Effect of Human Immunodeficiency Virus Type 1 (HIV-1) Nucleocapsid Protein on HIV-1 Reverse Transcriptase Activity in Vitro †

ARTICLE *in* BIOCHEMISTRY · FEBRUARY 1996

Impact Factor: 3.02 · DOI: 10.1021/bi951707e · Source: PubMed

3 AUTHORS, INCLUDING:

George Klarmann
The Geneva Foundation
24 PUBLICATIONS **1,420** CITATIONS

SEE PROFILE

Effect of Human Immunodeficiency Virus Type 1 (HIV-1) Nucleocapsid Protein on HIV-1 Reverse Transcriptase Activity *in Vitro*†

Xiaodong Ji,‡ George J. Klarmann,‡ and Bradley D. Preston*

Departments of Biochemistry and Radiation Oncology, Eccles Institute of Human Genetics, University of Utah, Salt Lake City, Utah 84112, and Laboratory for Cancer Research, Rutgers University, Piscataway, New Jersey 08854

Received July 24, 1995; Revised Manuscript Received October 20, 1995⊗

ABSTRACT: Conversion of human immunodeficiency virus type 1 (HIV-1) genomic RNA to viral DNA is a requisite step in the virus life cycle. This conversion is catalyzed by reverse transcriptase (RT) associated with a large nucleoprotein complex composed of several viral proteins including nucleocapsid (NC). To better characterize the biochemical mechanisms of viral DNA synthesis, we overexpressed and purified recombinant HIV-1 NC and studied its effect on the activity and processivity of HIV-1 RT during polymerization of HIV-1 template sequences *in vitro*. The effect of NC on steady-state RT activity was dependent on the order of addition of reaction components. Addition of NC prior to formation of RT−primer·template−dNTP ternary complexes inhibited primer extension and reduced total product yields by slowing steady-state RT turnover. In contrast, addition of NC to preformed ternary complexes resulted in efficient primer extension and increased RT processivity at specific DNA template sites. NC stimulated polymerization (2−4 times) through eight of 13 sites examined in the cRRE region of HIV-1 *env* and increased the rate of polymerization through the D3/CTS region of HIV-1 *pol* 10 times. The data suggest that NC affects RT processivity by facilitating polymerization through regions of template secondary structure. Thus, NC functions as a single-strand binding (SSB)-like accessory replication factor for RT *in vitro* and may be part of a multicomponent retroviral replication complex.

Retroviral replication requires the conversion of single-stranded viral RNA to double-stranded DNA (Coffin, 1990). This conversion involves a series of complex DNA polymerization steps on both RNA and DNA templates and is thought to occur within a cytoplasmic nucleoprotein complex derived, at least in part, from the virion core. Reverse transcriptase (RT)[1] and integrase (IN) are required for the synthesis and subsequent chromosomal integration of viral DNA (Katz & Skalka, 1994), and these proteins are found associated with viral RNA/DNA preintegration complexes isolated from the cytoplasm of infected cells (Bowerman et al., 1989; Bukrinsky et al., 1993; Farnet & Haseltine, 1991; Lee & Coffin, 1991). In addition, other viral proteins are bound to viral preintegration complexes, including matrix (MA), nucleocapsid (NC), capsid (CA), p6, and Vpr (Bowerman et al., 1989; Bukrinsky et al., 1993; M. Stevenson and M. Bukrinsky, personal communication). Thus, the conversion of viral RNA to DNA likely occurs in association with catalytic (RT and IN) as well as other (MA, NC, CA, p6, and Vpr) viral proteins.

Studies in cell-free systems indicate that retroviral DNA synthesis is biochemically complex. The synthesis of full-length viral DNA *in vitro* has been achieved only in permeabilized virions (i.e., "endogenous" RT reactions; Borroto-Esoda & Boone, 1991, and references therein) or starting with partially purified ribonucleoprotein complexes prepared from virions (Chen et al., 1980). However, these cell-free systems are very sensitive to small changes in reaction conditions (particularly ionic strength and detergent concentration; Borroto-Esoda & Boone, 1991) and result in low yields of full-length viral DNA, suggesting that the viral replication machinery is labile and/or requires additional replication factors. Studies with purified RTs *in vitro* show that both RNA and DNA templates are copied with low processivity (∼10−300 nucleotides; Klarmann et al., 1993, and references therein) at fidelities significantly lower than those observed in infected cells in culture (Varela-Echavarría et al., 1992) and with very low efficiency (Collett & Faras, 1977). This failure to reconstitute viral DNA synthesis using purified RT alone also suggests that additional replication factors and/or unique structural elements are required for efficient viral DNA synthesis *in vivo*.

Several studies have shown that both viral and cellular proteins stimulate the overall activity of purified retroviral RTs *in vitro*. CA from murine leukemia virus (Bandyopadhyay, 1977; Bandyopadhyay & Levy, 1978), NC from avian myeloblastosis virus (Sykora & Moelling, 1981), and the p15 NC precursor from HIV-1 (Volkmann et al., 1993) all increase the activities of their cognate RTs in conventional

† This work was supported by grants from the National Institutes of Health (R29 CA48174, RO1 AI34834, P30 ES05022, and P30 CA42014) and the Leukemia Research Foundation. B.D.P. was a Henry Rutgers Fellow and recipient of an American Cancer Society Junior Faculty Research Award (JFRA-245).

* To whom correspondence should be addressed at the present address: Program in Human Molecular Biology and Genetics, Eccles Institute of Human Genetics, University of Utah, Salt Lake City, UT 84112. Tel.: (801) 585-6342. FAX: (801) 585-3501. E-mail: bpreston@corona.med.utah.edu.

‡ These authors contributed equally to this paper.

⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1995.

[1] Abbreviations: bp, base pairs; CA, capsid protein; cPPT, central polypurine tract; cRRE, complement of the Rev response element; CTS, central termination sequence; DTT, dithiothreitol; HIV-1, human immunodeficiency virus type 1; IPTG, isopropyl 1-thio-β-D-galactopyranoside; IN, integrase; MA, matrix protein; NC, nucleocapsid protein; NC_{55}, fully mature HIV-1 NC, 55 amino acids in length; *n*-mer, synthetic DNA oligomer, "*n*" nucleotides in length; nt, nucleotide; PEI, polyethylenimine; PCR, polymerase chain reaction; RT, reverse transcriptase; SSB, single-strand binding protein.

0006-2960/96/0435-132$12.00/0

DNA polymerization assays *in vitro*. Unidentified viral and cellular proteins (Das et al., 1976; Hung & Lee, 1976; Lee & Hung, 1977; Leis & Hurwitz, 1972; Padhy et al., 1976) as well as cellular topoisomerase I (Takahashi et al., 1995) have also been shown to stimulate RT activities *in vitro*. Although the mechanisms of these stimulatory effects are not known, these studies show that retroviral RTs, like other replicative DNA polymerases (Kornberg & Baker, 1992), respond to and probably function in coordination with accessory replication factors during DNA synthesis.

NC is an abundant viral core protein (~2,000−3,000 molecules per virion; Henderson et al., 1992) that is processed from Gag precursor peptides in the virion prior to cellular infection and initiation of reverse transcription. NC binds to and coats the genome in free virus particles (Chen et al., 1980) and is associated with viral DNA preintegration complexes in infected cells (M. Stevenson and M. Bukrinsky, personal communication). Studies *in vitro* show that NC proteins bind nucleic acids with high affinity, low to moderate cooperativity, defined stoichiometry, no strong sequence specificity, and with a preference for single strands (Karpel et al., 1987; Khan & Giedroc, 1992; Surovoy et al., 1993; You & McHenry, 1993, and references therein). Moreover, retroviral NCs facilitate denaturation and renaturation of nucleic acids (Allain et al., 1994; Dib-Hajj et al., 1993; Khan & Giedroc, 1992; Peliska et al., 1994; Rodríguez-Rodríguez et al., 1995; Tsuchihashi & Brown, 1994; You & McHenry, 1994). The abundance and location of NC *in vivo* and its properties *in vitro* implicate this protein as a factor affecting viral DNA synthesis. A role for NC in viral DNA synthesis is also indicated from the phenotypes of viruses mutated in NC and from studies using specific inhibitors in culture (Gorelick et al., 1988, 1993; Méric & Goff, 1989; Nagy et al., 1994; Rice et al., 1993).

To better understand the biochemical mechanisms of viral DNA synthesis, we purified recombinant human immunodeficiency virus type 1 (HIV-1) NC protein in its fully mature 55-amino acid form (designated here as NC_{55})[2] and studied its effects on the activity and processivity of HIV-1 RT *in vitro*. We show that the effect of NC_{55} on DNA synthesis is dependent on the order of addition of reaction components and that, when added to preformed RT−primer·template−dNTP ternary complexes, NC_{55} increases HIV-1 RT processivity by facilitating polymerization through regions of template secondary structure. These data, together with those of others, suggest that retroviral NCs may function as single-strand binding (SSB)-like accessory proteins during viral DNA synthesis.

MATERIALS AND METHODS

Materials. Recombinant HIV-1 RT (p66/p51 heterodimer) and HIV-1 [5′-^{32}P]primer·templates were prepared as described (Klarmann et al., 1993). pNL4-3 was kindly provided by Arnold Rabson (New Jersey Center for Advanced Biotechnology and Medicine, Piscataway, NJ).

[2] The fully mature 55-amino acid HIV-1 NC protein is derived from the $Pr55^{gag}$ protein via a series of proteolytic events and forms intermediate precursors that are ~130 amino acids (p15 protein) and 71−72 amino acids in length (Henderson et al., 1988, 1992; Veronese et al., 1987). The smaller peptides (55, 71, and 72 amino acids) have all been called p7. To distinguish among these, we refer to the fully mature 55-amino acid form of NCp7 as NC_{55} and its precursor peptides as NC_{71} and NC_{72} (subscripts indicate amino acid lengths).

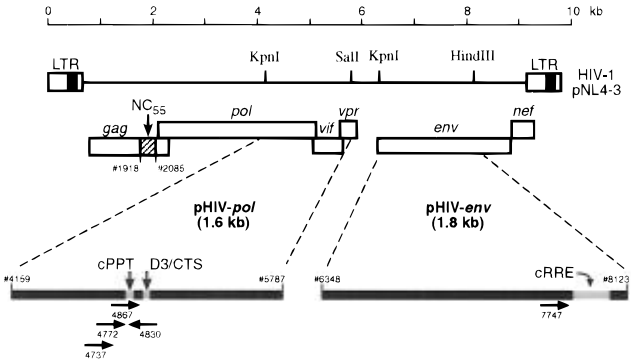

FIGURE 1: Schematic of the HIV-1_{NL4-3} proviral genome. The locations of the NC_{55} coding sequence (nucleotides 1918−2085; striped box) and the *Kpn*I/*Sal*I (nucleotides 4159−5187) and *Kpn*I/*Hin*dIII (nucleotides 6348−8123) fragments used to obtain pHIV-*pol* and pHIV-*env* are indicated. HIV-1 plus-strand RNA and minus-strand DNA templates were generated from these subclones as previously described (Klarmann et al., 1993). The DNA oligonucleotide primers used in this study (numbered according to the position of the 3′-terminal nucleotide in HIV-1_{NL4-3} proviral DNA; Klarmann et al., 1993) are indicated by horizontal lines (not to scale) with arrows positioned in approximate relation to the template elements cPPT, D3/CTS, and cRRE. Arrows pointing left and right indicate primers hybridizing to the plus-strand RNA and minus-strand DNA templates, respectively. For simplicity, the *tat*, *rev*, *tev*, and *vpu* HIV-1 genes are not shown.

pGEM-3zf(+) and pKK223-3 were obtained from Promega and Pharmacia LKB Biotechnology Inc., respectively. Ultrapure 2′-deoxyribonucleoside 5′-triphosphates (dNTPs) were from Pharmacia LKB Biotechnology Inc. All other reagents were of the highest available grade from Fisher Scientific or Gibco BRL.

HIV-1 RT active sites were determined by titrating RT (10 nM) with primer·template (1−100 nM) essentially as described (Kati et al., 1992) except a rapid quench apparatus was not used and burst amplitudes were measured in the presence of poly(rA)·oligo$(dT)_{12-18}$ (0.05 mg/mL) to restrict enzyme turnover to a single cycle (Klarmann et al., 1993). This analysis yielded a $K_{d(primer\cdot template)} = 14 \pm 2.6$ nM and showed that ~33% of the HIV-1 RT p66/p51 heterodimers in our preparation were catalytically active. Similar values are reported by others (Kati et al., 1992).

Overexpression and Purification of Recombinant HIV-1 NC_{55}. The NC_{55} coding sequence (nucleotides 1918−2085) from the infectious HIV-1_{NL4-3} clone (Adachi et al., 1986; Figure 1) was subcloned into the expression vector pET-3a (Novagen) using a polymerase chain reaction (PCR)-based strategy. PCR primers were designed to contain an *Nde*I site (underlined) and start codon (bold) in the 5′-primer (5′-CGCGGATCCCAT ***ATG*** *CAG AAA GGC AAT TTT AG*-3′; NC_{55} coding nucleotides shown in italics) and a *Bam*HI site (underlined) and stop anticodon (bold) in the 3′-primer (5′-GGAATTCGGATCC ***CTA*** *ATT AGC CTG TCT CTC AG*-3′). Noncoding nucleotides (shown in plain text) were included 5′ of each restriction site to ensure efficient *Nde*I and *Bam*HI cleavage of the resultant PCR product. The methionine coded by the start codon corresponds to the methionine found naturally at the amino-terminus of viral NC_{55} *in vivo* (Adachi et al., 1986; Henderson et al., 1992). This PCR strategy generates a 190-bp product with an open reading frame encoding a 55-amino acid protein identical to that predicted for NC_{55} from HIV-1_{NL4-3} (H_2N-MQKGNFRNQRKTVKCFNCGKEGHIAKNCRAPRKK-

GCWKCGKEGHQMKDCTERQAN-COOH; Adachi et al., 1986; Henderson et al., 1992).

PCR reactions (100 μL total volume containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 50 ng of pNL4-3, 2.5 μM of each PCR primer, 200 μM of each dNTP, and 2.5 units of *Taq* DNA polymerase) were overlaid with 50 μL light mineral oil and subjected to 30 thermal cycles consisting of three steps: denaturation at 94 °C for 1.5 min, primer annealing at 55 °C for 2 min, and primer extension at 72 °C for 3 min. Reactions were incubated at 72 °C for 15 min after completion of the last cycle. Negative control DNAs were amplified in parallel to ensure PCR specificity.

The 190-bp PCR product was purified by gel electrophoresis in 2% low-melting agarose and then digested with *Nde*I and *Bam*HI and ligated into the corresponding sites downstream of the T7 promoter in the expression vector pET-3a (Studier et al., 1990) using standard procedures (Sambrook et al., 1989). To obtain stable recombinants, competent HB101 *Escherichia coli* were transformed with the ligated samples and ampicillin-resistant colonies were selected. Recombinant plasmids were isolated, and the complete NC_{55} coding region from several independent clones was sequenced to ensure the absence of PCR errors. A clone, designated pET3a-NC_{55} and containing the wild-type NC_{55} coding sequence, was selected for all subsequent manipulations and was maintained in HB101 *E. coli*.

For protein production, pET3a-NC_{55} was transferred to BL21(DE3)LysE *E. coli* (Studier et al., 1990). Single transformed colonies were inoculated into modified ZB medium (Studier et al., 1990) in the presence of ampicillin (100 μg/mL) and chloramphenicol (25 μg/mL) and grown for 10−12 h at 30 °C. The culture was then diluted 10-fold with M9ZB medium (Studier et al., 1990) and grown at 37 °C to an $OD_{600} \approx 0.5$. Protein expression was induced by addition of isopropyl 1-thio-β,D-galactopyranoside (IPTG; 1 mM final concentration). Cells were harvested by centrifugation after growing another 3 h at 37 °C, and cell paste was stored in ∼5 gram portions at −70 °C.

All subsequent steps of purification were conducted at 0−4 °C in the presence of $ZnCl_2$ and dithiothreitol (DTT) using degassed buffers (helium-sparged) in order to minimize protein oxidation and degradation. A 5-g amount of cell paste from ∼5 L of culture was thawed and resuspended in 40 mL of degassed buffer X (75 μM $ZnCl_2$, 50 mM Tris·HCl (pH 7.0), 50 mM NaCl, 10% glycerol, 5 mM DTT) containing proteinase inhibitors (35 μg of phenylmethylsulfonyl fluoride/mL, 0.3 mg of EDTA/mL, 0.5 μg of leupeptin A/mL, 0.7 μg of pepstatin A/mL). Resuspension and cell lysis were achieved by vigorous agitation on a vortex mixer for a total of 5 min in 1-min bursts with 2-min incubations on ice between bursts. The lysate was mixed with 0.3%−0.4% polyethylenimine [PEI; concentration optimized for each lysate as described by Burgess (1991)], incubated on ice for 5 min, and centrifuged two times for 15 min at 17 000*g*. The clear amber supernatant was loaded at 1.5 mL/min onto a 100-mL DEAE (Whatman DE52) column (15 mm × 60 cm) attached to a Waters model 650E chromatography system using degassed buffers continuously sparged with helium. The column was washed with 360 mL of buffer X. The flow-through fractions containing NC_{55} were pooled and applied directly onto a 24-mL heparin-Sepharose CL-6B column (10 mm × 30 cm; Pharmacia LKB Biotechnology Inc.) at a flow rate of 0.15 mL of buffer X/min. Elution of NC_{55} was achieved with a 90-mL linear gradient of 0.05−1.0 M NaCl in buffer X. Fractions containing the 6.4-kDa NC_{55} protein [eluting at ∼0.7 M NaCl as detected by SDS−polyacrylamide gel electrophoresis (PAGE; Figure 2)] were pooled and dialyzed against 20 L of buffer A (75 μM $ZnCl_2$, 50 mM Tris·HCl (pH 7.0), 50 mM NaCl, 10% glycerol, 1 mM DTT, 50 mM EDTA). The dialyzed material was further purified by chromatography through a 1-mL MonoS column (5 mm × 5 cm; Pharmacia LKB Biotechnology Inc.) at 0.5 mL/min flow rate. The column was washed with 10 mL of buffer X, and the bound proteins were eluted with a 40-mL linear gradient of 0.05−1.0 M NaCl in buffer X. The NC_{55} eluted at about 0.9 M NaCl. For some preparations the MonoS column step was repeated to further increase purity. Peak fractions were pooled, divided into multiple aliquots, and stored at −70 °C until further use.

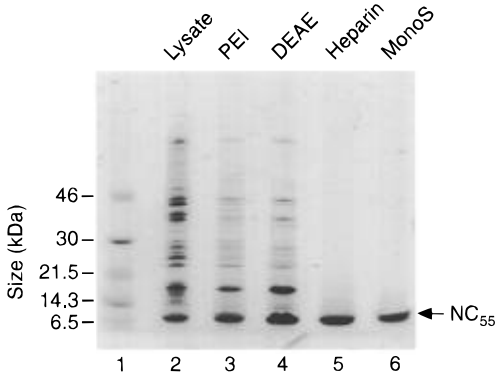

FIGURE 2: Overexpression and purification of recombinant HIV-1 NC_{55}. The 6.4-kDa NC_{55} was purified from soluble lysates of IPTG-induced BL21(DE3)LysE *E. coli* harboring pET3a-NC_{55} by sequential steps of PEI fractionation followed by DEAE, heparin-Sepharose, and MonoS chromatography as described in the text. Aliquots from appropriate fractions were removed after each step (lanes 2−6) and analyzed by SDS 8%−25% polyacrylamide gradient Phastgel electrophoresis (Pharmacia LKB Biotechnology Inc.). Protein bands were detected by Coomassie Blue staining. The band corresponding to NC_{55} (as determined from partial amino acid sequencing) is indicated at the right. This band was absent in lysates from noninduced cells as well as from cells harboring the parental pET3a vector (data not shown). The lanes shown here were loaded with similar amounts of NC_{55} peptide and do not reflect absolute yields during purification. Lane 1, molecular weight markers with sizes indicated at the left.

To avoid degradation of NC activity (Dib-Hajj et al., 1993), all preparations were used within 3 months of purification and individual aliquots were frozen and thawed only once. Protein concentrations were determined using the Bradford protein assay (Bio-Rad) with bovine serum albumin as the standard.

DNA Polymerization in Vitro. Polymerization reactions were conducted essentially as described (Klarmann et al., 1993). Reactions (20−100 μL total volume) contained 3−54 nM [5′-^{32}P]primer·template (corresponding to 6−50 μM template nucleotides), 0−6 μM recombinant HIV-1 NC_{55}, 0−8 nM HIV-1 RT active sites, 100 μM each of dATP, dCTP, dGTP, and dTTP, 10 mM $MgCl_2$, 25 mM Tris·HCl (pH 8.0), 30 mM KCl, 2.5 mM NaCl, 2 mM DTT, and 4 μM $ZnCl_2$. Where indicated, RT−primer·template binary and RT−primer·template−dNTP ternary complexes were preformed prior to addition of NC_{55} as described under Results. Polymerizations were conducted at 37 °C for 0−15 min, and products were analyzed by denaturing 8 M urea−

8% PAGE, autoradiography, and densitometry essentially as described (Klarmann et al., 1993) but using a Sun SPARCstation IPC equipped with a Howtek Scanmaster 3+ flatbed scanner and Millipore Visage Electrophoresis Gel Analysis software for densitometric analyses.

Nucleic Acid Binding Assay. *Hin*dIII-linearized pHIV-*env* (Figure 1) was used as a template for T7 RNA polymerase-catalyzed transcription *in vitro* in the presence of [α-^{32}P]CTP (400 Ci/mmol) to produce the 1.78-kb HIV-1 *env* [^{32}P]RNA (specific activity = 3×10^7 cpm/μg). Transcription reactions were conducted according to the manufacturer's protocol (Promega).

To measure nucleic acid binding, 0−1 μM NC_{55} was incubated for 30 min at 37 °C with 0.2 nM [^{32}P]RNA (corresponding to 0.4 μM RNA nucleotides) in 20 μL of binding buffer [25 mM Tris·HCl (pH 8.0), 30 mM KCl, 10 mM $MgCl_2$, 2 mM DTT]. After incubation, each sample was immediately transferred into a well of a Filtration Manifold Apparatus (Schleicher & Schuell) and vacuum filtered through a nitrocellulose membrane filter (0.45 μ; Schleicher & Schuell) that was first prepared by wetting with deionized water, followed by soaking in 0.4 M KOH (15 min, 23 °C), rinsing in deionized water (five times), and soaking in binding buffer (30 min, 23 °C). After adsorption of sample, each well was slowly rinsed with 250 μL of binding buffer four times under gentle vacuum. The filter was dried and overlaid with X-ray film. After autoradiography, each well position on the filter was excised, placed in 5 mL of Econofluor, and counted using a liquid scintillation spectrometer.

Helix Destabilization Assay. Single-stranded DNA (4.8 kb; prepared from the phagemid pHIV-*pol*; Figure 1) and single-stranded RNAs [1.63 or 0.27 kb, prepared by *in vitro* transcription of *Sal*I-linearized pHIV-*pol* or *Pvu*II-linearized pGEM-3zf(+), respectively] were hybridized to synthetic [5′-^{32}P]DNA oligonucleotides (20- or 22-mers) as previously described (Klarmann et al., 1993). The resultant [5′-^{32}P]-DNA oligonucleotide·DNA and [5′-^{32}P]DNA oligonucleotide·RNA hybrids each consisted of a long stretch of single-stranded nucleic acid (0.27−4.8 kb) with a short duplex region (20−22 bp). These partial duplexes (6−16 μM nucleic acid nucleotides) were incubated (37 °C for 5 min) with 0−4 μM NC_{55} in 20 μL of 25 mM Tris·HCl (pH 8.0), 30 mM KCl, 10 mM $MgCl_2$, 2 mM DTT. Reactions were stopped by addition of loading dye (0.25% bromophenol blue, 0.25% xylene cyanole, 25% Ficoll) and immediately fractionated by native 5% or 12% PAGE. Substrate duplexes and product single strands were visualized by autoradiography and quantified by densitometry.

RESULTS

Recombinant HIV-1 NC_{55}. To obtain large amounts of HIV-1 NC for biochemical studies, we used a PCR-based strategy to subclone and overexpress the NC_{55} coding sequence from the infectious HIV-1 clone pNL4-3 (Figure 1). Initial attempts to express NC_{55} under control of the pKK223-3 *tac* promoter in JM105 *E. coli* or the pET3a T7 promoter in BL21(DE3)LysS *E. coli* resulted in unstable clones and low protein yields with or without IPTG induction. Both of these systems lack stringent promoter control (Sambrook et al., 1989; Studier et al., 1990), thus suggesting that NC_{55} expression affects the stability of *E. coli* transformants. To overcome this difficulty, the pET3a-NC_{55} plasmid was maintained as a stable clone in HB101 *E. coli*, which lacks T7 RNA polymerase and thus does not express the NC_{55} gene. Subsequent transformation of pET3a-NC_{55} into BL21(DE3)LysE *E. coli*, which tightly controls T7 promoter activity (Studier et al., 1990), resulted in stable transformants when maintained at low density ($OD_{600} \leq 0.5$) in the absence of IPTG. After induction with IPTG, NC_{55} became a major cellular protein comprising 10%−30% of total protein in crude lysates (Figure 2, lane 2). Greater than 90% of the induced NC_{55} was soluble in neutral aqueous buffer.

Most of the NC_{55} in crude lysates was bound to cellular nucleic acids, as evidenced by anomalous retention of the highly basic NC_{55} protein on anion exchange resins (data not shown). Therefore, nucleic acids were first removed from cellular lysates by precipitation with PEI, and the resultant free NC_{55} was purified by sequential chromatography through DEAE, heparin-Sepharose, and MonoS columns (Figure 2, lanes 2−6). All steps of purification were conducted under reducing conditions using degassed buffers to minimize protein oxidation, and the last chromatography step was conducted in the presence of excess $ZnCl_2$ and absence of EDTA to enrich for the zinc-bound form of NC_{55}. The final purified protein was >95% homogeneous (Figure 2, lane 6) and free of detectable DNase and RNase activity (data not shown).

Ten cycles of Edman degradation showed that the purified NC_{55} had an unmodified amino-terminal sequence (H_2N-MQKGNFRNQR). Thus, the terminal methionine residue is not cleaved in the *E. coli*. On the basis of these data, our cloning strategy, and the size and homogeneity of the purified protein, the amino acid sequence of the recombinant NC_{55} should precisely match that of fully mature NC_{55} in HIV-1_{NL4-3} virions (Adachi et al., 1986; Henderson et al., 1992). Nitrocellulose filter binding assays showed that the recombinant NC_{55} binds to single-stranded RNA and DNA *in vitro* (data not shown). The recombinant NC_{55} also exhibited helix destabilizing activity, as evidenced by the release of single-stranded [5′-^{32}P]DNA oligonucleotides upon incubation of NC_{55} with preformed [5′-^{32}P]DNA·RNA (Figure 3) and [5′-^{32}P]DNA·DNA (data not shown) partial duplexes *in vitro*. NC_{55} preferentially destabilized some duplexes over others, indicating that helix destabilization is dependent on the sequence of the nucleic acid substrate (data not shown).

Effect of HIV-1 NC_{55} on HIV-1 RT Activity. To determine the effect of HIV-1 NC_{55} on viral DNA synthesis, we examined DNA polymerization in a partially reconstituted *in vitro* system using HIV-1 *env* and *pol*/*vif*/*vpr* sequences as templates and synthetic DNA oligonucleotides as primers (Figure 1). These templates were chosen because they are relatively long and thus representative of the HIV-1 genome, because they are copied inefficiently by HIV-1 RT alone, and because they include regions that are likely to form higher order secondary structures that disrupt processive DNA synthesis *in vitro* (Klarmann et al., 1993). In this *in vitro* system, purified HIV-1 RT is used to catalyze DNA synthesis on preformed [5′-^{32}P]primer·templates, and polymerization products are analyzed by urea−PAGE. Reactions contain limiting RT and excess substrate (primer·template, Mg^{2+}, and dNTPs) and are conducted for short incubation times (≤30 min) so that DNA synthesis occurs in the steady state (Klarmann et al., 1993).

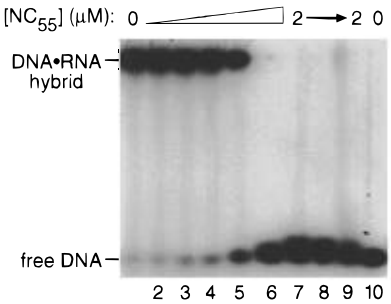

FIGURE 3: Helix destabilization by recombinant NC_{55}. Preformed [5′-^{32}P]DNA (20 nucleotides)•*pol* RNA (1630 nucleotides) hybrid (3.8 nM corresponding to 5.6 μM nucleotides) was incubated at 37 °C for 5 min with increasing concentrations of NC_{55} (0, 0.04, 0.1, 0.2, 0.4, 1, 2, or 2 μM NC_{55}; lanes 1−8, respectively), and products were analyzed by nondenaturing 5% PAGE and autoradiography to detect the release of free [5′-^{32}P]DNA from the original hybrid (indicated on the left). The position of hybridization of the DNA oligonucleotide (primer 4830; 5′-TCTGTTGCTATTATGTCTAC-3′) is shown in Figure 1. Lane 9: 2 μM NC_{55} was incubated with unhybridized [5′-^{32}P]DNA alone. Lane 10: unhybridized [5′-^{32}P]DNA in the absence of NC_{55}. The slight shift in electrophoretic mobility observed at high concentrations of NC_{55} presumably results from nonspecific binding of NC_{55} to the free DNA oligonucleotide (lanes 7−9).

Effect on Steady-State DNA Synthesis. As previously observed (Klarmann et al., 1993), steady-state DNA polymerization by HIV-1 RT alone resulted in the accumulation of multiple products at specific template regions (designated "pause sites" R1, R2, etc. and D1, D2, etc. on RNA and DNA templates, respectively; Figures 4 and 5). Addition of HIV-1 NC_{55} reduced the total yield of polymerization products on both RNA (Figure 4) and DNA (Figures 5 and 6) templates in a dose-dependent manner. Essentially 100% inhibition occurred at concentrations greater than one NC_{55} molecule per two template nucleotides (Figure 4B). However, with the exception of the D2 pause site (discussed below), NC_{55} had little effect on the overall distribution of polymerization products within the time frame of these reactions ($\leq$5 min; Figures 4A and 5). The inhibitory effect of NC_{55} could not be explained by primer•template helix destabilization, since inhibition occurred on several primer•templates regardless of their sensitivity to denaturation by NC_{55} (data not shown).

Time-course reactions using a single concentration of NC_{55} (one NC_{55} molecule per 15 template nucleotides, corresponding to ~50% coating of the template; You & McHenry, 1993) showed that NC_{55} slows the rate of steady-state DNA synthesis and confirmed that product distribution is minimally effected during the first 5 min of polymerization (Figure 5). When reactions containing NC_{55} were conducted for longer times, total product yields increased very slowly (data not shown). This contrasts with the normal pattern of steady-state polymerization by HIV-1 RT in the absence of NC_{55} which shows a relatively rapid linear increase in total extension products up to about 40 min (Klarmann et al., 1993; data not shown).

Effect of NC_{55}:RT Ratio and Addition Order. The effect of NC_{55} on the efficiency of steady-state DNA synthesis was influenced by the ratio and order of addition of reaction components. Inhibition of DNA synthesis by NC_{55} was dependent on NC_{55} concentration (Figure 4 and Figure 6A, lanes 1−5) and was reduced by addition of increasing

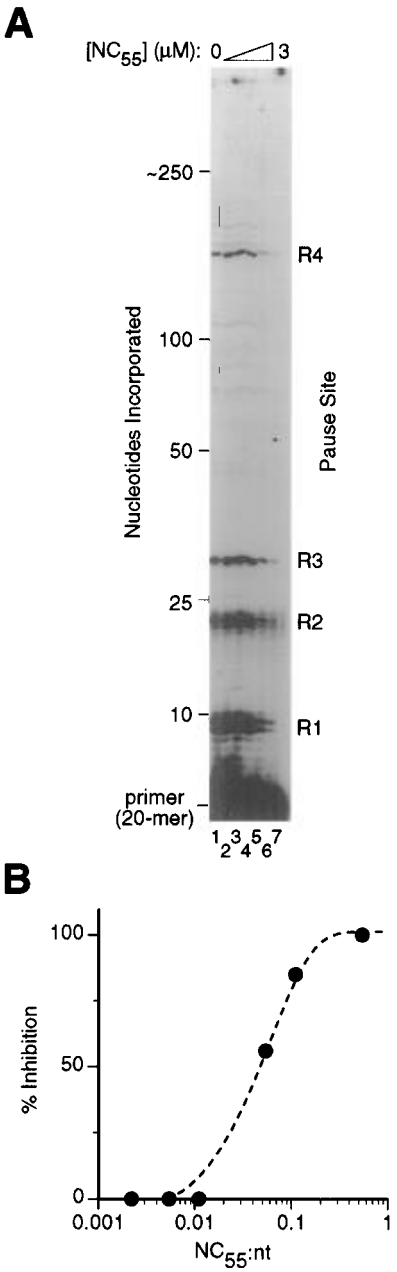

FIGURE 4: Effect of NC_{55} on HIV-1 RT activity. Single-stranded RNA corresponding to plus-strand HIV-1_{NL4-3} nucleotides 4159−5787 of the *pol*/*vif*/*vpr* genes was hybridized to a complementary [5′-^{32}P]DNA oligonucleotide [20-mer primer 4830; see Figure 1 and Klarmann et al. (1993)] and used as a template in DNA polymerization reactions catalyzed by HIV-1 RT with increasing concentrations of HIV-1 NC_{55}. [5′-^{32}P]Primer•template (3.4 nM corresponding to 5.6 μM nucleotides) was first incubated with HIV-1 RT (0.1 nM active sites) for 5 min at 37 °C. Polymerizations were then initiated by addition of NC_{55} (0−3.1 μM), $MgCl_2$, and dNTPs, and incubations were continued at 37 °C for an additional 5 min. (A) Autoradiograph of products separated by urea−PAGE after polymerization in the presence of 0, 0.01, 0.03, 0.06, 0.3, 0.6, or 3.1 μM NC_{55} (lanes 1−7, respectively). The number of nucleotides incorporated and template pause sites R1, R2, R3 and R4 are indicated in the margins. (B) Percent inhibition of total product formation as a function of NC_{55}:template nucleotide ratio. Total products were quantified by densitometry and are expressed relative to the product yield in reactions containing no NC_{55} (lane 1 in panel A).

amounts of HIV-1 RT (Figure 6A, lanes 6−9). When NC_{55} was incubated with primer•template prior to addition of RT and Mg^{2+}-dNTPs, DNA synthesis was inhibited (Figure 6B,

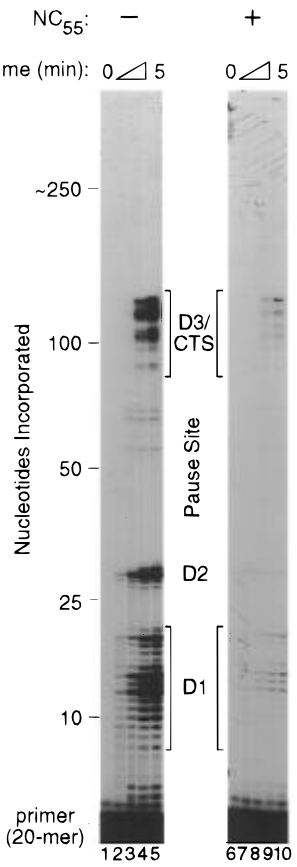

FIGURE 5: Effect of NC_{55} on the rate of steady-state DNA synthesis. Single-stranded DNA corresponding to minus-strand HIV-1_{NL4-3} nucleotides 4159−5787 of the *pol*/*vif*/*vpr* genes was hybridized to a complementary [5′-^{32}P]DNA oligonucleotide primer [20-mer primer 4772; see Figure 1 and Klarmann et al. (1993)] and used as template in DNA polymerization reactions catalyzed by HIV-1 RT with and without HIV-1 NC_{55}. HIV-1 RT (0.1 nM active sites) was first bound to [5′-^{32}P]primer•template (3.0 nM corresponding to 15 μM nucleotides) by incubation for 5 min at 37 °C in the absence of $MgCl_2$ and dNTPs, and polymerizations were initiated by addition of $MgCl_2$ and dNTPs alone (lanes 1−5) or together with 0.9 μM HIV-1 NC_{55} (lanes 6−10). Polymerizations were then continued at 37 °C for an additional 0, 0.5, 1, 3, or 5 min, and the reactions were analyzed by urea−PAGE and autoradiography. The number of nucleotides incorporated and template pause sites D1, D2, and D3/CTS are indicated. D3/CTS corresponds to the HIV-1 central termination sequence (CTS) recently identified *in vivo* and *in vitro* (Charneau et al., 1994; Klarmann et al., 1993).

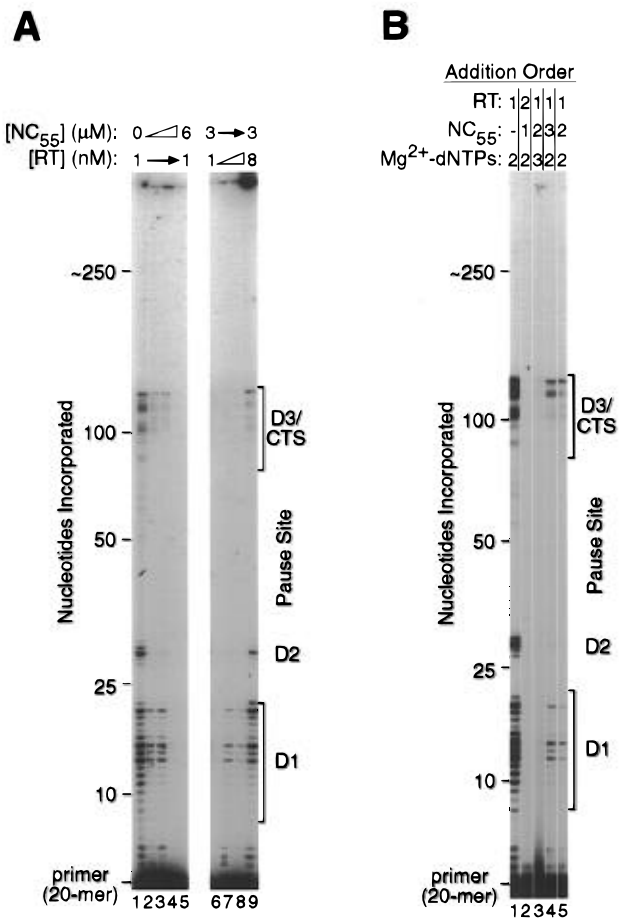

FIGURE 6: Effect of NC_{55}:RT ratio and addition order on DNA synthesis. (A) Effect of NC_{55}:RT ratio. Polymerization reactions were conducted essentially as in Figure 5 except 10 nM primer•template (corresponding to 49 μM nucleotides) was used, the amounts of NC_{55} and HIV-1 RT were varied, and all polymerizations proceeded for 5 min after addition of the last reaction component. In the first set of reactions, HIV-1 RT concentration was constant (0.7 nM active sites) and NC_{55} concentrations were 0, 0.7, 1.4, 2.7, or 5.5 μM (lanes 1−5, respectively). In the second set, NC_{55} concentration was constant (2.7 μM) and HIV-1 RT concentrations were 0.7, 1.7, 3.3, or 8.3 nM active sites (lanes 6−9, respectively). (B) Effect of addition order. Polymerization reactions were conducted as described in Figure 5 except the order of addition of reaction components was varied and all polymerizations proceeded for 5 min after addition of the last reaction component. In the first two reactions, either HIV-1 RT (lane 1) or NC_{55} (lane 2) was prebound to primer•template by incubation for 5 min at 37 °C, and then polymerization was initiated by addition of $MgCl_2$ and dNTPs alone (lane 1) or $MgCl_2$ and dNTPs together with HIV-1 RT (lane 2). In the next three reactions, HIV-1 RT was prebound to primer•template as in lane 1, and then NC_{55} was added prior to (lane 3), after (lane 4), or simultaneously with (lane 5) $MgCl_2$ and dNTPs. In lanes 3 and 4, the interval between addition of NC_{55} and Mg^{2+}-dNTPs was 10 s.

compare lanes 1 and 2). Product formation was also inhibited when preformed RT−primer•template binary complexes were incubated with NC_{55} prior to addition of Mg^{2+}-dNTPs (Figure 6B, lane 3). In contrast, an intermediate level of product formation was observed when NC_{55} was added to RT−primer•template complexes either after (Figure 6B, lane 4) or simultaneously (Figure 6B, lane 5, and Figure 5, lanes 6−10) with Mg^{2+}-dNTPs.

Taken together, these data suggest that NC_{55} slows steady-state cycling of RT *in vitro* (see Discussion). Moreover, these data indicate that preformed RT−primer•template−dNTP ternary complexes are active in the presence of NC_{55} and that NC_{55} restricts polymerization to essentially a single processive cycle starting from these ternary complexes.

Effect of HIV-1 NC_{55} on HIV-1 RT Processivity. The experiments described above define conditions that permit DNA polymerization when HIV-1 NC_{55} is present at levels approximating the NC_{55}:nucleotide stoichiometry in virions (Henderson et al., 1992). Under these conditions using incubation times ≤5 min, NC_{55} minimally affected the relative distribution of most reaction products. One noteworthy exception was the D2 product. In the absence of NC_{55}, D2 was formed at levels similar to bands in D1 and D3/CTS (Figure 5, lanes 1−5, and Figure 6A,B, lane 1). In contrast, when polymerizations were conducted in the presence of NC_{55}, the yield of D2 was reduced relative to D1 and D3/CTS (Figure 5, lanes 6−10, and Figure 6B, lanes 4 and 5). This NC_{55}-dependent reduction in relative D2 yields was consistently observed in independent experiments and was not an artifact of autoradiography (D1:D2:D3 ratios

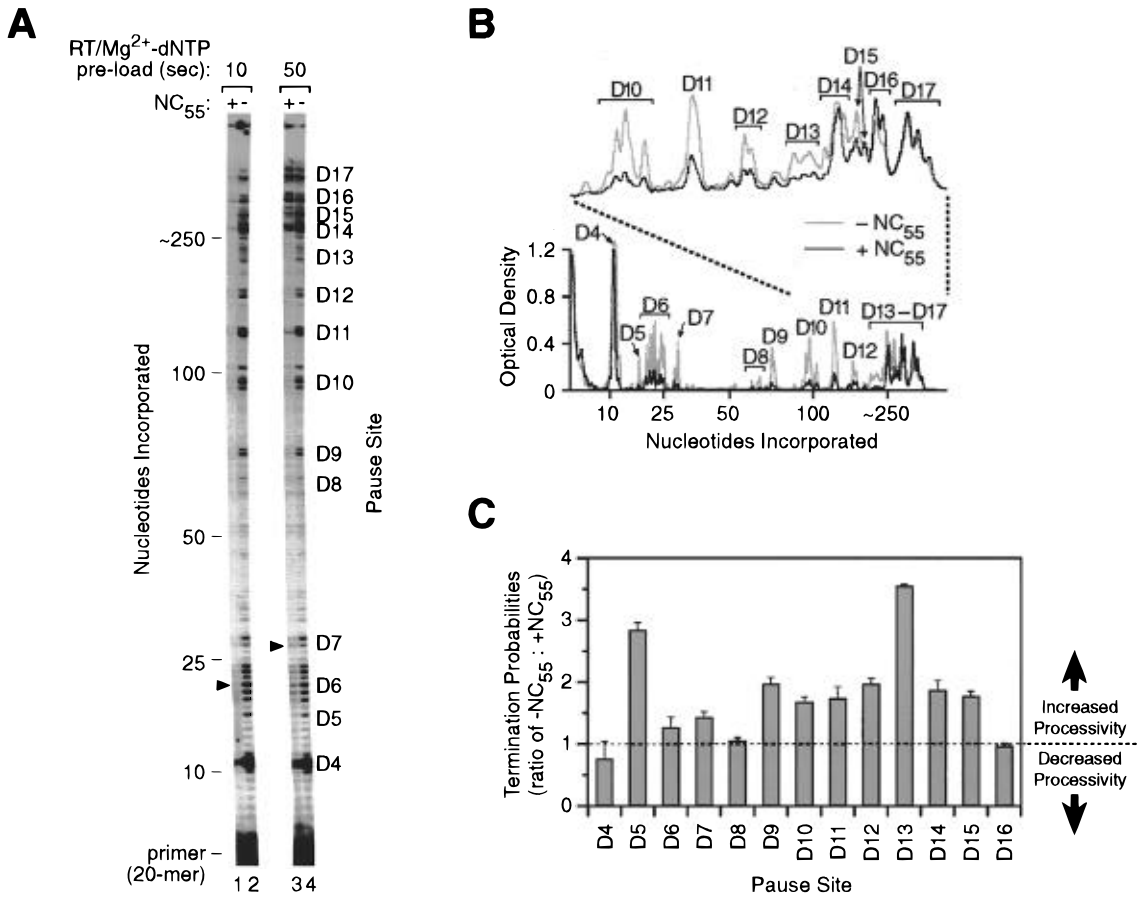

FIGURE 7: Effect of NC_{55} on polymerization at cRRE. (A) Autoradiograph of products separated by urea−PAGE after HIV-1 RT-catalyzed polymerization through the HIV-1 cRRE template region. Single-stranded DNA corresponding to minus-strand HIV-1_{NL4-3} nucleotides 6348−8123 of the *env* gene was hybridized to a [5′-^{32}P]DNA oligonucleotide primer (20-mer) such that the 3′-base of the primer was positioned 12 nucleotides upstream of the start of cRRE [primer 7747; see Figure 1 and Klarmann et al. (1993)]. DNA polymerization on this [5′-^{32}P]primer·template was initiated in three stages. HIV-1 RT (0.1 nM active sites) was first prebound to primer·template (3.2 nM corresponding to 16 μM nucleotides) by incubation for 5 min at 37 °C. $MgCl_2$ and dNTPs were then added, and incubation was continued at 37 °C for 10 s (lanes 1 and 2) or 50 s (lanes 3 and 4). This "preload" with $MgCl_2$ and dNTPs resulted in the formation of RT−primer·template−dNTP ternary complexes and the extension of primer by 21−28 nucleotides (indicated by arrowheads; data not shown). Finally, NC_{55} was added to 0.9 μM final concentration, and polymerization proceeded for an additional 5 min at 37 °C. Reactions with and without NC_{55} are indicated above each lane by "+" (lanes 1 and 3) and "−" (lanes 2 and 4), respectively. (B) Densitometer tracings of lanes 3 and 4 in panel A showing D4−D17 product distributions from reactions conducted in the absence (gray line) and presence (black line) of NC_{55}. (C) Effect of NC_{55} on HIV-1 RT termination probabilities. Termination probabilities (Abbotts et al., 1993) were calculated for each of the template pause site regions D4−D16 after polymerizations with and without NC_{55} under steady-state conditions with a 30-molar excess of primer·template over enzyme as in panel A (lanes 3 and 4). The data are presented as the ratios of termination probabilities without (−) and with (+) NC_{55} at each pause site. Thus, decreases in termination and concomitant increases in processivity imparted by NC_{55} are reflected by an increase in this ratio. Each datum point represents the mean ± SD of two independent experiments.

were unchanged among multiple autoradiographic exposure times falling within the linear response range of the X-ray film). Thus, NC_{55} prevents pausing of DNA synthesis at D2 presumably by a mechanism that increases HIV-1 RT processivity at this site.

To explore the effect of NC_{55} on HIV-1 RT processivity in greater detail, we examined polymerization using primers that resisted helix destabilization by NC_{55} and that were positioned upstream of known strong polymerization pause sites on templates with likely intramolecular secondary structures (Klarmann et al., 1993). A limited survey of primers failed to identify convenient primer·RNA template combinations that were stable in the presence of NC_{55} (e.g., see Figure 3). Thus, for practical reasons, we focused on select primer·DNA templates that resisted denaturation by NC_{55}. All reactions were conducted at an NC_{55}:template nucleotide ratio of 1:15. HIV-1 RT and Mg^{2+}-dNTPs were "preloaded" onto the primer·templates to enrich for RT−primer·template−dNTP ternary complexes prior to addition of NC_{55}. These preloaded complexes were formed by first incubating HIV-1 RT with primer·template at 37 °C for 5 min (to form RT−primer·template binary complexes) and then adding $MgCl_2$ and all four dNTPs and continuing incubation at 37 °C for 10−50 s (to initiate polymerization and thus form RT−primer·template−dNTP ternary complexes). Aliquots were removed to determine the extent of polymerization during the preload, NC_{55} was added to the reactions, and polymerization proceeded for an additional 0−15 min at 37 °C.

Effect at cRRE. Polymerization of the cRRE region of the *env* gene by HIV-1 RT alone resulted in the accumulation of multiple products at specific template sites (pause sites D4−D17 in Figure 7A, lanes 2 and 4). The total yield of products increased with time as expected in these steady-state reactions where RT frequently dissociates from the growing primer·template at pause sites and reinitiates synthesis on unextended primer·templates that are present in excess [Klarmann et al. (1993) and data not shown].

Inclusion of poly(rA)·oligo(dT) as an RT trap (Klarmann et al., 1993) confined synthesis to a single processive cycle but did not change the relative distribution of products at sites D4−D17 (data not shown). This is consistent with previous studies showing that the products of steady-state synthesis by HIV-1 RT alone reflect the accumulation of multiple cycles of processive synthesis (Klarmann et al., 1993; a processive cycle starts with RT binding to primer·template and ends when RT dissociates from the growing strand; processivity is defined as the number of nucleotides polymerized per cycle).

As observed on the *pol* primer·templates (Figures 4−6), polymerization of *env* in the presence of NC_{55} resulted in lower yields of total extended products compared to polymerizations with HIV-1 RT alone (Figure 7A, compare lanes 1 and 3 with lanes 2 and 4). Thus, NC_{55} restricts RT cycling on primer·templates of different sequence (i.e., both *pol* and *env*). A comparison of the distribution of products generated with and without NC_{55} (Figure 7B) shows that the presence of NC_{55} decreased the relative yields of shorter products (D5−D13) and concomitantly increased the relative yields of longer products (D14−D17). Thus, NC_{55} shifted the distribution of products to longer lengths, reflecting an apparent increase in the overall processivity of HIV-1 RT. This increase in processivity was observed using different preload conditions (10−50 s at 37 °C or 5 min at 0 °C) and in multiple independent experiments.

To quantify the effect of NC_{55} and to correct for differences in total product yields, HIV-1 RT termination probabilities were calculated for each of the pause site regions in reactions with and without NC_{55}. Termination probability is a measure of the probability that a polymerase will stall or dissociate at a given template site (Abbotts et al., 1993). Thus, increases in the rate of polymerization relative to the rate of polymerase stalling or dissociation will result in decreased termination probabilities and *vice versa*. A comparison of termination probabilities throughout the cRRE region of *env* shows that the effect of NC_{55} is site-specific (Figure 7C). NC_{55} reduced the termination probability of HIV-1 RT by 2−4-fold at eight different template sites in the cRRE sequence (D5 and D9−D15) while having little or no effect at five of the pause sites (D4, D6−D8, and D16). On average, NC_{55} reduced HIV-1 RT termination at the 13 sites examined by a factor of 2. This resulted in a more than 2-fold increase in the percent of products >300 nucleotides in length.

Effect at D3/CTS. Polymerization of the D3/CTS region of the *pol* gene by HIV-1 RT alone resulted in strong pausing of DNA synthesis at three template locations: D1, D2, and D3/CTS (Figure 8A, lanes 1−4, and Figures 5 and 6). The strongest pausing occurred at D3/CTS, where essentially 100% of the growing strands terminate and RT dissociates from the DNA (Klarmann et al., 1993). Termination of DNA synthesis at this template site *in vitro* is paradoxical because D3/CTS is positioned immediately downstream of the central polypurine tract (cPPT) which is an obligatory initiation site for HIV-1 plus-strand DNA synthesis *in vivo* (Charneau et al., 1992; Charneau & Clavel, 1991; Hungnes et al., 1991). Although D3/CTS appears to be copied efficiently after initiation at the cPPT *in vivo* (Charneau & Clavel, 1991; Hungnes et al., 1991), it is an extreme block to polymerization by purified HIV-1 RT *in vitro* (Figure 5, lanes 1−5; Charneau et al., 1994; Klarmann et al., 1993). This paradox makes the D3/CTS region well-suited for biochemical studies of factors that affect plus-strand DNA polymerization.

Initial experiments evaluating the effect of NC_{55} on this template were conducted for short incubation times (≤5 min) and revealed a reduction in the relative yields of the D2 pause product with little change in D1 and D3/CTS (Figures 5 and 6). However, when polymerizations were conducted for >5 min, NC_{55} increased polymerization through D3/CTS, resulting in products >350 nucleotides in length (Figure 8A, lanes 5−8, and Figure 8B). Quantitation of product yields as a function of time showed that NC_{55} increases the rate of polymerization through D3/CTS about 10-fold (Figure 8C). However, polymerization through D3/CTS in the presence of NC_{55} is still relatively slow as evidenced by the absence of detectable long products in reactions conducted for <5 min (Figure 8A, lanes 5 and 6, and Figure 5, lanes 6−10). As observed on the cRRE *env* template, the effect of NC_{55} varied from site to site. Thus, NC_{55} minimally affected the rate of polymerization through D1 and progressively increased polymerization of D3/CTS as RT advanced through subregions a−d (data not shown). The processivity of HIV-1 RT (i.e., the average number of nucleotides incorporated per RT−primer·template binding event) on this primer·template was 68 ± 17 and 160 ± 43, without and with NC_{55}, respectively ($n = 3$; 15-min incubations). When polymerization was initiated from a primer positioned immediately upstream of D3/CTS (primer 4867 in Figure 1), the processivity of HIV-1 RT increased from ∼15 nucleotides in the absence of NC_{55} to ∼90 nucleotides in the presence of NC_{55} (5-min incubations; data not shown).

DISCUSSION

To better characterize the biochemistry of HIV-1 DNA synthesis, we examined the effect of HIV-1 NC_{55} on DNA polymerization catalyzed by purified HIV-1 RT on HIV-1 template sequences *in vitro*. We overexpressed and purified recombinant NC_{55} identical in sequence to the fully processed, 55-amino acid viral peptide (Figure 2). Using a partially reconstituted *in vitro* system, we showed that recombinant NC_{55} slows steady-state HIV-1 RT turnover during DNA polymerization (Figures 4−6). Moreover, when added to preformed RT−primer·template−dNTP ternary complexes, NC_{55} increased RT processivity by facilitating DNA polymerization through template regions that were copied inefficiently by RT alone (Figures 7 and 8).

A critical first step in reconstituting HIV-1 DNA synthesis *in vitro* is the isolation of large amounts of viral replication proteins. Purified HIV-1 RT is already readily available in both virion and recombinant forms (Katz & Skalka, 1994; Klarmann et al., 1993; Preston et al., 1988, and references therein). The NC precursor peptides NCp15 and NC_{71} have also been isolated as recombinant proteins (Fitzgerald & Coleman, 1991; Khan & Giedroc, 1992; Tsuchihashi & Brown, 1994), but these NC precursors are not the predominant form present in mature virions (Henderson et al., 1992). To obtain large amounts of the mature form of NC (i.e., NC_{55}), we found it necessary to use the pET expression system in *E. coli* engineered to strictly control recombinant gene expression (Studier et al., 1990). Using this system, HIV-1_{NL4-3} NC_{55} was produced at high levels after IPTG induction and was easily purified to near homogeneity by conventional chromatography (Figure 2). You and McHenry

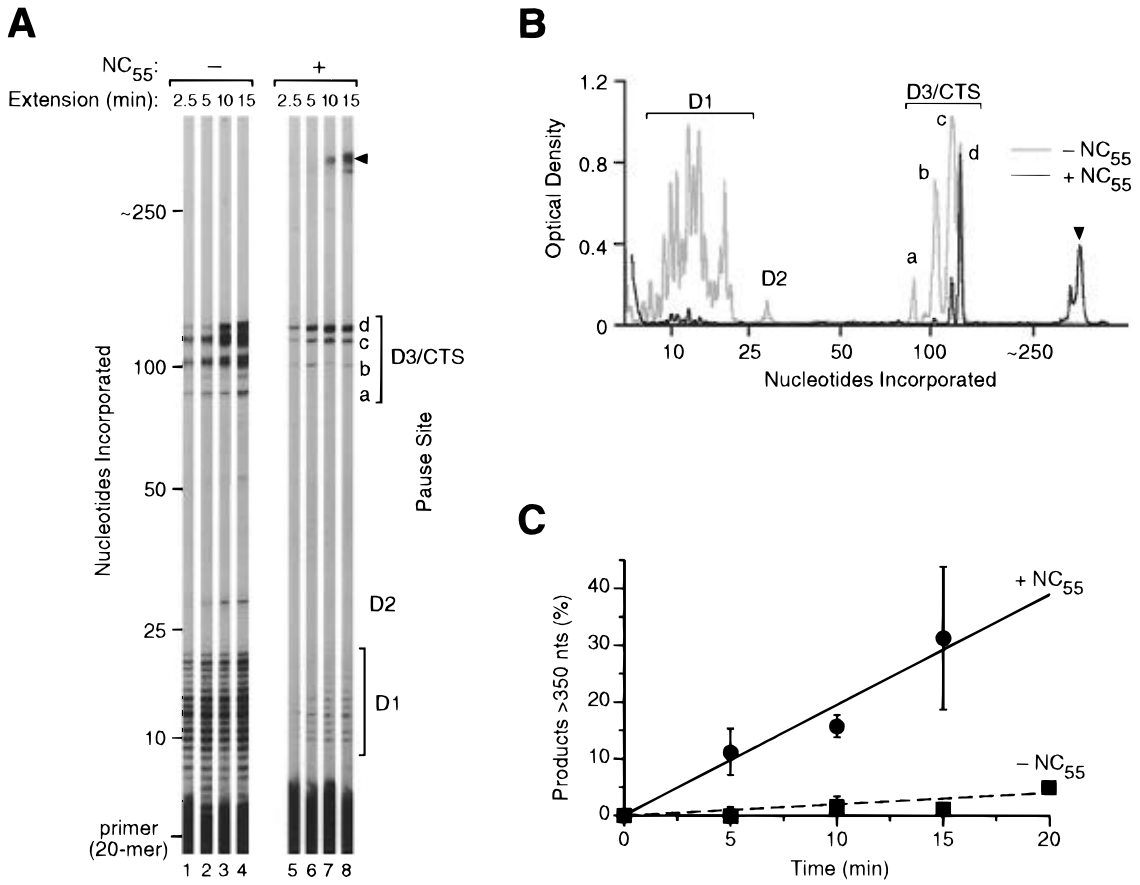

FIGURE 8: Effect of NC_{55} on polymerization at D3/CTS. (A) Autoradiograph of products separated by urea−PAGE after HIV-1 RT-catalyzed polymerization through the HIV-1 D3/CTS template region. The [5′-^{32}P]primer·template described in Figure 5 was used in these reactions. The primer is located immediately upstream of pause site D1 and about 100 nucleotides from D3/CTS. HIV-1 RT (0.1 nM active sites) was first bound to primer·template (3.4 nM corresponding to 16 μM nucleotides) by incubation at 37 °C for 5 min. Polymerizations were initiated by incubation with $MgCl_2$ and dNTPs at 37 °C for 10 s followed by addition of NC_{55} (0.9 μM) and continued incubation at 37 °C for up to 15 min (indicated above each lane). Lanes 1−4, no NC_{55}; lanes 5−8, with NC_{55}. The major products >350 nucleotides in length are indicated by the arrowhead. (B) Densitometer tracings of lanes 4 (without NC_{55}; gray line) and 8 (with NC_{55}; black line) from panel A. (C) Rate of polymerization through D3/CTS. The percent of products extended beyond D3/CTS were quantified by densitometry and plotted as a function of time. Data from reactions with (+NC_{55}; circles, solid line) and without (−NC_{55}; squares, dashed line) NC_{55} are shown. Each point represents the mean ± SD of three independent experiments except the 20-min, minus-NC_{55} time point which is from a single experiment. In some experiments and time points, products >350 nucleotides in length were quantified from overexposed autoradiographs in order to facilitate detection of trace products (e.g., panel A, lane 6).

(1993) recently reported a similar strategy for the purification of recombinant NC_{55} from HIV-$1_{ARV/8a}$.

Our recombinant HIV-1_{NL4-3} NC_{55} binds both RNA and DNA *in vitro* as predicted from its properties *in vivo* and as observed for other retroviral NCs and NC precursors (Khan & Giedroc, 1992; Surovoy et al., 1993; You & McHenry, 1993, and references therein). It was recently demonstrated that the precursor peptide NC_{71} stimulates denaturation and renaturation of nucleic acids *in vitro* (Khan & Giedroc, 1992; Tsuchihashi & Brown, 1994). We now show that the fully mature NC_{55} peptide also destabilizes nucleic acid duplexes (Figure 3). This is consistent with studies showing that both NC_{55} (Rodríguez-Rodríguez et al., 1995; You & McHenry, 1994) and NC_{71} (Peliska et al., 1994) stimulate strand switching during DNA polymerization *in vitro*, presumably by facilitating transitions between double- and single-stranded nucleic acid conformers (Tsuchihashi & Brown, 1994). It is not clear whether these peptides have quantitatively identical helix destabilizing/annealing activities and whether one or both function in this capacity during viral DNA synthesis and recombination in the cell.

Addition of NC_{55} to HIV-1 RT-catalyzed, steady-state DNA polymerization reactions reduced product yields on both RNA and DNA templates (Figures 4−6). The steady-state rate of processive polymerization by HIV-1 RT is largely determined by the rate of RT dissociation from growing strands (Klarmann et al., 1993). Thus, our observation that NC_{55} reduces product yield but not length (Figure 5) suggests that NC_{55} interferes with steady-state RT turnover. This mechanism is further supported by experiments showing that the effect of NC_{55} on product yield is dependent on RT concentration and on the order of addition of reaction components (Figure 6). HIV-1 RT catalyzes DNA polymerization by an ordered kinetic mechanism in which RT first binds to primer·template to form a binary complex which then binds dNTP to form a ternary complex prior to covalent nucleotide addition (Hsieh et al., 1993; Kati et al., 1992; Majumdar et al., 1988; Reardon, 1993). In our experiments, addition of NC_{55} to preformed RT−primer·template binary complexes prior to addition of Mg^{2+} and dNTPs inhibited primer extension (Figure 6B). Binding of HIV-1 RT to primer·template is stabilized by inclusion of Mg^{2+} and the next correct dNTP (Hsieh et al., 1993; Kati et al., 1992; Perrino & Mekosh, 1992). Thus, addition of NC_{55} to reactions containing only RT−primer·template may disrupt these less stable binary complexes. NC_{55} also

inhibited primer extension when added prior to RT (Figure 6B). The observation that inhibition is dependent on NC_{55} concentration and is relieved by addition of excess RT (Figure 6A) suggests that NC_{55} competes with RT for primer·template binding. Together these data indicate that NC_{55} reduces product yield by destabilizing RT−primer·template binary complexes and preventing or slowing their reformation in the steady state. When NC_{55} is added to preformed RT−primer·template−dNTP ternary complexes, polymerization proceeds but is still restricted to essentially a single processive cycle.

The inhibition of RT cycling by NC_{55} may have important ramifications *in vivo*. It implies that a strict order of addition is required for the initiation of viral DNA synthesis from tRNA primers and that strand switching after normal retroviral "strong stop DNA" synthesis requires either a processive mechanism or disassembly and ordered reassembly of the replication complex. Reinitiation of polymerization at pause sites where RT dissociates from the primer·template (Klarmann et al., 1993) may similarly be affected by NC_{55} *in vivo*. The virus must somehow circumvent these inhibitory effects of NC_{55} to ensure efficient initiation and propagation of viral DNA synthesis. Preferential binding of retroviral RTs to their cognate tRNA primers may provide a mechanism for ordered addition during initiation of viral DNA synthesis (Barat et al., 1993). It is not clear whether another loading factor, a processivity factor, or some other mechanism functions to overcome the inhibitory effects of NC_{55} after strand switching and paused synthesis *in vivo*.

To determine the effect of NC_{55} on HIV-1 RT processivity, we examined DNA polymerization on two HIV-1 minus-strand DNA template sequences: the D3/CTS region in the *pol* gene and the cRRE region in the *env* gene. These templates were chosen (1) because they are copied inefficiently by HIV-1 RT alone and likely form intramolecular secondary structures (Charneau et al., 1994; Klarmann et al., 1993; Le et al., 1988), and (2) because primer·template combinations were identified with primers both proximal to template regions of interest and resistant to helix destabilization by NC_{55}. Addition of NC_{55} to preformed RT−primer·template−dNTP complexes increased the yields of longer products relative to shorter products on both the *env* and *pol* templates (Figures 7 and 8). Because these reactions were conducted under steady-state conditions and because NC_{55} restricts polymerization to essentially one steady-state cycle (see above), this increase in product length reflects an increase in HIV-1 RT processivity. Quantitation of RT termination probabilities and first-order rate constants show that the stimulatory effect of NC_{55} varies from site to site, ranging from no effect (R1−R4, D4, D6−D8, and D16) to about a 10-fold increase (D3/CTS; Figures 4A, 7C, and 8C). Stimulatory effects have also recently been observed for the p15 precursor of HIV-1 NC_{55} during RNA-templated DNA polymerization (Volkmann et al., 1993), and earlier reports show that NC from avian myeloblastosis virus stimulates both RNA- and DNA-templated polymerization by RT as well as DNA-templated polymerization by mammalian DNA polymerase α (Sykora & Moelling, 1981). In accord with our data on HIV-1 NC_{55}, stimulation by the avian retroviral NC varies at different template sequences (Sykora & Moelling, 1981). While this manuscript was in preparation, Bambara and colleagues (Rodríguez-Rodríguez et al., 1995) reported a minimal effect of NC_{55} on the overall processivity of HIV-1 RT *in vitro* but noted a redistribution in the relative yields of specific pause products over the 90-nucleotide RNA template examined. Tanchou et al. (1995) also recently showed that the NC_{72} precursor peptide increases HIV-1 RT processivity at specific RNA template sites *in vitro*. The site-specificity of stimulation by NCs may reflect different mechanisms contributing to paused synthesis (Abbotts et al., 1993; Klarmann et al., 1993) and/or preferential binding of NC to specific template sequences (Sakaguchi et al., 1993).

Our studies provide some clues about the mechanism of action of NC_{55} on RT processivity. It is unlikely that NC_{55} stimulates polymerization through D2 (Figure 5) by functioning as a processivity clamp (Kornberg & Baker, 1992), since HIV-1 RT already binds persistently to paused D2 primer·templates even in the absence of NC_{55} (Klarmann et al., 1993) and any increase in primer·template binding affinity is expected to have no measurable effect under these experimental conditions. Another possible mechanism is that NC_{55} induces longer product formation by stimulating the transfer of nascent strands to partially homologous template regions that are relatively free of structures that impede polymerization. However, the fact that NC_{55} induces shifts to longer products without changing pause site identities (Figures 5 and 7B) argues against a template switching mechanism. The presence of secondary structures in cRRE, D3/CTS, and D2 and the observation that NC_{55} (Figure 3) and its precursors (Khan & Giedroc, 1992; Tsuchihashi & Brown, 1994) destabilize helices suggest that NC_{55} functions, at least in part, by removing template secondary structures. In our reactions, NC_{55} was in large excess over RT at a concentration sufficient to coat ∼50% of template nucleotides (assuming one NC_{55} binds approximately seven nucleotides; You & McHenry, 1993), suggesting that NC_{55} imparts its effect by direct interaction with the template. However, recent studies indicate that the NC_{71} precursor peptide interacts with HIV-1 RT *in vitro* (Peliska et al., 1994). Similar interactions between mature NC_{55} and HIV-1 RT could bring about conformational or other biophysical changes that also increase RT processivity. Taken together, the data indicate that NC_{55} increases RT processivity (1) by coating template nucleotides and removing secondary structures that block RT progression and/or (2) by directly stimulating RT itself to copy through secondary structures.

The activities of retroviral NCs are remarkably similar to those of single-strand binding proteins (SSBs) that function as accessory DNA replication factors in other organisms [reviewed by Chase and Williams (1986) and Kornberg and Baker (1992)]. SSBs, like retroviral NCs, preferentially and stoichiometrically bind single-stranded nucleic acids and facilitate denaturation and renaturation of double-stranded helices. Although NCs appear to bind nucleic acids with lower cooperativity (Karpel et al., 1987; Khan & Giedroc, 1992; You & McHenry, 1993), both SSBs and NCs stimulate polymerization to a similar extent (2−10-fold for NC_{55} and 3−10-fold for T4 gene 32 protein) and in a manner dependent on addition order, protein:nucleotide stoichiometry, and template site (Huang & Hearst, 1980; Huang et al., 1981). Both also function in part by destabilizing template secondary structures that impede polymerase progression (Huang & Hearst, 1980; Huang et al., 1981; LaDuca et al., 1983). When considered together, the data suggest

that retroviral NCs are functional homologues of SSBs from other organisms.

Like other SSBs, NCs have multiple effects on nucleic acids and may play several overlapping roles in viral DNA synthesis, including genomic folding and packaging (Herschlag et al., 1994), primer selection (Barat et al., 1993; De Rocquigny et al., 1992), promotion of strand transfers (Allain et al., 1994; Peliska et al., 1994; You & McHenry, 1994), modulation of polymerase fidelity (Kunkel et al., 1979; Varela-Echavarría et al., 1992), and stimulation of DNA polymerization through regions of template secondary structure. Selective destabilization of short nucleic acid duplexes (Figure 3; Khan & Giedroc, 1992; Tsuchihashi & Brown, 1994) may also influence strand displacement synthesis and the availability of primers for plus-strand DNA synthesis *in vivo*. Additional genetic and biochemical studies are required to clearly define the role(s) of NC during viral DNA synthesis.

The presence of multiple viral proteins associated with viral DNA *in vivo* suggests that DNA synthesis occurs as part of a multicomponent complex. Our studies show that NC_{55} stimulates DNA synthesis *in vitro* up to 10-fold. However, this effect is site-specific, and RT is still unable to produce viral genomic-length products (~9.5 kb). Thus, other accessory replication factors may be required for viral DNA synthesis *in vivo* (Bandyopadhyay, 1977; Lee & Hung, 1977; Leis & Hurwitz, 1972; Padhy et al., 1976; Takahashi et al., 1995). A complete understanding of HIV-1 DNA synthesis requires knowledge of the activities of the viral DNA replication complex and the functions, interactions, and stoichiometries of its individual components. Detailed biochemical studies of HIV-1 DNA synthesis may also identify new strategies for the design of antiviral therapies. HIV-1 NC_{55} is an attractive antiviral target because it is multifunctional, virally encoded, and may be required for viral DNA synthesis.

ACKNOWLEDGMENT

We thank Cherylene Schauber for purification of the HIV-1 RT, Sandy Price for technical assistance, Mario Stevenson, Lorna Rodríguez-Rodríguez, and Bob Bambara for communicating unpublished data, and Janet Lindsley, Wes Sundquist, Mallika Singh, Tim Formosa, David Virshup, and Barbara Graves for critical reading of the manuscript.

REFERENCES

Abbotts, J., Bebenek, K., Kunkel, T. A., & Wilson, S. H. (1993) *J. Biol. Chem. 268*, 10312−10323.

Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabson, A., & Martin, M. A. (1986) *J. Virol. 59*, 284−291.

Allain, B., Lapadat-Tapolsky, M., Berlioz, C., & Darlix, J.-L. (1994) *EMBO J. 13*, 973−981.

Bandyopadhyay, A. K. (1977) *J. Biol. Chem. 252*, 5883−5887.

Bandyopadhyay, A. K., & Levy, C. C. (1978) *J. Biol. Chem. 253*, 8285−8290.

Barat, C., Schatz, O., Le Grice, S., & Darlix, J.-L. (1993) *J. Mol. Biol. 231*, 185−190.

Borroto-Esoda, K., & Boone, L. R. (1991) *J. Virol. 65*, 1952−1959.

Bowerman, B., Brown, P. O., Bishop, J. M., & Varmus, H. E. (1989) *Genes Dev. 3*, 469−478.

Bukrinsky, M. I., Sharova, N., McDonald, T. L., Pushkarskaya, T., Tarpley, W. G., & Stevenson, M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6125−6129.

Burgess, R. (1991) *Methods Enzymol. 208*, 3−10.

Charneau, P., & Clavel, F. (1991) *J. Virol. 65*, 2415−2421.

Charneau, P., Alizon, M., & Clavel, F. (1992) *J. Virol. 66*, 2814−2820.

Charneau, P., Mirambeau, G., Roux, P., Paulous, S., Buc, H., & Clavel, F. (1994) *J. Mol. Biol. 241*, 651−662.

Chase, J. W., & Williams, K. R. (1986) *Annu. Rev. Biochem. 55*, 103−136.

Chen, M.-j., Garon, C. F., & Papas, T. S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1296−1300.

Coffin, J. M. (1990) in *Virology* (Fields, B. N., Knipe, D. M., Chanock, R. M., Melnick, J. L., Roizman, B., & Shope, R. E., Eds.) pp 1437−1500, Raven Press, Ltd., New York.

Collett, M. S., & Faras, A. J. (1977) *J. Virol. 22*, 86−96.

Das, M. R., Padhy, L. C., Koshy, R., Sirsat, S. M., & Rich, M. A. (1976) *Nature 262*, 802−805.

De Rocquigny, H., Gabus, C., Vincent, A., Fournié-Zaluski, M.-C., Roques, B., & Darlix, J.-L. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6472−6476.

Dib-Hajj, F., Khan, R., & Giedroc, D. P. (1993) *Protein Sci. 2*, 231−243.

Farnet, C. M., & Haseltine, W. A. (1991) *J. Virol. 65*, 1910−1915.

Fitzgerald, D. W., & Coleman, J. E. (1991) *Biochemistry 30*, 5195−5201.

Gorelick, R. J., Henderson, L. E., Hanser, J. P., & Rein, A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8420−8424.

Gorelick, R. J., Chabot, D. J., Rein, A., Henderson, L. E., & Arthur, L. O. (1993) *J. Virol. 67*, 4027−4036.

Henderson, L. E., Copeland, T. D., Sowder, R. C., Schultz, A. M., & Oroszlan, S. (1988) in *Human Retroviruses, Cancer, and AIDS: Approaches to Prevention and Therapy* (Bolognesi, D., Ed.) pp 135−147, Alan R. Liss, Inc., New York.

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., & Fenselau, C. (1992) *J. Virol. 66*, 1856−1865.

Herschlag, D., Khosla, M., Tsuchihashi, Z., & Karpel, R. L. (1994) *EMBO J. 13*, 2913−2924.

Hsieh, J.-C., Zinnen, S., & Modrich, P. (1993) *J. Biol. Chem. 268*, 24607−24613.

Huang, C.-C., & Hearst, J. E. (1980) *Anal. Biochem. 103*, 127−139.

Huang, C.-C., Hearst, J. E., & Alberts, B. M. (1981) *J. Biol. Chem. 256*, 4087−4094.

Hung, P. P., & Lee, S. G. (1976) *Nature 259*, 499−502.

Hungnes, O., Tjotta, E., & Grinde, B. (1991) *Arch. Virol. 116*, 133−141.

Karpel, R. L., Henderson, L. E., & Oroszlan, S. (1987) *J. Biol. Chem. 262*, 4961−4967.

Kati, W. M., Johnson, K. A., Jerva, L. F., & Anderson, K. S. (1992) *J. Biol. Chem. 267*, 25988−25997.

Katz, R. A., & Skalka, A. M. (1994) *Annu. Rev. Biochem. 63*, 133−173.

Khan, R., & Giedroc, D. P. (1992) *J. Biol. Chem. 267*, 6689−6695.

Klarmann, G. J., Schauber, C. A., & Preston, B. D. (1993) *J. Biol. Chem. 268*, 9793−9802.

Kornberg, A., & Baker, T. A. (1992) *DNA Replication*, W. H. Freeman, New York.

Kunkel, T. A., Meyer, R. R., & Loeb, L. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6331−6335.

LaDuca, R. J., Fay, P. J., Chuang, C., McHenry, C. S., & Bambara, R. A. (1983) *Biochemistry 22*, 5177−5188.

Le, S. Y., Chen, J. H., Braun, M. J., Gonda, M. A., & Maizel, J. V. (1988) *Nucleic Acids Res. 16*, 5153−68.

Lee, S. G., & Hung, P. P. (1977) *Nature 270*, 366−369.

Lee, Y. M. H., & Coffin, J. M. (1991) *Mol. Cell. Biol. 11*, 1419−1430.

Leis, J. P., & Hurwitz, J. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2331−2335.

Majumdar, C., Abbotts, J., Broder, S., & Wilson, S. H. (1988) *J. Biol. Chem. 263*, 15657−15665.

Méric, C., & Goff, S. P. (1989) *J. Virol. 63*, 1558−1568.

Nagy, K., Young, M., Baboonian, C., Merson, J., Whittle, P., & Oroszlan, S. (1994) *J. Virol. 68*, 757−765.
Padhy, L. C., Kar, S. K., Rao, K. K., & Das, M. R. (1976) *Nature 262*, 805−807.
Peliska, J. A., Galasubramanian, S., Giedroc, D. P., & Benkovic, S. J. (1994) *Biochemistry 33*, 13817−13823.
Perrino, F. W., & Mekosh, H. L. (1992) *J. Biol. Chem. 267*, 23043−23051.
Preston, B. D., Poiesz, B. J., & Loeb, L. A. (1988) *Science 242*, 1168−1171.
Reardon, J. E. (1993) *J. Biol. Chem. 268*, 8743−8751.
Rice, W. G., Schaeffer, C. A., Graham, L., Bu, M., McDougal, J. S., Orloff, S. L., Villinger, F., Young, M., Oroszlan, S., Fesen, M. R., Pommier, Y., Mendeleyev, J., & Kun, E. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9721−9724.
Rodríguez-Rodríguez, L., Tsuchihashi, Z., Fuentes, G. M., Bambara, R. A., & Fay, P. J. (1995) *J. Biol. Chem. 270*, 15005−15011.
Sakaguchi, K., Zambrano, N., Baldwin, E. T., Shapiro, B. A., Erickson, J. W., Omichinski, J. G., Clore, G. M., Gronenborn, A. M., & Appella, E. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5219−5223.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60−89.
Surovoy, A., Dannull, J., Moelling, K., & Jung, G. (1993) *J. Mol. Biol. 229*, 94−104.
Sykora, K. W., & Moelling, K. (1981) *J. Gen. Virol. 55*, 379−371.
Takahashi, H., Matsuda, M., Kojima, A., Sata, T., Andoh, T., Kurata, T., Nagashima, K., & Hall, W. W. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5694−5698.
Tanchou, V., Gabus, C., Rogemond, V., & Darlix, J.-L. (1995) *J. Mol. Biol. 252*, 563−571.
Tsuchihashi, Z., & Brown, P. O. (1994) *J. Virol. 68*, 5863−5870.
Varela-Echavarría, A., Garvey, N., Preston, B. D., & Dougherty, J. P. (1992) *J. Biol. Chem. 267*, 24681−24688.
Veronese, F. D., Rahman, R., Copeland, T. D., Oroszlan, S., Gallo, R. C., & Sarngadharan, M. G. (1987) *AIDS Res. Hum. Retroviruses 3*, 253−264.
Volkmann, S., Dannull, J., & Moelling, K. (1993) *Biochimie 75*, 71−8.
You, J. C., & McHenry, C. S. (1993) *J. Biol. Chem. 268*, 16519−16527.
You, J. C., & McHenry, C. S. (1994) *J. Biol. Chem. 269*, 31491−31495.

BI951707E