The nucleocapsid protein (NC) of all animal retroviruses, encoded by the gag gene, is the major structural protein of the core ribonucleoprotein complex, bound to genomic RNA in mature virions. NC is also thought to play one or more accessory roles in reverse transcription. Mature NC (p7\textsuperscript{18}) from human immunodeficiency virus type 1 (HIV-1) is a 71-amino acid, basic protein which contains two Cys-His Zn(II) retroviral-type zinc finger domains. Herein, we describe the subcloning and expression of HIV-1 NC, denoted NC71, from an inducible phage T7 RNA polymerase promoter in Escherichia coli. Purified NC71 can be reversibly reconstituted with 2 g-at Zn(II) determined by atomic absorption. Ultraviolet circulation dichroism spectroscopy has been used to characterize the complexes between highly purified NC71 and the RNA homopoly-nucleotide poly(A) and E. coli tRNA\textsuperscript{mixed}. On poly(A), Zn\textsubscript{2} NC71 is characterized by an apparent size \( n = 15 \pm 3 \) nucleotides and high affinity (\( K_{pp} = 3 \times 10^7 \text{ M}^{-1} \)) and moderately cooperative (\( \omega = 170 \pm 25 \)) binding. A mixture of E. coli tRNA species (tRNA\textsuperscript{mixed}) was used to probe the conformational changes induced in tRNA upon binding of HIV-1 NC71. Two structural forms of tRNA\textsuperscript{mixed}, which differ in their degree of tertiary structure, were assayed for their susceptibility to denaturation by NC71. Five molar monomer equivalents of NC71 are required to denature the "inactive" tRNA in the absence of Mg\textsuperscript{2+}. A Zn(II)-free, oxidized form of NC71 was also shown to unwind inactive tRNA with the same efficiency and stoichiometry. The detailed spectral changes which occur on NC-induced denaturation closely mimic temperature-induced denaturation of inactive tRNA\textsuperscript{mixed}. The prototype helix-stabilizing protein, T4 gene 32 protein, is unable to unwind this form of tRNA under the same conditions. The stoichiometry of unwinding of inactive tRNA by NC71 is consistent with the site size determined with poly(A). An "active" form of tRNA\textsuperscript{mixed}, prepared by thermal denaturation and refolding of the inactive form with Mg\textsuperscript{2+}, proved less susceptible to both temperature and NC71-induced unwinding. The mechanistic implications of these findings on the reported biochemical activities of RNA:RNA annealing and replication primer tRNA positioning by NC are discussed.

The gag gene of all known retroviruses encodes a multifunctional polyprotein precursor product which has been shown to play fundamental roles in virus genomic RNA encapsidation and replication (1, 2). These RNA binding activities are mediated by the nucleocapsid (NC)\textsuperscript{1} domain of the precursor or processed NC subunit, respectively (1–3). Correct encapsidation involves formation of the genomic RNA dimer, composed of two identical 35 S viral RNA molecules, and requires both major and minor cis-acting RNA elements found toward the 5' end of the genome (1, 4–6). Genomic dimerization appears to aid in the switch from genomic RNA translation of gag precursor polyproteins to genomic packaging and virus assembly in the production of progeny virus (7). In vitro, viral NC from several retroviral sources appears able to promote dimerization (oligomerization) of viral genomic fragments containing major cis-acting packaging signals (7–10). In the initiation of proviral cDNA synthesis by reverse transcriptase, NC from both HIV-1 and MoMLV have been hypothesized to promote the base-pairing or annealing of the cognate replication primer tRNA onto genomic viral fragments containing the complementary primer binding site (7–12).

NC from all animal retroviruses contains one or two conserved Cys-Xaa\textsubscript{2}-Cys-Xaa\textsubscript{2}-His-Xaa\textsubscript{2}-Cys retroviral-type zinc-finger Cys\textsubscript{3}His motifs (13, 14), which can be reconstituted with Zn(II), Cd(II), and Co(II) in vitro (15–18) (see Fig. 1). These domains are thus reminiscent of "zinc-finger" domains in other nucleic acid binding proteins (19). The role or functional significance of metal binding by NC in the virion remains poorly defined. Mutagenesis of Zn(II) coordinating ligands and adjacent amino acids in infectious proviral clones from a number of retroviruses results in a variety of replicative defects. The most severe completely block genomic encapsidation, while less severe mutations result in reduced levels of correctly dimerized genomic RNA (20–26). Interestingly, all such mutants show dramatically reduced infectivities (by \( \geq 10^5 \)-fold), apparently independent of the amount of genomic RNA packaged (20–26). These studies suggest that this region of NC is important in RNA recognition at some point in the retroviral life cycle, and/or plays a structural role in eventual formation of the reverse transcriptionally active ribonucleoprotein complex (cf. Ref. 27). It is generally accepted that processed NC is bound nonspecifically in a histone-like condensation of the diploid RNA genome in mature virus preparations (27). These functional activities are consistent with the biochemical activities of purified NC. Zn(II) coordination however, appears to have little affect on this type of binding in vitro (18, 28, 29). It was recently demonstrated that neither dimerization of genomic RNA fragments nor tRNA annealing

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\‡ Recipient of American Cancer Society Junior Faculty Research Award JFRA-270. To whom correspondence should be addressed.

\textsuperscript{1} The abbreviations used are: NC, nucleocapsid; ss, single-stranded; Na\textsubscript{2}EDTA, trisodium ethylenediaminetraacetic acid; MoMLV, Moloney murine leukemia virus; HIV-1, human immunodeficiency virus; kh, kilobase(s); bp, base pair(s); DTT, dithiothreitol.
purified from the overproducing plasmid, pPLg32-A.wt (32), and treated with Klenow fragment and dNTPs to form blunt ends, and pIBI.HK22. This fragment was then purified and used as a template and GG400 (5'-TGGATCCGGTCTGCmTAAAAATTCCC-') precipitation from ethanol, and exhaustive dialysis against 10 mM protein. The significance of this cleavage is unknown.

The pol open reading frame from pEcoB was then cloned between the stop codon (underlined). These manipulations produce a trans-

One report (40) suggests that there is an additional C-terminal coordination chelate (10), in contrast to an earlier report (9).

In this report, we present experiments directed toward elucidating the mechanism of RNA:RNA annealing by HIV-1 NC. The mode by which NC interacts with model single-stranded as well as duplex and tertiary structure-containing RNA molecules of defined structure must be determined before experiments with more complex retroviral RNAs are undertaken. Herein, we describe the subcloning, bacterial overexpression, and purification of mature, correctly processed HIV-1 NC (30, 31), denoted NC71, and CD spectrscopic studies aimed at identifying the types of complexes NC71 forms with the model ssRNA polynucleotide, poly(A), and two structural forms of tRNA. We conclude that NC71 is a potent RNA double-helix unwinding protein which derives from its ability to bind stoichiometrically and cooperatively to single-stranded nucleic acids.

EXPERIMENTAL PROCEDURES

Materials

All buffers were prepared with doubly distilled and deionized Milli-Q water. ssDNA-cellulose was prepared as described (32, 55). Di-thiothreitol(nitrobenzoic acid), isopropyl-1-thio-β-D-galactopyranoside, and chromatographically purified DNase were obtained from Sigma. Poly(A) was purchased from the Midland Certified Reagent Company (Midland, TX) and used following exhaustive dialysis into 10 mM Tris-HCl, pH 8.1. tRNA<sup>met</sup> from <i>Escherichia coli</i> MRE 600, obtained from Boehringer-Mannheim (Indianapolis, IN), was used following phenol:chloroform extraction, precipitation from ethanol, and exhaustive dialysis against 10 mM Tris-HCl, pH 8.1, 0.1 mM Na<sub>2</sub>EDTA, 0.05 M NaCl, pH 8.1. This is the "inactive" tRNA<sup>met</sup> preparation (33). An aliquot of this material (2.2 × 10<sup>2</sup> M molecules) was heated to 75 °C for 2 min and quick-cooled on ice (33). The CD spectrum of this material was indistinguishable from the starting material. The "active" tRNA<sup>met</sup> was prepared by adding MgCl<sub>2</sub> to 10 mM, followed by heating to 60 °C for 5-6 min and quick-cooled on ice (33). Molecular biologists were obtained from either New England Biolabs (Boston, MA), Boehringer-Mannheim, or Promega (Fisher Scientific). Recombinant T4 gene 32 protein lacking the C-terminal 49 amino acids (g32P-A) was purified from the overproducing plasmid, pFL322-A.wt (32), and purified essentially as described for the wild-type protein (32).

Methods

Plasmid Constructions—All molecular biological methods were carried out according to standard methods (35). The plasmid pBluHS-R5 (kindly provided by H. Z. Streicher, National Cancer Institute, and used with permission) was the source of the coding region for HIV-1 NC (30). A 4.2-kb 5' EcorI fragment was cloned into the EcoRI site of pUC8 to make pEcoB. A 2.2-kb HindIII-KpnI fragment containing the 5'-GG300 primer was added to 0.1 mM, and this solution was lyophilized to dryness. This acidified NC71 fraction was dissolved in 20 ml of 100 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 50 μM ZnCl<sub>2</sub>, 0.1 mM phenylmethylsulfonyl fluoride, 29 μg/ml DNase) was added at a ratio of 10 of buffer/g wet weight cells to complete the resuspension of the cells. This suspension was incubated at 37 °C for 30 min, solid NaCl was added to 0.8 M, incubated an additional 30 min, 37 °C, and then centrifuged at 6000 rpm for 10 min. This low-speed lysis pellet contained most of the NC71. The NC71 was then solubilized by dissolving the low-speed pellet in 20% (v/v) tris(2-carboxyethyl)phosphine and 10 mM Tris-HCl, pH 8.0 (40). This fraction was loaded directly onto a 2.5 × 10-cm C<sub>5</sub> column (Waters preparative C<sub>5</sub>, 25-Å pore, 55-105 μm particle size) and washed with 0.05% trifluoroacetic acid, 40% acetonitrile. The protease inhibitor phenylmethylsulfonyl fluoride was added to 0.1 mM, and this solution was lyophilized to dryness.

This acidified NC71 fraction was dissolved in 20 ml of 100 mM Tris-HCl, pH 8.0, 0.1 mM DTT, 0.2 mM ZnCl<sub>2</sub>, 50 mM NaCl, and loaded onto a ssDNA-cellulose column (1.5 × 15 cm) equilibrated with 100 mM Tris-HCl, pH 8, 0.01 mM DTT, 0.2 mM ZnCl<sub>2</sub>, 50 mM NaCl. After washing, the column was developed with a 100-ml linear gradient from 50 mM to 1 mM NaCl in the same buffer. Generally, two distinct fractions of NC71 were eluted from the column during the NC71 gradient (Fig. 1A). N-terminal sequencing and amino acid analysis revealed that the first fraction was enriched in two of the products of NC71, corresponding to NC 11-71 and NC 4-71, resulting from cleavage following Arg<sup>23</sup> and Arg<sup>29</sup>, respectively (Fig. 1B). The second, tighter binding fraction was primarily authentic 1-71 with variable amounts of NC71 4-71. In some cases, to further purify NC 1-71 and to remove the adenovirus (Zn<sup>II</sup>) vector DNA, the NC pool was subjected to dialysis against 10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 2 mM β-mercaptoethanol, and was subsequently loaded onto a TSK-Gel CM-550M carboxymethyl-Septadex (1 × 9 ml) column equilibrated with 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.1 mM DTT. Following a wash with equilibration buffer, the column was developed with a step to 0.2 M NaCl and a 25/25 ml linear 0.2/0.5 M NaCl gradient in the same buffer. The major pool fraction, eluting last in the NaCl gradient (at ~0.4 M NaCl), was greater than 95% pure by amino acid analysis and N-terminal sequencing. NC71 was 95% pure by amino acid analysis and N-terminal sequencing. NC71 was 95% pure by amino acid analysis and N-terminal sequencing. NC71 was 95% pure by amino acid analysis and N-terminal sequencing. NC71 was 95% pure by amino acid analysis and N-terminal sequencing. NC71 was 95% pure by amino acid analysis and N-terminal sequencing. NC71 was 95% pure by amino acid analysis and N-terminal sequencing. NC71 was 95% pure by amino acid analysis and N-terminal sequencing.
no reactive Cys by 5,5'-dithiobis(nitrobenzoic acid) titration.

**CD Spectroscopy—**Near UV-CD spectra were collected on a Jasco C-600 spectropolarimeter in a 10-mm pathlength rectangular cuvette (volume = 1.65–1.7 ml) thermostatted at 25 ± 0.1 °C in 10 mM Tris-HCl, 0.1 mM DTT, pH 8.3 (buffer A), 0.1 M NaCl, with or without 10 mM MgCl$_2$. CD spectra of poly(A) were recorded from 300–240 nm at the indicated concentrations of poly(A), quantitated by $e_{260}$ = 10,300 M$^{-1}$cm$^{-1}$ (38). For each acquisition, two scans were digitally averaged at a scan rate of 20 nm/min, 2-s time constant, 1-nm bandwidth, and 20-ndeg full scale. CD spectra of tRNA$_{\text{loaded}}$ were recorded from 310–210 nm with the same acquisition parameters as used for poly(A). In some cases, four spectra recorded from 270 to 250 nm were digitally averaged. [tRNA$_{\text{loaded}}$] was determined with $e_{260}$ = 4.8 × 10$^3$ M$^{-1}$cm$^{-1}$ or 19.9 (mg molecule/ml)$^{-1}$ (34). Use of this molar extinction coefficient gives a mean weight residue ellipticity $[\theta]_w$, at 262 nm (assuming average values of 320 g/mol nucleotide and 75 nucleotides per tRNA) for “inactive” tRNA$_{\text{loaded}}$ of 1.74 × 10$^4$ deg-cm$^2$.dmol$^{-1}$ and 1.95 × 10$^4$ deg-cm$^2$.dmol$^{-1}$ for “activated” tRNA, both within 5% of their published values (34). In titrations of poly(A) and tRNA with protein, aliquots of protein were added directly to the CD cuvette and continuously stirred for 2 min. The stirring was then ceased, and the CD spectrum was recorded as indicated. During the course of multiple scans, or upon re-scanning 10–20 min later, the CD spectrum of the protein-tRNA complex did not significantly change (≤0.5 mdeg), evidence that equilibrium had been reached. [NC71] was determined by UV absorption as described above. Free NC71 is devoid of optical activity in the 300–240 nm region at the protein concentrations used for the binding experiments, and does not contribute to the CD spectrum of the complex. In the g52P-A/tRNA titration experiments, the small (≤5%) spectral contribution of the aromatic CD of g52P-A was digitally subtracted from the UV-CD spectra of g52P-A-tRNA mixtures to obtain the corrected spectrum of the tRNA-g52P complex. All nucleic acid spectra were then corrected for dilution and the resulting corrected CD spectrum obtained. Fractional saturation (θ) at the ith addition of NC71 [NC71], to poly(A) was calculated on the positive Cotton peak (264 nm) from $\theta = \frac{(m_{\text{deg}} - m_{\text{deg}_0})}{m_{\text{deg}_0}}$ (data not shown). The far-UV CD spectrum of Z$_{\text{NN}}$ NC71 was determined with given values of $K_i$ (intrinsic monomer affinity), $\omega$ (cooperativity parameter), and $n$ (occluded site size) from Equation 1 (39) as follows,

$$\frac{\nu}{L\nu} = \frac{K_m(1 - \nu^j)}{[2(\omega(1 - \nu))][2(1 - \nu)]} \times \left[1 - (n + 1)\nu + R/[2(1 - \nu)]\right]$$

(1)

where $R = \theta/n$ and $L\nu = L - L_0$, where $L_0$ is free ligand [NC71] and $L$ is the bound NC71, $L\nu = \nu(\{\text{poly}A\})$. These curves were superimposed on the experimental data. The site size $n$ was determined independently at low [NaCl] and was assumed not to change with [NaCl]. Several nonstoichiometric titrations were subjected to nonlinear least-squares regression using the program NONLIN for Macintosh (Robelko Software) in order to obtain estimates of $\nu$ and $K_i$ with $n$ fixed at 15. The error given in these values reflects the statistical confidence limits.

**RESULTS**

**Bacterial Expression and Characterization of Mature HIV1 NC$^3$.** NC71—Fig. 1A shows that when pT7nc71.hiv is transformed into E. coli BL21(DE3)/pLysS, expression of a protein of apparent molecular mass less than 11 kDa is observed following induction of T7 RNA polymerase. NC71 has a predicted molecular mass of 8300 daltons. A typical chromatography run of the ssDNA-cellulose column is also shown (Fig. 1A). Note that at least two major fractions of NC are eluted from ssDNA-cellulose at different [NaCl] over the course of elution with a NaCl gradient (see “Experimental Procedures”). These two NC fractions differ in their primary structures in the N-terminal region (see “Experimental Procedures”). The tightest binding material, which contained authentic 1-71, with trace amounts of NC 4-71, was characterized in this study.

**Authentic NC71 as well as the two proteolyzed forms of NC, all contain ≥2 g at Zn(II) by atomic absorption and 6 cysteine thioes at titration with DTNB. A metal-free form of NC71, prepared by dialysis against EDTA, results in facile oxidation of most or all of the sulfhydryls, not unexpected based on previous findings with a fusion protein NC$^{15,16}$ (16).** Analytical gel filtration chromatography reveals that both forms of NC71 cochromatograph with RNase T$_1$, the expected position of the monomeric or dimeric form of the protein. This suggests that in the metal-free oxidized form of NC71, most or all of the Cys in NC71 participate in intramolecular disulfide bonds (28). One- and two-dimensional $^1$H NMR spectra of Z$_{\text{N}}$ NC71 show extensive similarities and chemical shift dispersion of previously published spectra of Z$_{\text{N}}$ HIV-1 NC 1-55 from virions (40), suggesting that both the recombinant and viral molecules have very similar conformations (data not shown). The far-UV CD spectrum of Z$_{\text{N}}$ NC71 shows the following transitions: at 238 nm, −500 deg-cm$^{-2}$.dmol$^{-1}$; 221 nm, +1400 deg-cm$^{-2}$.dmol$^{-1}$; 203 nm, −8300 deg-cm$^{-2}$.dmol$^{-1}$; and 189 nm, +1900 deg-cm$^{-2}$.dmol$^{-1}$. Analysis of these data suggest little standard a or b structure, and indicates that nonregular turn and coil secondary structures must predominate. These CD spectral features are roughly similar to those previously found for NC preparations from HIV-1 and other retroviral sources (16–18).

**NC71 Interacts Cooperatively with Poly(A)—Complex formation between NC71 and the homopolymer poly(A) was monitored by diminution of the intense Cotton bands in the UV circular dichroism spectrum of poly(A)$^3$ (see Fig. 3A, inset). NC71 unstacks or otherwise reduces the electronic coupling of adjacent bases of the helical poly(A) single strand; similar structural changes characterize binding by T4 gene 32 protein (41). **

$^3$ It proved problematic to measure NC71-poly-nucleotide complex formation by monitoring the quenching of NC71 protein Trp fluorescence since free NC71 exhibited a variable time-dependent loss in fluorescence. Quantum yield at low [NaCl] and protein concentrations required for these experiments. In addition, while the residual fluorescence of the Zn(II) complex was strongly quenched by poly(A) binding, the fluorescence of the metal-free apoprotein was quenched much less so (D. Giedroc, unpublished results).

**Fig. 1.** A, purification of HIV-1 NC71 by ssDNA-cellulose chromatography. $L_\nu$ and $z = 2$ h, total cellular lysates of E. coli BL21(DE3)/ pT7nc71.hiv before ($L_0$) and after (2 h) induction; LOAD (S), protein fraction loaded onto ssDNA-cellulose column; 12–35, fractions eluted from ssDNA-cellulose column with a linear 0.05 to 1.0 M NaCl gradient. See “Experimental Procedures” for details. B, deduced primary structure of recombinant HIV-1 NC71.

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**References**

The apparent site-size converted to fractional saturation (see "Experimental Procedures"). The CD intensity at 264 nm was recorded as a function of \( R, \) the molar ratio of total [NC71] to total poly(A) nucleotide, and the McGhee-von Hippel model (39) with the product \( K_{\text{app}(\infty)} \) held constant \( K_{\text{app}(\infty)} = K_{\alpha}(\infty) = 4.4 \times 10^{8} \, \text{M}^{-1} \), with moderate cooperativity (\( \omega = 170 \pm 25 \)) and \( n \) fixed at 15. Theoretical isotherms calculated by holding the product \( K_{\alpha}(\infty) \) constant with \( K_{\alpha} \) and \( \omega \) varied reciprocally reveal that \( \omega \) values 3-fold greater (\( \omega = 500 \), curve a) or 3-fold less (\( \omega = 60 \), curve c) than \( \omega = 170 \) do not fit the experimental data as well.

Proteins which associate with nucleic acids can derive much of their binding free energy from an increase in entropy which occurs upon the release of cations thermodynamically associated with the nucleic acid lattice in the formation of electrostatic interactions (43). The salt-dependence of the binding of NC71 to poly(A) was estimated by carrying out titrations like those described at various [NaCl] with all other buffer components held constant (Fig. 2C). Here we collect the data for three representative titrations carried out at 0.1, 0.21, and 0.29 M NaCl with [poly(A)] held at 1.3 \times 10^{-5} \, \text{M}. The smooth curves are the best-fit isotherms described by the McGhee-von Hippel model with \( n = 15 \) held at 170 and 15, respectively, and \( K_{\alpha} \) given by 1.4 \times 10^{6}, 4800, and 2600 \, \text{M}^{-1} \) at 0.1, 0.21, and 0.29 M NaCl. The inset plots the NaCl dependence in the form of a plot of log \( (K_{\alpha}, \omega) \) versus log [NaCl] or a log-log plot (43).

The NC71/poly(A) complex shows a moderate Na\(^{+}\) dependence with \( \partial \log (K_{\alpha}, \omega)/\partial \log [\text{NaCl}] = -3.8 \pm 0.7 \). Since the contribution of anion release from NC71 to this salt-dependence has not yet been determined but may be significant (cf. Ref. 44), this slope corresponds to \( -4.5 \pm 1.0 \) Na\(^{+}\) ions released from poly(A) upon the binding of a NC71 monomer (43). The binding affinity estimated at 1 M NaCl, \( K_{\text{app}(\infty)} = K_{\alpha}(\infty) \), remains significant, \( =3 \times 10^{8} \, \text{M}^{-1} \), suggesting that electrostatic interactions, while important, are supplemented by a significant nonelectrostatic free energy term.

**NC71 Can Denature tRNA**—The optical activity of tRNA in the near UV-CD spectrum originates with the A-like duplex regions of the molecule (34). Two structural forms of a mixture of *E. coli* tRNA (lysine, phenylalanine, serine, and valine-specific) were prepared which differ in the amount of tertiary structure present (33, 34). An “inactive” form was prepared by dialyzing tRNA in a buffer containing low monovalent salt concentration (50 mM NaCl) and no Mg\(^{2+}\). An “activated” tRNA form was prepared from this material by heat-denatured

represent theoretical isotherms described by the following parameters:

- **A**: \( n = 15, \omega = 170 \) for all three curves with \( K_{\alpha} = 1.4 \times 10^{6}, 4800, \) and 2600 \, \text{M}^{-1} \) for the 0.1, 0.21, and 0.29 M NaCl titrations, respectively. **Inset**, \( \partial \log (K_{\alpha}, \omega)/\partial \log [\text{NaCl}] \) (log-log plot) of the [NaCl] dependence of \( K_{\alpha}, \omega \) obtained from data in the main body of the figure.
ation, followed by refolding in the presence of 10 mM MgCl₂ at elevated temperature (33). The complete UV-CD spectra of these two forms indicate that the activated form is characterized by a positive Cotton band centered at 263 nm of slightly greater (11%) intensity than the inactive conformer, coupled with an extensive negative transition centered at 212 nm (Fig. 3A). These features mirror those reported previously (34). The activated form is much less susceptible to thermal denaturation than the inactive tRNA (Fig. 3A, inset), additional evidence that the Mg²⁺ complex has greater tertiary structure content (34).

Fig. 3B compares the relative efficacy of Zn₂ NC71 and recombinant bacteriophage T4 g32P-A, a C-terminal deletion product of the prototype helix-destabilizing protein, T₄ gene 32 protein, of denaturing both forms of tRNA<sub>mixed</sub>. This fragment, unlike native g32P itself, is able to efficiently unwind duplex regions in natural DNAs, provided a single-stranded nucleation site is present (38). NC71 induces a complete collapse of the Cotton effect in the near UV-CD spectrum of the inactive tRNA conformer at NC71/tRNA nucleotide ratio (R) of ~0.065. This represents physical evidence that NC71 can denature or unwind tRNA. The optical activity at 262 nm of the Mg²⁺ form, in contrast, is reduced by only 40–50%, even when twice as much NC71 is added. G32P-A is completely unable to reduce or otherwise alter the CD spectrum of either form of tRNA over the same range of protein concentration. The Cotton band of tRNA in the protein-tRNA complex is fully restored to its uncomplexed intensity by increasing the [NaCl] to greater than 0.7 M (data not shown). The spectral changes which result from NC71-induced denaturation of tRNA closely mimic the temperature-induced denaturation of the molecule. These changes can be summarized by an initial red shift in the positive Cotton band, followed by gradual diminution of the positive and negative CD bands (spectra not shown).

Fig. 3C compares the efficiency of tRNA unwinding by NC71 with g32P-A with the data expressed on a per tRNA molecule basis (taking an average of 75 nucleotides per E. coli tRNA molecule). From this plot it is clear that 5–6 monomer equivalents of NC71 are required to unwind inactive tRNA. G32P-A is far less effective; similar results were obtained for native g32P (data not shown). Also shown is the unwinding of tRNA by the zinc-free and thiol-oxidized form of NC71. The relative efficacies of tRNA unwinding by Zn₂ and apo-oxidized NC appear indistinguishable, a result consistent with the poly(A) titrations of the apo-oxidized protein (data not shown; see also Refs. 18, 19). Although the binding of NC71 to tRNA appears to result in a sigmoidal binding curve, suggestive of cooperativity and potentially consistent with the poly(A) data (Fig. 2B), the relationship between fractional change in the tRNA CD spectrum at a single wavelength (262 nm) and fractional saturation of the tRNA molecule by NC71 is not expected to be linear, since the overall shape of the CD spectrum is changed significantly upon NC71 binding (data not shown).

**DISCUSSION**

Darlix and coworkers (7–12) have recently reconstituted a replication complex capable of accurate minus strand strong stop cDNA synthesis in vitro from a variety of retroviral systems. The reaction minimally requires a 5’ primer binding site-containing genomic RNA fragment, tRNA primer, purified reverse transcriptase, and processed NC, which upon formation of a 16–18-bp heteroduplex, primes cDNA synthesis (1, 7–12). Although proviral synthesis occurred in the absence of added NC, addition of a few molecules of NC per viral RNA strand appeared to greatly increase the yield of cDNA products (11). Other bona fide single-strand binding proteins including E. coli recA and phage T₄ gene 32 protein (g32P) proved inactive in this activity (7–12). All physiochem-
ical studies published to date suggest that mature NC purified from many retroviral sources binds preferentially to ss nucleic acids (18, 28, 29). It is well established that stoichiometric unwinding by helix-destabilizing proteins, like T4 gene 32 protein (as opposed to catalytic unwinding by helicases), can promote intermolecular RNA duplex formation (annealing) by destabilizing intramolecular duplex regions (38, 42). Another aspect of this activity is the facilitation by helix-destabilizing proteins of correct intermolecular base-pairing by melting out incorrect structures. In contrast, a direct annealing activity, like that of a recombinase, for example (45), requires that the local concentration of complementary single-stranded sequences be greatly increased. With NC71, this could potentially be achieved with more than one RNA binding site per NC monomer, or NC-NC protein interactions. Recently, mammalian heterogeneous nuclear ribonucleoprotein A1, a ssRNA binding protein, was shown to facilitate intermolecular basepairing of RNA (46), apparently through a facilitated annealing rather than an unwinding mechanism.

Recombinant HIV-1 NC71 has the intrinsic capacity to unwind or denature duplex regions of tRNA in a stoichiometric fashion, with virtue of its affinity for ssRNA. Analytical experiments with the partially double-stranded DNA and RNA alternating copolymers (d[poly(A-T)] and r[poly(A-U)]) further confirm this. The occluded site size of 15 nucleotides per NC monomer determined with poly(A) predicts that one tRNA molecule could bind up to five to six NC monomers. This binding would occur concomitant with a collapse of the CD spectrum which derives from the A-form duplex structure. This is consistent with the stoichiometry that we observe with the inactive tRNA conformer, indicating that the binding is quite tight (Fig. 3C). T4 g32P is unable to unwind either form of tRNA (Fig. 3C). Thus, it is not necessary to invoke NC-RNA specificity to explain why g32P proved inactive in the primer tRNA annealing assay (8).

The Mg$^{2+}$ form of tRNA is less susceptible to NC-induced unwinding, with approximately 40–50% of the optical activity relatively resistant to NC binding. However, even this level of unwinding may be sufficient for intermolecular tRNA-primer binding site duplex formation since $^1H$ NMR studies of tRNA melting under similar solution conditions generally reveal that the tertiary structural basepairs and the anticodon and acceptor helices open first, followed by complete denaturation of these helices, then the TΨC helix, and finally the D helix (47). Thus the region that would be complementary to the viral primer binding site, e.g. the tertiary structural contacts at the elbow of “L”, and the acceptor helix in the Mg$^{2+}$ form, become single-stranded first. It is possible that these are the regions that NC is most efficient at unwinding. Finally, given the site size for NC71 of 15 ± 3 nucleotides, potentially as few as one or perhaps two bound NC71 molecules would be required to sufficiently denature even the active Mg$^{2+}$-tRNA conformer.

Complete collapse of CD signal of the inactive tRNA conformer is observed, indicative of uniform denaturation of a mixed population of tRNA molecules (Fig. 3B). Thus, the unwinding by NC71 is unlikely to be specific for the replication primer packaged by HIV-1, tRNA$^{\text{A1-71}}$ (12, 36). The reverse transcriptase may play a larger role in primer tRNA selectivity (11). The unwinding reaction clearly does not require Zn(II) coordination by NC71 (Fig. 3C), consistent with a recent study which showed the tRNA positioning in vitro is zinc-independent (10). The precise role of Zn(II)-binding by NC or the polyprotein continues to remain undefined, and awaits identification of a Zn(II)-dependent viral RNA ligand(s) (e.g. derived from the cis-acting encapsidation signal locus) (48) and/or detailed physiochemical characterization of the gag polyprotein itself or intermediate processed forms of gag (cf. Ref. 12).

Recombinant Zn$_2$ NC71 binds to ss nucleic acids with moderate cooperativity, $\omega = 170 ± 25$ (39). Previously reported binding properties of viral and synthetic NC preparations from other retroviral sources are generally indicative of relatively weak and weakly or noncooperative binding ($\omega = 1–20$) to homopolymers at low salt ($K_{m_p} = 10^4 M^{-1}$) and a moderately smaller site size, $n = 5 ± 1$ (18, 28, 29). These NC molecules previously characterized however, differ from HIV-1 NC in that in HIV-1 and other complex retroviruses, the nucleocapsid domain of gag is further processed just C-terminal to the second Cys-His region, to yield a p6 C-terminal subunit of unknown function (30). The nucleic acid binding properties of this fragment or that of the p15$^{\text{NC}}$ (16) progenitor remain unreported, but may well differ from mature NC71. Indeed, the first viral proteolytic cleavage event in HIV-1 maturation occurs at the mature N terminus of NC71, followed by liberation of the C-terminal p6 fragment (31). Thus the 108-amino acid p7–p6 peptide is transiently present prior to mature NC71 in virions; this suggests the possibility that the nucleic acid binding activities that we observe for NC71 may be regulated by the processing at the p7–p6 junction (49). This type of regulation is not without precedent. The C-terminal region of T4 gene 32 protein is a kinetic barrier for unregulated DNA duplex unwinding, the equilibrium binding parameters unchanged (38).

It is of interest to compare the conformational changes induced in tRNA by NC71 with those induced by another helix-destabilizing protein, mammalian UP1 (33, 42), now known to be a proteolytic fragment of the hnRNP A1 protein (50). UP1 was previously shown to bind to the inactive form of yeast tRNA$^{\text{A1-71}}$ and maximally depress the UV-CD Cotton band by 40–50%. Addition of 0.01 M MgCl$_2$ to the preformed NC71-inactive complex quickly brought about return of the tRNA spectrum to that indistinguishable from the native, fully folded active tRNA$^{\text{A1-71}}$. UP1 thus facilitates the renaturation of tRNA from an inactive to an active conformer in the presence of Mg$^{2+}$; these two conformers are nominally separated by a very large free energy barrier (33). The presence of Mg$^{2+}$, by virtue of stabilizing the native form of tRNA, must bring about dissociation of the transiently formed UP1-tRNA complex since UP1 has much lower affinity for duplex structures (33). A kinetic model was developed (42), whereby UP1 binds transiently to the inactive form, reducing the energy barrier to renaturation. UP1 therefore reduces the lifetime of the denatured intermediate, in promoting the correct intramolecular basepairing.

We find that NC71 can completely depress the Cotton effect of inactive tRNA, and also shows some ability to bind to and unwind the active or Mg$^{2+}$ form, unlike UP1. Furthermore, addition of 20 mM Mg$^{2+}$ to the preformed NC71-inactive tRNA complex formed in the absence of Mg$^{2+}$ only results in a partial (≈20%) return of the optical activity even after extended periods of incubation time (data not shown). It is possible that NC71 denatures tRNA and in so doing, forms a

4 R. Khan, unpublished results.
5 In the annealing experiments previously reported (7–12), it is unclear if the tRNA molecule contained the full complement of tertiary structure.
NC71-tRNA complex which, in contrast to the UP1 complex, is resistant to Mg\(^{2+}\) dissociation, with the result that intramolecular basepairing within tRNA is slow. If a complementary RNA molecule is present, intermolecular annealing would appear favored (7-12). This resistance might lie on the tRNA nucleic acid (51).

Quantitative studies of the interaction of NC71 with a single recombinant RNA species, as well as other partially double-stranded model RNAs of defined structure are required to further elucidate the molecular aspects of this and other NC-RNA complexes, the formation of which appear important for one or more stages in retroviral replication.

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