an accessory protein during reverse transcription. To investigate this possibility, we tested the activity of HIV-1 NC, a 55-amino-acid basic protein (Fig. 3), in our primer extension assay (Fig. 4).

Figure 4A displays the DNA products synthesized in reaction mixtures containing doses of HIV-1 NC protein ranging from 0.5 μM (~9.4 nt per NC molecule) to 4 μM (~1 nt per NC molecule). Radioactivity in the gel was quantified with a PhosphorImager (see Materials and Methods); the relative amounts of the 75- and 321-nt (full-length) DNAs were plotted as percentages of the total DNA products (Fig. 4B) to normalize the data. The results show that as the dose of NC was increased, the relative amount of the 75-nt DNA was reduced by ~10-fold; the relative amount of full-length DNA reached a level of over 80% with the highest dose of NC. Density values obtained by PhosphorImager analysis indicated a close correlation between the relative and actual amounts of the 75-nt pause product. Thus, compared with reactions without NC, the reduction in the actual amount of the 75-nt DNA was about sevenfold at 4 μM NC (data not shown). The maximum increase in the actual amount of the 321-nt DNA (twofold at 4 μM NC) was accompanied by a corresponding increase (1.5-fold) in the amount of total radioactivity (data not shown).

The experiment shown in Fig. 4A was performed with reaction mixtures incubated for 10 min. It was also of interest to determine how the effect of NC is manifested over time (Fig. 4C and D). To examine this question, we incubated reaction mixtures over a 60-min period without NC and with two different doses of NC protein: 1 μM (~4.7 nt per NC molecule) and 2.5 μM (~2 nt per NC molecule). The 1 μM dose was chosen because at this concentration, the ratio of nucleotides of RNA template to NC molecules is close to the ratio of seven nt per NC molecule, which corresponds to the size of the binding site in vitro (21, 44, 79) and is compatible with the estimated nucleotide-binding sites and NC protein molecules in the retrovirus particle (36). In addition, at the 1 μM concentration, there was a demonstrable effect on RT pausing in the dose-response experiment (Fig. 4A and B).

At each time point, addition of NC reduced the relative amount of the 75-nt DNA (Fig. 4C) and increased the relative amount of full-length DNA (Fig. 4D), compared with the control without NC. In addition, reduction of the amount of 75-nt DNA and stimulation of full-length DNA synthesis occurred at faster rates with NC and in a dose-dependent manner. The greatest effect was achieved with an NC dose of 2.5 μM.

The data presented in Fig. 4 are representative of results from multiple experiments. We also performed dose-response and time course experiments with reaction mixtures containing NC and lower concentrations of RT (e.g., ratios of RT to primer-template of 1:1 and 1:10) and obtained results (data not

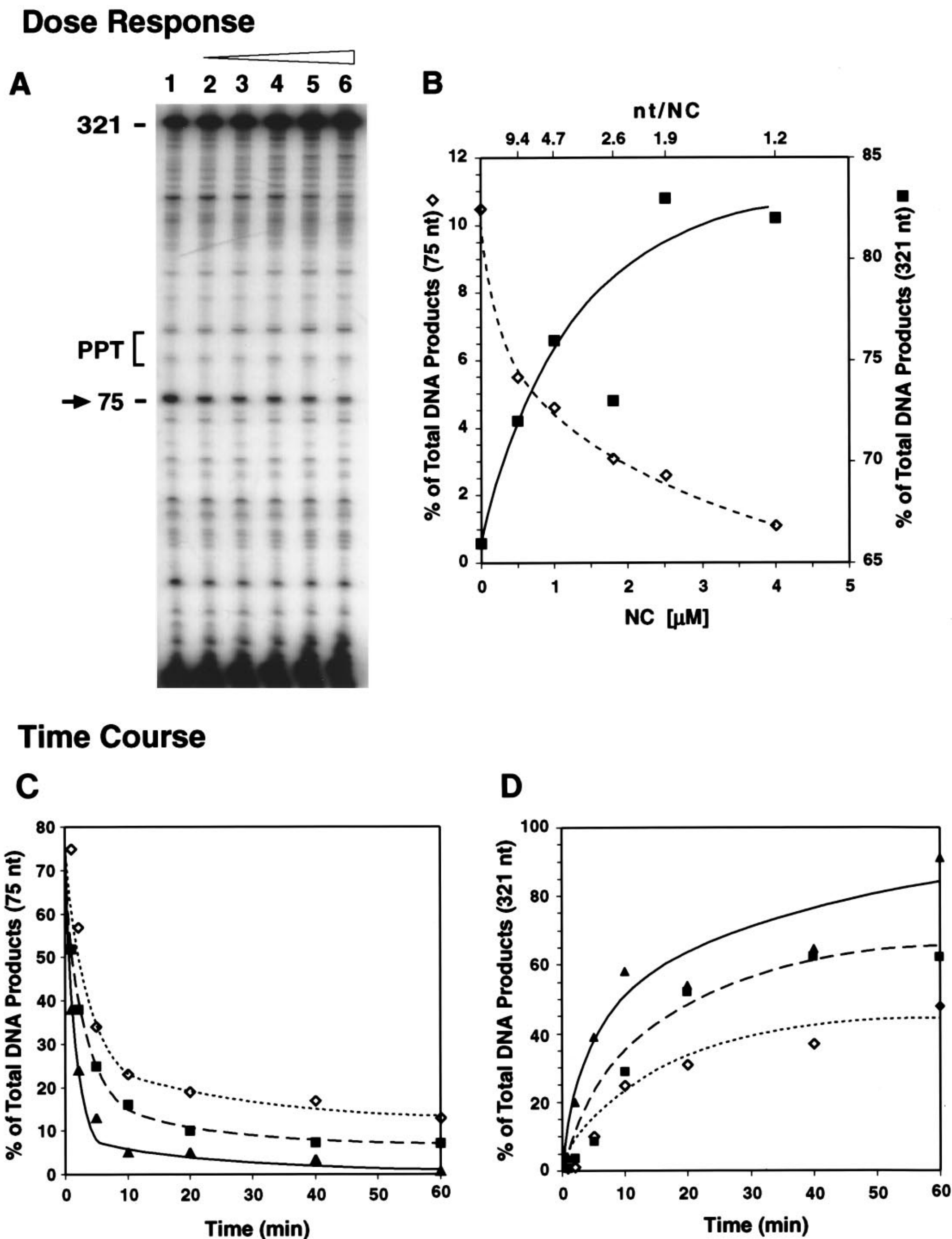


FIG. 4. Effect of HIV-1 NC on the formation of the 75-nt pause product and synthesis of 321-nt full-length DNA. (A and B) Dose response. Reaction mixtures were incubated with increasing amounts of NC for 10 min and analyzed as described in Materials and Methods. (A) Gel analysis. The concentrations of NC were as follows: lane 1, 0 μM; lane 2, 0.5 μM (9.4 nt per NC molecule); lane 3, 1 μM (4.7 nt per NC molecule); lane 4, 1.8 μM (2.6 nt per NC molecule); lane 5, 2.5 μM (1.9 nt per NC molecule); lane 6, 4 μM (1.2 nt per NC molecule). The positions of the 321-nt full-length product, the PPT, and the 75-nt pause product (arrow) are indicated on the left. Note that after 10 min of incubation with increasing concentrations of NC, some of the minor DNA products (>75 nt) were somewhat increased; however, upon further incubation (up to 60 min), the amounts of these DNAs were reduced (data not shown). (B) Quantitative PhosphorImager analysis of gel data. The percentage of total DNA products represented in the 321-nt (closed square, solid line) and 75-nt (open diamond, dashed line) DNA products is plotted against the NC concentration expressed in micromolar units or as the ratio of nucleotides to NC molecules. (C and D) Time course. Five-microliter aliquots from each reaction mixture were removed at each time point and processed and analyzed as described in Materials and Methods. The percentages of total DNA products present in the 75-nt (A) and 321-nt (B) DNAs were plotted against the time of incubation. Symbols: open diamond and stippled line, no NC; closed square and dashed line, 1 μM NC; closed triangle and solid line, 2.5 μM NC.

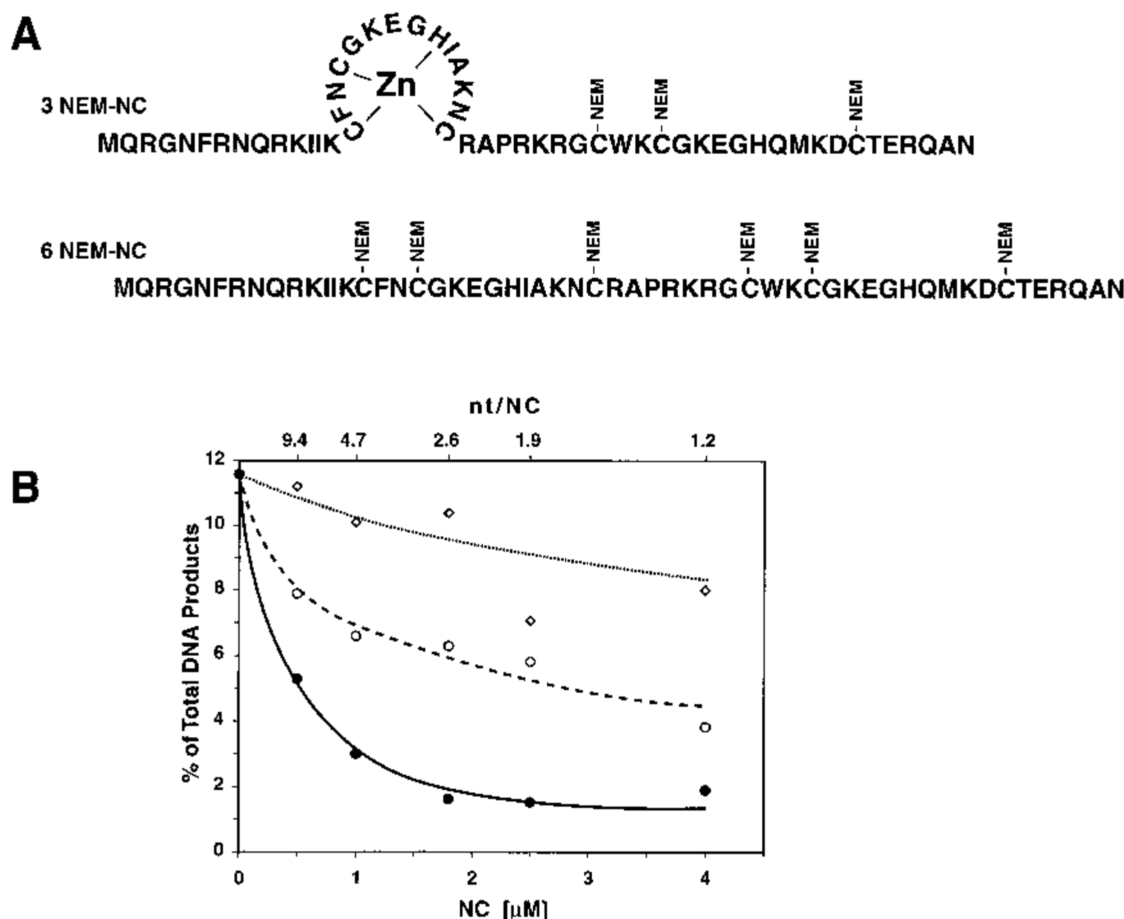


FIG. 5. Effect of HIV-1 NC chemically modified with NEM on formation of the 75-nt pause product. (A) Schematic diagram showing three or six molecules of NEM bound to one of NC. Note that binding of three NEM molecules is exclusively to the cysteines in the second finger. (B) Analysis of reaction mixtures containing 3NEM-NC or 6NEM-NC as described in Materials and Methods. The percentage of total DNA products present in the 75-nt pause product was plotted against the NC concentration, expressed as micromolar units or as the ratio of nucleotides to NC molecules. Symbols: closed circle and solid line, untreated NC; open circle and dashed line, 3NEM-NC; open diamond and stippled line, 6NEM-NC.

shown) very similar to those illustrated in Fig. 4. Taken together, these findings demonstrate that NC promotes more-efficient DNA synthesis by reducing RT pausing, presumably by destabilizing secondary structure in the template RNA.

Effect of NEM on NC function. HIV-1 NC has two zinc fingers (Fig. 3), and both are required for virus replication (22, 32, 34). It was therefore of interest to determine whether the zinc fingers contribute to the ability of NC to reduce pausing. The first approach we considered was to use a drug capable of disrupting the zinc finger structure. Recently, it has been shown that compounds which oxidize the sulfur atoms in the zinc finger can inactivate HIV-1 (65) or MuLV (64) virions and dramatically inhibit virus replication. These compounds would also inactivate RT in an in vitro assay, since the presence of a reducing agent like dithiothreitol is required for activity (52, 76). To solve this problem, we tested the activity of HIV-1 NC proteins which were modified chemically with either 3 or 6 equivalents of NEM and then purified by HPLC to remove any residual free NEM (data not shown). These modified NCs were designated 3NEM-NC and 6NEM-NC, respectively. Sequence analysis of the purified NCs showed that in the 3NEM-NC protein, all of the NEM was bound exclusively to the three cysteine residues in the second finger; in the 6NEM-NC protein, NEM was bound to all six cysteine residues (data not shown; see schematic diagram in Fig. 5A).

Figure 5B shows dose-response curves for the reduction of the 75-nt pause product in the primer extension assay. The data show that at the highest concentration of 3NEM-NC which was tested, the relative amount of the 75-nt DNA was reduced about threefold; at the corresponding dose, reduction of the 75-nt DNA by wild-type NC was about eightfold. In contrast, 6NEM-NC had little or no activity at low concentrations of NC; at the highest dose of protein, the relative amount of 75-nt DNA was decreased only 1.5-fold. These results indicate that HIV-1 NC, having a normal N-terminal zinc finger and an inactive second finger, could still reduce pausing near the PPT, although not as efficiently as wild-type NC; inactivation of both zinc fingers had a more severe effect on NC activity.

Interestingly, when binding of NC to ^{32}P-labeled template RNA was measured as a function of the NC dose in a filter binding assay, the results showed that 3NEM-NC had impaired binding activity compared with wild-type NC (data not shown). At high concentrations, 6NEM-NC had minimal binding activity, but at low concentrations, no binding could be detected (data not shown). These results suggest that the inhibitory

TABLE 1. Summary of activities of mutant and chemically modified NC proteins in a primer extension assay

NC protein	Ability to reduce pausing
Wild type	++++[a]
8(R, K)→A	−
6(C)→A	++
F16A	++
W37A	++
F16A/W37A	++
Δfinger 1[b]	++
Δfinger 2[c]	++
1:1[d]	+++
2:2[e]	+++
2:1[f]	+++
3NEM-NC	++
6NEM-NC	+

[a] Reduction in relative amount of 75-nt pause product compared with control without NC: ++++, 8- to 10-fold; +++, 5- to 8-fold; ++, 2- to 4-fold; +, 1.5-fold; −, not detectable. Assignment of activity values is based on results of multiple experiments.
[b] Residues 15 to 28 replaced by GG (see Fig. 3).
[c] Residues 36 to 49 replaced by GG (see Fig. 3).
[d] Residues 36 to 49 replaced by residues 15 to 28 (32).
[e] Residues 15 to 28 replaced by residues 36 to 49 (32).
[f] Residues 15 to 28 and 36 to 49 exchanged (32).

effect of NEM-induced inactivation of the zinc fingers on reduction of pausing may be due, at least in part, to a reduction in the ability to bind template RNA.

Effect of mutations in HIV-1 NC residues on the ability to reduce pausing. Modification of NC with NEM, especially in the case of the 6NEM-NC protein, could lead to a distortion of the normal conformation of NC due to steric hindrance. It was therefore of interest to carry out a more detailed analysis of NC function in our system with NC mutant proteins having less drastic changes. A summary of the mutant NC data is given in Table 1. The NC residues which were changed are shown in bold type in Fig. 3. Our initial results indicated that the eight basic (arginine and lysine) residues flanking the first zinc finger are essential, since changing these residues to alanine completely eliminated the ability of NC to reduce pausing (Fig. 6A).

To evaluate the relative importance of each zinc finger, we tested NCs having the following changes: (i) two N-terminal fingers (1:1), (ii) two C-terminal fingers (2:2), and (iii) the two zinc fingers in reversed order, i.e., the second finger sequence in the N-terminal position and the first finger sequence in the C-terminal position (2:1) (32). As shown in Fig. 6B, these changes had only a minimal effect on the ability of NC to reduce pausing; moreover, no significant differences could be detected in the activities of the mutants. Incubation at shorter times, i.e., for 1 to 5 min, gave essentially the same results (data not shown).

Figure 6C shows that when either of the zinc fingers was replaced with two glycine residues, pausing was reduced, but only ~2.5-fold. (In this experiment, wild-type NC reduced the relative amount of the 75-nt DNA ~11-fold.) Interestingly, the same results were obtained regardless of which zinc finger was deleted. As shown above with 3NEM-NC (Fig. 5B), these data demonstrate that removal of only one zinc finger allows partial expression of NC function. Moreover, in accord with the data of Fig. 6B, these findings also demonstrate that the two zinc fingers have essentially equivalent activity in our assay.

It is clear from these results that the presence of both zinc fingers is required for maximal NC activity in our assay. To determine whether both zinc finger structures are absolutely required for this effect, we tested an NC mutant in which the six cysteine residues were changed to alanine. Although this mutation presumably eliminates zinc ion coordination, NC was not completely inactivated. However, the mutant NC reduced pausing by ~2-fold, whereas reduction of pausing by the wild-type protein was ~10-fold (Fig. 6D). (In some experiments, the difference between mutant and wild-type activities was slightly less, i.e., about fourfold.) Results obtained with reaction mixtures containing NC protein treated with aldrithiol, a disulfide cross-linking reagent which oxidizes the cysteine residues and eliminates zinc (64, 65), were consistent with the findings obtained with the mutant having the cysteines replaced with alanine (data not shown). Taken together, these results imply that the zinc finger structures and the ability to coordinate zinc enhance NC ability to reduce pausing but are not absolutely required for this activity.

HIV-1 NC contains an aromatic residue after the first cysteine in each zinc finger (Fig. 3). To determine whether these residues play any role in the NC activity being measured, we tested mutants in which either the phenylalanine or tryptophan in the first and second zinc fingers, respectively, or both residues were changed to alanine. In all cases, the NC mutants could reduce pausing, but about fourfold less than with the wild-type protein (Fig. 6E). These results show that the aromatic residues influence NC activity in our system, but the contribution of each of the residues is not additive.

DISCUSSION

In the present report, we describe the ability of the HIV-1 NC protein to reduce RT pausing near the PPT during minus-strand DNA synthesis on an MuLV RNA template. We also provide new information, not previously reported, on features of NC which are required for this function. Since genomic RNA is associated with RT and NC as a ribonucleoprotein complex in virions (11, 16, 26, 50), the system used here may be viewed as a model for DNA synthesis in a viral replication complex.

Computer analysis of template sequences predicts that a stable, 24-nt stem-loop structure can form through interaction of bases within the PPT, including the six G's and bases downstream of the PPT, which include a run of four C's (Fig. 2A). By using mutational analysis, we were able to demonstrate experimentally that the predicted secondary structure is responsible for formation of a prominent 75-nt pause product (Fig. 2B). In earlier work, we showed that in reactions containing a competitor [poly(rA)-oligo(dT)], the 75-nt DNA can represent up to 50% of the total DNA products (35); with prolonged incubation (up to 1 h), the amounts of the pause and full-length products remained constant, indicating that only a single cycle of polymerization occurs under these conditions (data not shown). Taken together, these results indicate that the presence of the 24-nt stem-loop structure in the template leads to dissociation of RT from the primer-template and, consequently, a reduction in processive DNA synthesis.

If significant pausing were to occur near the PPT during reverse transcription of the viral RNA genome, then the efficiency of minus-strand and plus-strand DNA synthesis, and ultimately the efficiency of virus replication itself, would be reduced. How does the virus overcome this potential problem? We considered the possibility that NC functions as an accessory protein during reverse transcription, on the basis of its physical association with RT and viral RNA in the virus particle (see above) and its biochemical properties.

NC is a member of a class of proteins known as RNA

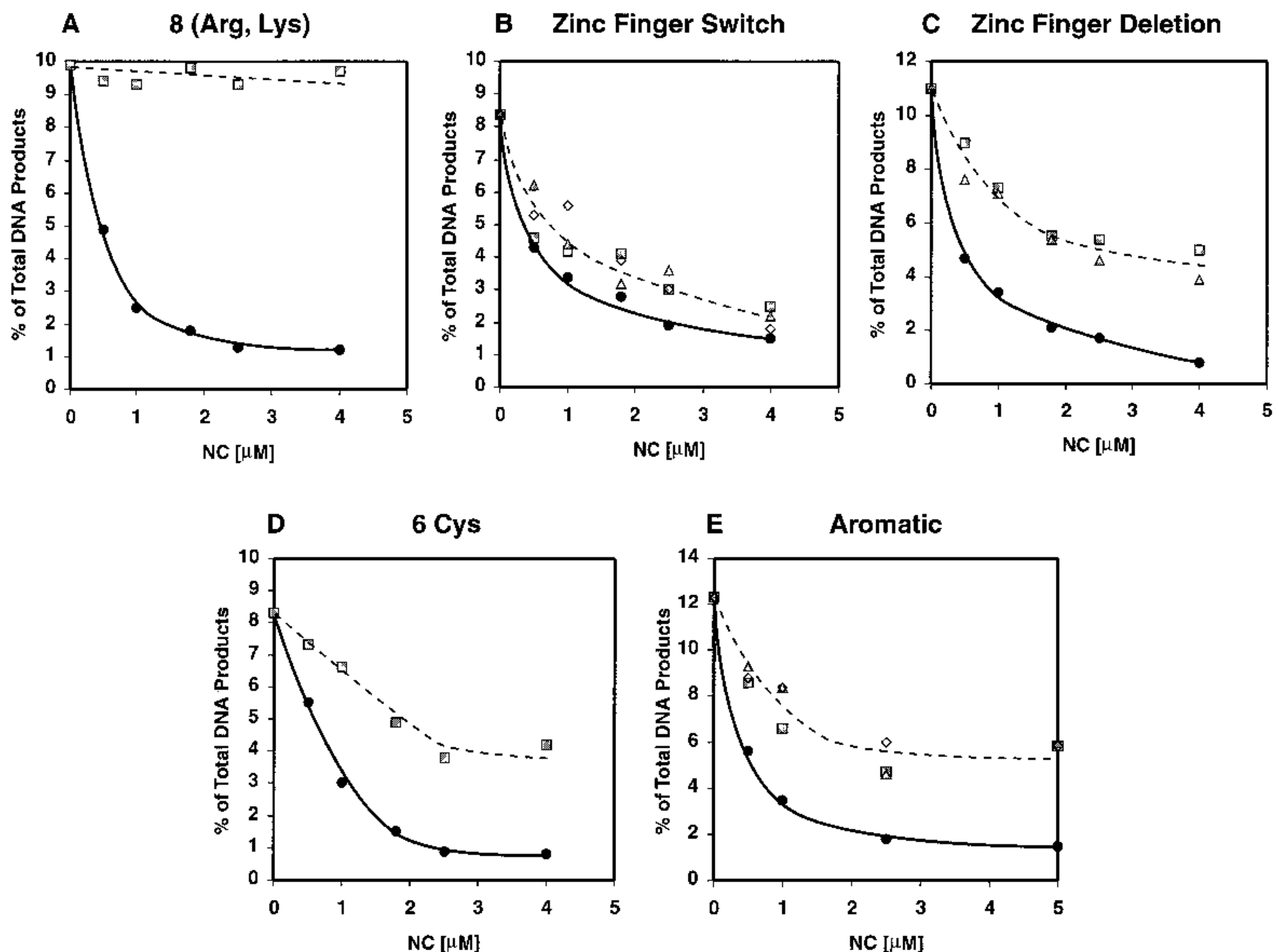


FIG. 6. Ability of HIV-1 mutant NC proteins to reduce formation of the 75-nt pause product. Reaction mixtures containing the wild-type and indicated mutant NC proteins were analyzed as described in Materials and Methods. The percentage of total radioactivity present in the 75-nt DNA is plotted against the NC concentration. The number of nucleotides per NC molecule for each of the NC concentrations is the same as that shown in Fig. 4B, except in the case of the aromatic mutants (E), where there is no point for 1.8 μM (2.6 nt per NC molecule) and the highest concentration is 5 μM (0.9 nt per NC molecule). In each panel, the wild-type points are represented by a solid line and closed circles; the mutant points are represented by a dashed line and the symbols described below. In panels B, C, and E, very similar data were obtained for each of the mutant sets; in these cases, a single dashed line was drawn. (A) 8 (Arg, Lys). The eight basic residues flanking the first zinc finger were changed to alanine (shaded square); (B) Zinc finger switch. The positions of the two fingers were exchanged (2:1, shaded square), the C-terminal finger was replaced with the N-terminal finger (1:1, shaded triangle), and the N-terminal finger was replaced with the C-terminal finger (2:2, open diamond). (C) Zinc finger deletion. The N-terminal (shaded triangle) or C-terminal (shaded square) finger was deleted and replaced with two glycine residues. (D) 6 Cys. All six cysteines were changed to alanine (shaded square). (E) Aromatic. The aromatic residues in the zinc fingers were changed to alanine. Symbols: shaded square, F16A; shaded triangle, W37A; open diamond, F16A/W37A.

chaperones (reviewed in reference 40): it binds to single-stranded nucleic acids (44) and catalyzes conformational changes which result in formation of the optimal, most thermodynamically stable structure (21, 25, 74, 79; reviewed in reference 40). Since NC has been shown to destabilize stem-loop structures (46, 74, 79), it might be expected to facilitate reduction of pausing near the PPT. Indeed, wild-type NC, acting in a dose-dependent manner, significantly reduced the amount of the 75-nt DNA, while also increasing the rate at which the pause product was reduced (Fig. 4A and C). In addition, increasing concentrations of NC stimulated the amount of full-length DNA detected in reaction mixtures, as well as overall primer extension (Fig. 4A, B, and D).

From these experiments, we concluded that NC increases the efficiency of viral DNA synthesis by allowing RT to traverse sites of secondary structure in viral RNA. A similar conclusion was reached by Ji et al. (43) in a report describing HIV-1 NC-stimulated reduction of pausing on HIV-1 templates, which appeared while this report was in preparation. Enhancement of retroviral DNA synthesis by HIV-1 NC proteins which contain some additional Gag sequences has also been reported (72, 77, 78).

While the chaperone activity of NC appears to be responsible, in large part, for the observed reduction in RT pausing, there could also be some interaction between NC and RT which contributes to an increase in the efficiency of DNA synthesis. It has been suggested (66) that NC stimulates synthesis of full-length DNA by promoting the rebinding of primer-template which has dissociated from the enzyme. This activity might involve an interaction with the RNase H domain, since it is known that the RNase H domain facilitates processive DNA synthesis by stabilizing the binding of RT to primer-template (35 [and references therein], 73). In studies on the first strand transfer event, Peliska et al. (60) noted that NC

affects the rate and specificity of RNase H cleavage. They postulated that in this system, interactions between NC and the RNase H domain may be important to reposition the RNA-DNA hybrid for 3′-OH-independent RNase H cleavage, i.e., cleavage in which the 3′-OH of the DNA is not bound to the polymerase active site (29, 30, 62). (This mode of cleavage is required for removal of 3′-terminal donor RNA bases (~14 to 18 nt) prior to strand transfer [12, 60, 61].)

In view of NC activity as a nucleic acid chaperone (40), it is not surprising that HIV-1 NC is active in a primer extension assay with an MuLV RNA template. Thus, we concluded that (i) the ability to reduce in vitro pausing by RT is not dependent on a homologous RNA-NC system and (ii) at least some of the interactions between NC and the RNA template are nonspecific. These conclusions are consistent with the ability of HIV-1 NC to induce stabilization of an in vitro-formed dimer of Harvey sarcoma virus RNA (25) and with in vitro NC activity with a wide variety of other viral and nonviral RNA and DNA sequences (7, 21, 41, 59, 74, 75, 79).

A major aspect of this study was concerned with identifying the elements within NC which are important for reduction of RT pausing. To put our findings in context, it is useful to first review what is known about the contribution of various NC residues to other NC functions. The importance of the basic amino acids for nucleic acid binding was demonstrated in assays which specifically measure binding or annealing (15, 18, 63, 67, 70). These residues are also required for in vitro stabilization of a Harvey sarcoma virus RNA dimer (25) and for infectivity (42).

Although both zinc fingers are required for HIV-1 infectivity (22, 32, 34), the molecular details of RNA binding and the role of the two zinc fingers are not understood. As a result, it is difficult to interpret the range of results obtained in various in vitro assays. For example, it has been suggested that NC may preferentially bind RNAs containing the packaging sequence (5, 13, 15). However, on the basis of studies of NC binding to such RNAs in vitro, one group found that an NC with either one of the zinc fingers has activity (5), while another reported that the more highly conserved first finger (32 and references therein) is required (15). On the other hand, neither the six cysteine residues nor the zinc finger structures per se are required for NC to induce in vitro maturation of an unstable viral RNA dimer (25). Deletion of both fingers also has no effect on NC-stimulated RNA dimerization in vitro or on the efficiency of the nucleic acid-annealing activities of NC (18, 51, 63; reviewed in reference 17). Moreover, the second zinc finger is completely dispensable for NC-enhanced catalytic activity of a hammerhead ribozyme, and deletion of the first zinc finger has only a minor effect on NC function in this assay (59).

What emerges from the mutational analysis of NC function in our primer extension assay is the importance of the conserved elements within the NC protein (Table 1). In the case of the basic residues flanking the first zinc finger, the requirement for activity is absolute, since changing these residues to alanine completely destroys NC activity (Fig. 6A). This observation reflects the critical role of the basic amino acids in nucleic acid binding.

The zinc finger structures and certain residues within the zinc fingers, which are highly conserved in NC proteins, also contribute to NC function. However, in this case, mutagenic changes reduce, rather than abolish, the ability of NC to decrease pausing near the PPT. The results obtained with the zinc finger deletion mutants (Fig. 6C) and with 3NEM-NC (Fig. 5B) show that NC can function with only one zinc finger, although not as efficiently as the wild-type protein. If the ability to coordinate zinc is completely eliminated, as in the mutant having the six cysteine residues replaced with alanine (Fig. 6D) or in 6NEM-NC (Fig. 5B), there is some activity, but it is considerably less than that of the wild type.

Taken together, our data demonstrate that the zinc finger structures and the ability to coordinate zinc are elements which enhance the activity of HIV-1 NC in our assay. On the basis of evidence that each of the zinc finger deletion mutants has the same level of activity (about threefold lower than that of the wild type) (Fig. 6C) and that the 1:1, 2:2, and 2:1 finger switch mutants (32) have essentially wild-type activity (Fig. 6B), it appears that the two zinc fingers have equivalent activity in our system. Furthermore, either finger can be in the first or second position.

In addition to the basic amino acids and the conserved zinc finger motif, all retroviral NC proteins have at least one zinc finger with an aromatic residue between the first and second cysteines (32 and references therein). The aromatic amino acids are required for infectivity (22, 23, 33, 55) and, in physical studies, have been shown to intercalate between the bases of the bound nucleic acid (48, 49, 69). Here, we found that mutation of phenylalanine in the N-terminal finger and/or tryptophan in the C-terminal finger to alanine led to partial loss of NC activity in our assay (Fig. 6E). (It is not clear why a change in both aromatic residues does not have an additive effect.)

Surprisingly, the activities of the aromatic NC mutants in the pausing assay are in sharp contrast to the effect of these same mutations on in vitro stabilization of a retroviral RNA dimer. In the assay for RNA maturation, the phenylalanine in the N-terminal finger is absolutely required for NC activity; however, when the tryptophan in the C-terminal finger is changed to alanine or the entire second finger is deleted, NC retains some activity (25). Thus, despite the fact that NC functions as an RNA chaperone in both assays, the requirements for activity are clearly not identical. This observation suggests that the two assays measure somewhat different properties of NC.

In summary, we have shown that HIV-1 NC increases the efficiency of viral DNA synthesis and can function as an accessory protein during reverse transcription of viral RNA. This activity of NC is completely dependent on the presence of the arginine and lysine residues which flank the first zinc finger. The N- and C-terminal zinc fingers and the cysteine and aromatic residues within the zinc fingers all contribute to this activity but are not absolutely essential. Presumably, the basic residues promote binding through electrostatic interactions with the phosphate backbone of the nucleic acid and the aromatic residues can contribute to the stability of the complex by intercalating between bases in the bound state. The zinc-coordinating residues are required for optimal conformational orientation of the protein (69). The observation that coordination of zinc is important for NC function during synthesis of minus-strand viral DNA is significant. One additional implication of this finding is that it provides a further rationale for anti-HIV therapy involving the use of drugs which inactivate the zinc fingers (64, 65).

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