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Retroviral nucleocapsid proteins possess potent nucleic acid strand renaturation activity

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Abstract

The nucleocapsid protein (NC) is the major genomic RNA binding protein that plays integral roles in the structure and replication of all animal retroviruses. In this report, select biochemical properties of recombinant Mason-Pfizer monkey virus (MPMV) and HIV-1 NCs are compared. Evidence is presented that two types of saturated Zn_2 NC–polynucleotide complexes can be formed under conditions of low [NaCl] that differ in apparent site-size ($n = 8$ vs. $n = 14$). The formation of one or the other complex appears dependent on the molar ratio of NC to RNA nucleotide with the putative low site-size mode apparently predominating under conditions of protein excess. Both MPMV and HIV-1 NCs kinetically facilitate the renaturation of two complementary DNA strands, suggesting that this is a general property of retroviral NCs. NC proteins increase the second-order rate constant for renaturation of a 149-bp DNA fragment by more than four orders of magnitude over that obtained in the absence of protein at 37 °C. The protein-assisted rate is 100–200-fold faster than that obtained at 68 °C, 1 M NaCl, solution conditions considered to be optimal for strand renaturation. Provided that sufficient NC is present to coat all strands, the presence of 400–1,000-fold excess nonhomologous DNA does not greatly affect the reaction rate. The HIV-1 NC-mediated renaturation reaction functions stoichiometrically, requiring a saturated strand of DNA nucleotide:NC ratio of about 7–8, rather than 14. Under conditions of less protein, the rate acceleration is not realized. The finding of significant nucleic acid strand renaturation activity may have important implications for various events of reverse transcription particularly in initiation and cDNA strand transfer.

Keywords: nucleic acid strand renaturation; nucleocapsid protein; retroviral replication; single-strand binding protein; zinc-finger

The *gag* gene product of all known retroviruses encodes a multifunctional polyprotein precursor product that has been shown to play fundamental roles in virus genomic RNA encapsidation and replication (Varmus & Brown, 1989). These RNA binding activities are mediated by the NC domain of the precursor or processed NC subunit (Leis et al., 1988; Varmus & Brown, 1989). Numerous groups have shown that NC from all animal retroviruses contains one or two conserved Cys-Xaa_2-Cys-Xaa_4-His-Xaa_4-Cys retroviral-type zinc-finger Cys_3His motifs (Henderson et al., 1981; Berg, 1986), which can be reconstituted with Zn(II), Cd(II), and Co(II) in vitro (Green & Berg, 1989; Roberts et al., 1989; South et al., 1989; Fitzgerald & Coleman, 1991; Khan & Giedroc, 1992). Recent studies provide evidence that these Zn(II) domains are present in vivo in mature virus preparations (Bess et al., 1992; Summers et al., 1992). These domains are thus reminiscent of "zinc-finger" domains in other nucleic acid-binding proteins (Berg, 1990). It is generally accepted that processed NC is bound nonspecifically in a histone-like condensation of the diploid RNA genome in mature virus preparations (Chen et al., 1980).

The molecular details of RNA binding of any kind by NCs and the role that Zn(II) plays remain poorly defined. Recent solution structural studies of the Zn(II)-complexed form of NC from HIV-1 virions (a 55-amino acid protein) and related synthetic peptides reveal that this molecule is remarkably lacking in stable secondary structure outside of the two structurally well-defined Zn(II)-coordination

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Abbreviations: ss, single-stranded; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; Na_3EDTA, trisodium ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(nitrobenzoic acid); DTT, dithiothreitol; MPMV, Mason-Pfizer monkey virus; HIV-1, human immunodeficiency virus; g32P, gene 32 protein; SSB, single-strand binding protein; PCR, polymerase chain reaction; MoMLV, Moloney murine leukemia virus; TFA, trifluoroacetic acid; NC, nucleocapsid protein; PMSF, phenylmethylsulfonylfluoride.

domains (Omichinski et al., 1991; Summers et al., 1992). The zinc-finger structure itself is probably highly conserved in the retroviral NC family (Berg, 1986). Because NC shows so little regular structure, a well-defined RNA binding groove or surface is difficult to identify. Recent studies have focussed on the zinc-fingers themselves as primary RNA binding determinants (Delahunty et al., 1992; Summers et al., 1992), but other biochemical and molecular genetic studies (Fu et al., 1988; Cornille et al., 1990; Secnik et al., 1990; Khan & Giedroc, 1992) suggest the highly positively charged N-terminus and/or interfinger regions may be involved. Indeed, these regions are perhaps ideally suited to potentially adopt one or more defined conformations in the presence of a wide range of RNA structural contexts, exactly the type of binding one would expect to find in sequence nonspecific genome condensation (Churchill & Travers, 1991).

It is generally the case that retroviral particles that are formed when Zn(II) coordinating ligands and adjacent amino acids are altered show dramatically reduced genomic RNA packaging efficiencies. At least some of this loss in packaging efficiency seems to be partially attributable to a loss in specific recognition of viral RNA sequences by Zn(II) NC in vivo (Dupraz & Spahr, 1992). All such defective particles have markedly reduced infectivities (by $\geq 10^5$-fold) and are nonreplicative, apparently independent of the amount of genomic RNA actually packaged (Méric & Spahr, 1986; Méric & Goff, 1989; Aldovini & Young, 1990; Dupraz et al., 1990; Gorelick et al., 1990). These studies additionally suggest that NC plays a central role in the structural and replicative integrity of the reverse transcriptionally active ribonucleoprotein complex, although the precise role of the Zn(II) domains remains undefined (Chen et al., 1980). One such function appears to occur in the initiation of proviral cDNA synthesis by reverse transcriptase. NC from both HIV-1 and MoMLV have been shown to promote the base-pairing or annealing of the cognate replication primer tRNA onto genomic viral fragments containing the complementary primer binding site (Prats et al., 1988; Bieth et al., 1990). RNA analysis of avian viral particles that contain a moderately defective mutant NC show that the replication primer is not stably base-paired to high molecular weight RNA as it is in wild-type virions (Prats et al., 1988). It is currently unknown whether NC directly influences other steps of reverse transcription, although a complete or near-complete abolition of infectivity in all mutants, even those that package significant amounts of highly structured virion RNA, suggests the possibility of one or more further defects in viral cDNA synthesis (Méric & Spahr, 1986; Méric & Goff, 1989).

Because tRNA annealing is a specialized case of nucleic acid strand renaturation, we have investigated the mechanism of renaturation of a completely homologous 149-nt DNA fragment in the presence and absence of highly purified recombinant NC proteins. We show here that both recombinant NC proteins from MPMV and HIV-1 have potent nucleic acid strand renaturation activities. The rate enhancements observed substantially exceed that expected for a simple melting of incorrectly paired intramolecular duplex regions by a single-strand binding protein. As the NC-mediated renaturation reaction remains second order, the data suggest that transient but productive interactions between NC-coated nucleic acid strands greatly enhance the bimolecular nucleation rate. The implications of this activity on fundamental aspects of reverse transcription other than in the initiation stage are discussed.

Results

The binding of MPMV Zn_2 NC96 and HIV-1 Zn_2 NC71 to model RNA polynucleotides

Forward or normal titrations

A forward or normal titration refers to the incremental addition of protein (ligand NC) to a polynucleotide lattice (macromolecule). In this titration, the protein binding density proceeds in a conceptually normal manner, from low to high binding density. Complex formation in a forward titration between NC96 and the ribohomopolymer poly(A) was monitored by diminution of the intensity of the UV CD spectrum of poly(A) (Fig. 1A) as well by monitoring the NC96 Trp fluorescence in the presence of polynucleotide (Fig. 1B) under solution conditions of low [NaCl] at pH 8.3 and 25 °C. These conditions proved to be tight binding conditions (see Materials and methods), thus permitting an estimate of the occluded site size (n_{app}) or saturation point. In Figure 1A, the area of the CD spectrum of poly(A) is incrementally reduced in intensity as NC96 binds until apparent saturation by the protein is obtained, defined as the point that the spectrum ceases to change. The isotherm shown plots the fractional change in ellipticity at 264 nm as a function of total added NC96. This technique has been widely used with other ss nucleic acid binding proteins and has been shown to give excellent estimates of site-size or saturation points consistent with other optical techniques (Karpel & Burchard, 1980; Nadler et al., 1991). Under these conditions, it appears that NC96 binds with an occluded site size of 13.6 nt/NC96 monomer.

Figure 1B shows that the same apparent value of n is obtained while monitoring the complex formation at the NC96 Trp fluorescence. In this "forward" or normal titration, the initial slope represents the protein fluorescence yield as all added protein binds to the poly(A). After all of the poly(A) is saturated with MPMV NC, a second slope is obtained that represents the fluorescence yield of uncomplexed protein. The intersection of the two lines represents the apparent saturation point, $n_{app} = 1/R$ (Lohman & Overman, 1985). This experiment reveals that the Trp fluorescence of NC96 is strongly quenched upon binding to poly(A) and that the apparent stoichio-

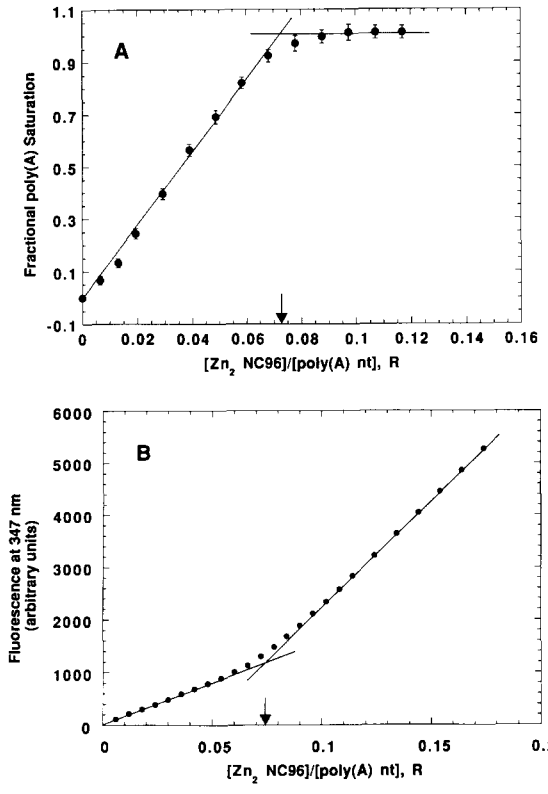

Fig. 1. Forward titrations of the binding of MPMV NC96 to poly(A) under tight binding conditions monitored by CD (A) and fluorescence (B) spectroscopies. **A:** The fractional change in the ellipticity of 23.6 μM (nucleotide) poly(A) at 264 nm was recorded as a function of added Zn_2 NC96. The intersection of the two lines representing apparent saturation of the poly(A) lattice occurs at $R = 1/n_{app}$ (indicated by the arrow), giving $n_{app} = 13.6$. **B:** The intrinsic fluorescence of increasing concentrations of NC96 in the presence of 20.0 μM poly(A) was measured and expressed as a function of molar ratio, R, as in A. The intersection of the two lines (indicated by the arrow; see text) occurs at $R = 1/n_{app}$, giving $n_{app} = 13.7$. Conditions: 10 mM Tris-HCl, pH 8.3, 0.1 M NaCl, 0.1 mM DTT, 25 °C. Other experiments carried out at 20 mM NaCl gave n_{app} 13.3 nt.

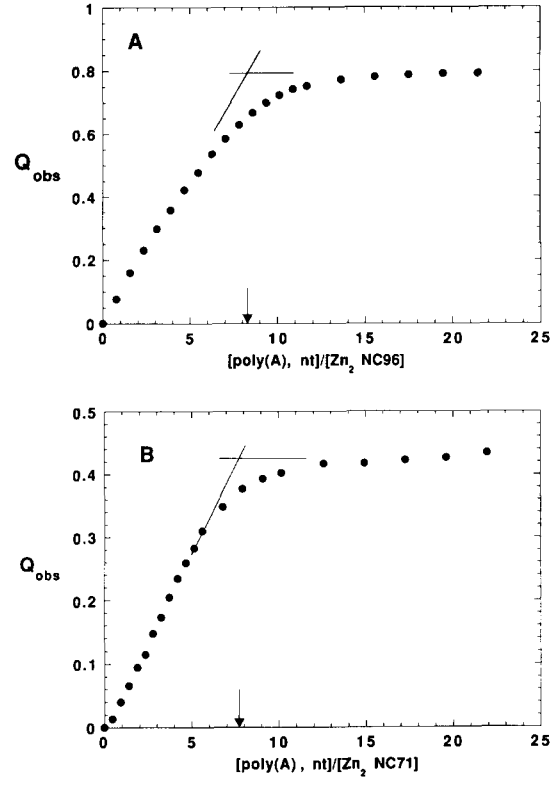

Fig. 2. Reverse fluorescence titrations monitoring the binding of Zn_2 NC96 (**A**) and Zn_2 HIV-1 NC71 (**B**) to poly(A) under tight binding conditions. The quenching of the intrinsic fluorescence of 1.6 μM NC96 or 2.89 μM NC71 (Q_{obs}) was determined as a function of added poly(A), expressed as the poly(A) nucleotide/NC molar ratio. The intersection of the two solid lines obtained by extrapolation yields n_{app} under these conditions (see arrow; approximately 8 nt in each case). Conditions are identical to those of Figure 1: 10 mM Tris-HCl, pH 8.3, 0.1 M NaCl, 0.1 mM DTT, 25 °C.

metric point is about 13–14 nt/NC96 monomer, consistent with the CD experiment (Fig. 1A).

Reverse titrations

Figure 2A shows a representative binding isotherm obtained for MPMV NC96 with complex formation again monitored by the quenching of Trp fluorescence (cf. Fig. 1B). In this case, however, a "reverse" titration under tight binding conditions (0.1 M NaCl) is shown. In a reverse titration, fractional saturation of the *protein* is monitored; RNA binding proceeds from high binding density, conditions where excess free protein is initially present, to low binding density, in which the concentration of free protein is dramatically reduced (Giedroc et al., 1990). Strikingly, the apparent saturation point is slightly more than *one-half* ($n_{app} = 8.2$) that found in the forward fluorescence titration (low to high binding density; Fig. 1B). The same findings characterize HIV-1 NC71 as well ($n_{app} = 7.7$; Fig. 2B). These data suggest the novel finding that NC proteins are capable of binding to poly(A) such that at least two types of distinguishable, apparently saturated complexes that differ in site-size by a factor of ≈ 2 can be formed.

Both MPMV NC96 and HIV-1 NC possess potent nucleic acid strand renaturation activity

The previously reported ability of NC proteins to facilitate the intermolecular base-pairing between replication primer tRNA and complementary RNA or DNA sequences (Prats et al., 1988) can be viewed as a specialized case of a general nucleic acid strand renaturation activity. Proteins that bind preferentially to ss rather than duplex DNA can, under certain solution conditions, exhibit the ability to catalyze the rate of renaturation of nucleic acid strands. *Escherichia coli* SSB and T4 gene 32 protein have both been shown to catalyze the renaturation rate under de-

fined solution conditions (Alberts & Frey, 1970; Christiansen & Baldwin, 1977). In this section, we address to what extent and with what characteristics NC proteins catalyze strand renaturation in general.

If NC is functioning as these classical helix-destabilizing proteins, it should accelerate the intermolecular base-pairing of two perfectly complementary single strands with the following characteristics: (1) The overall kinetic order will be second order in DNA concentration in the presence (Alberts & Frey, 1970; Pontius & Berg, 1990), as it is in the absence (Wetmur & Davidson, 1968), of protein. This is so because duplex formation is a bimolecular reaction, with the slow or rate-determining step being nucleation of helix, followed by fast zippering up of the duplex. (2) The maximum rate enhancement due to protein binding requires sufficient NC to *saturate* both strands, which requires knowledge of the occluded site size (Khan & Giedroc, 1992) (see above). Saturated strands are required because protein binding presumably melts out adventitious secondary intramolecular structures, thereby increasing the probability that any productive nucleation event will occur. A first-order reaction (half-time for renaturation is independent of [DNA]), on the other hand, is known to characterize the strand renaturation activity of recA protein (Weinstock et al., 1979), β protein of bacteriophage λ (Muniyappa & Radding, 1986) and presumably other recombinases.

The kinetics of renaturation of a denatured 149-bp end-labeled duplex were obtained in the presence and absence of NC71 and MPMV NC96 essentially as described by Pontius and Berg (1990). Duplex formation from isolated single strands was monitored by nondenaturing polyacrylamide gel electrophoresis, which easily resolves ss and double-stranded molecules. Figure 3 compares the time-course obtained (37 °C, 80 mM NaCl, pH 7.0) for the renaturation of the DNA duplex from isolated single strands in the absence of protein, in the presence of 30 nM NC71, and proteinase K-treated NC71 as indicated. The facilitation of this reaction by NC71 is quite pronounced under these conditions with the $t_{1/2}$ for renaturation reduced to less than 30 s under conditions where there is very little non-protein-mediated reaction. The reaction requires the covalent structure of NC71 as proteinase K pretreatment abolishes the rate enhancement. As these experiments are carried out in the presence of 1 mM EDTA, the metal-free form of NC71 is likely to be present in all cases (Khan & Giedroc, 1992). In another experiment, the renaturation reaction was carried out in the presence of 10 μM exogenous Zn(II) in the absence of EDTA with apparently little or no effect on the renaturation rate (Table 1).

Figure 4 shows a quantitation of representative renaturation reactions where the concentration of ssDNA remaining is plotted as a function of incubation time. In Figure 4A,B, NC71 and MPMV NC96 are present at 30 and 90 nM, respectively, with 0.45 nM and 0.6 nM ssDNA at 37 °C, 0.08 M NaCl. In Figure 4C, the renaturation of 9.0 nM ssDNA is carried out in 1 M NaCl, 68 °C. These are conditions under which strand–strand electrostatic repulsion is minimized, and adventitious secondary structures are essentially melted out (Wetmur & Davidson, 1968). Figure 4D shows an experiment carried out with 45 nM ssDNA in the absence of any protein at 37 °C, 80 mM NaCl. The solid lines superimposed on the experimental data are theoretical curves that describe a second-order rate of decay of ssDNA. As expected, the second-order rate constant derived from these experiments over a 20-fold range in initial [ssDNA] (c_0) proved to be independent of the [ssDNA] (Table 1) (Wetmur & Davidson, 1968). Further support for a second-order reaction is given in Figure 5. Here, the $\ln(t_{1/2})$ is plotted against the $\ln(c_0)$ in the presence of NC71 and MPMV NC96 relative to the reaction carried out under 68 °C and 1 M NaCl conditions. A slope of -1 in this plot is indicative of second-order behavior. This is clearly the case for

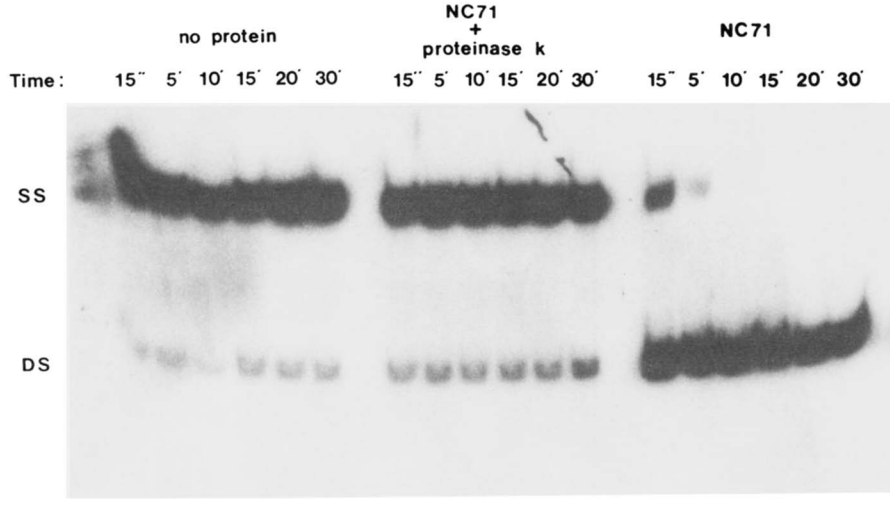

Fig. 3. NC71 catalyzes the renaturation of complementary 149-nt DNA single strands. 5′-^{32}P-end-labeled DNA at 4.5 nM ssDNA (expressed in nucleotides) was prepared as described in Materials and methods and incubated at 37 °C in the presence of no added protein, 30 nM NC71 pretreated with proteinase K, and 30 nM NC71 in 80 mM NaCl, pH 7.0, as indicated. Following a 2-min preincubation, individual aliquots were withdrawn from the reaction mixtures at the indicated times, prepared for electrophoresis, and subjected to electrophoresis as described through a 10% nondenaturing polyacrylamide gel. The migration of the fully duplex (DS) and single-strand (SS) are indicated.

Table 1. *Second-order rate constants (k_2) obtained for the renaturation of a 149-bp DNA fragment*[a]

[DNA] (M nt) ($\times 10^9$)	[Protein]	Conditions[b]	Temperature	k_2 ($M^{-1} \cdot s^{-1}$)
Standard conditions				
23	–	A, 80 mM NaCl	37 °C	870
45	–	A, 80 mM NaCl	37 °C	850
[DNA] dependence in 1 M NaCl, 68 °C				
9.0	–	A, 1 M NaCl	68 °C	7.2×10^4
4.5	–	A, 1 M NaCl	68 °C	6.9×10^4 (± 0.2)
1.8	–	A, 1 M NaCl	68 °C	6.2×10^4
0.9	–	A, 1 M NaCl	68 °C	6.2×10^4
Average		A, 1 M NaCl	68 °C	6.7×10^4 (± 0.5)
[NC71] dependence				
4.5	7.5 nM NC71	A, 80 mM NaCl	37 °C	3.3×10^4
4.5	15 nM NC71	A, 80 mM NaCl	37 °C	1.2×10^5
4.5	60 nM NC71	A, 80 mM NaCl	37 °C	2.4×10^7
4.5	120 nM NC71	A, 80 mM NaCl	37 °C	1.7×10^7
[DNA] dependence in presence of saturating NC71				
4.5	30 nM NC71	A, 80 mM NaCl	37 °C	1.3×10^7 (± 0.8)
1.8	30 nM NC71	A, 80 mM NaCl	37 °C	1.7×10^7
0.90	30 nM NC71	A, 80 mM NaCl	37 °C	1.9×10^7
0.45	30 nM NC71	A, 80 mM NaCl	37 °C	1.1×10^7 (± 0.1)
0.30	30 nM NC71	A, 80 mM NaCl	37 °C	1.0×10^7 (± 0.7)
0.23	30 nM NC71	A, 80 mM NaCl	37 °C	1.1×10^7
Average	30 nM NC71	A, 80 mM NaCl	37 °C	1.4×10^7 (± 0.6)
4.5	30 nM NC71	A, 80 mM NaCl, 10 mM $MgCl_2$	37 °C	4.9×10^6
NC71 Zn(II) dependence				
4.5	30 nM NC71	B, 80 mM NaCl	37 °C	7.3×10^6 (± 2.5)
[NC96] dependence				
4.5	30 nM NC96	A, 80 mM NaCl	37 °C	3.5×10^5 (± 0.5)
4.5	60 nM NC96	A, 80 mM NaCl	37 °C	9.8×10^6
4.5	90 nM NC96	A, 80 mM NaCl	37 °C	1.4×10^7 (± 0.6)
4.5	180 nM NC96	A, 80 mM NaCl	37 °C	2.0×10^7 (± 0.3)
4.5	270 nM NC96	A, 80 mM NaCl	37 °C	1.9×10^7 (± 0.4)
[DNA] dependence in the presence of saturating NC96				
1.8	180 nM NC96	A, 80 mM NaCl	37 °C	2.2×10^7
0.9	180 nM NC96	A, 80 mM NaCl	37 °C	2.1×10^7
0.6	180 nM NC96	A, 80 mM NaCl	37 °C	1.8×10^7
0.45	180 nM NC96	A, 80 mM NaCl	37 °C	2.2×10^7
T4 g32P				
45	60 nM g32P	A, 80 mM NaCl	37 °C	1,070
45[c]	500 nM g32P	C, 10 mM KCl	37 °C	1,000
45[c]	500 nM g32P	C, 10 mM KCl, 40 mM $MgSO_4$	37 °C	1.2×10^4

[a] Second-order rate constants were determined as described in Figure 6 and Materials and methods.

[b] Buffer A is 10 mM potassium phosphate, 1 mM Na_3EDTA, pH 7.0. Buffer B is 10 mM potassium phosphate, 0.1 mM DTT, 10 μM $ZnCl_2$, pH 7.0. Buffer C is 2 mM Tris-HCl, pH 8.0, 1 mM β-mercaptoethanol, 0.1 mM Na_3EDTA, 2% glycerol (solution conditions of Alberts & Frey, 1970).

[c] Renaturation of the indicated concentration of a 136-bp *Ban*I fragment (see Materials and methods). For comparison, k_2 was found to be 1.0×10^4 $M^{-1} \cdot s^{-1}$ at 68 °C, 1 M NaCl; 1.3×10^6 $M^{-1} \cdot s^{-1}$ in the presence of 30 nM NC71; and 1.5×10^6 $M^{-1} \cdot s^{-1}$ in the presence of 90 nM MPMV NC96 with this fragment.

ssDNA alone in 1 M NaCl and 68 °C (−1.04) and also seems to be the case for the protein-mediated reaction (slope = -1.07 ± 0.20).

Second-order rate constants for the renaturation of this ssDNA are compiled in Table 1 under a variety of different conditions. HIV-1 NC71 has potent strand renaturation activity, increasing k_2 by some 200-fold over the 68 °C, 1 M NaCl, conditions and by four or more orders of magnitude over the uncatalyzed rate at 37 °C. Analogous findings were obtained with another DNA molecule of comparable size (136 nt) but different sequence (see Materials and methods), evidence that the renaturation

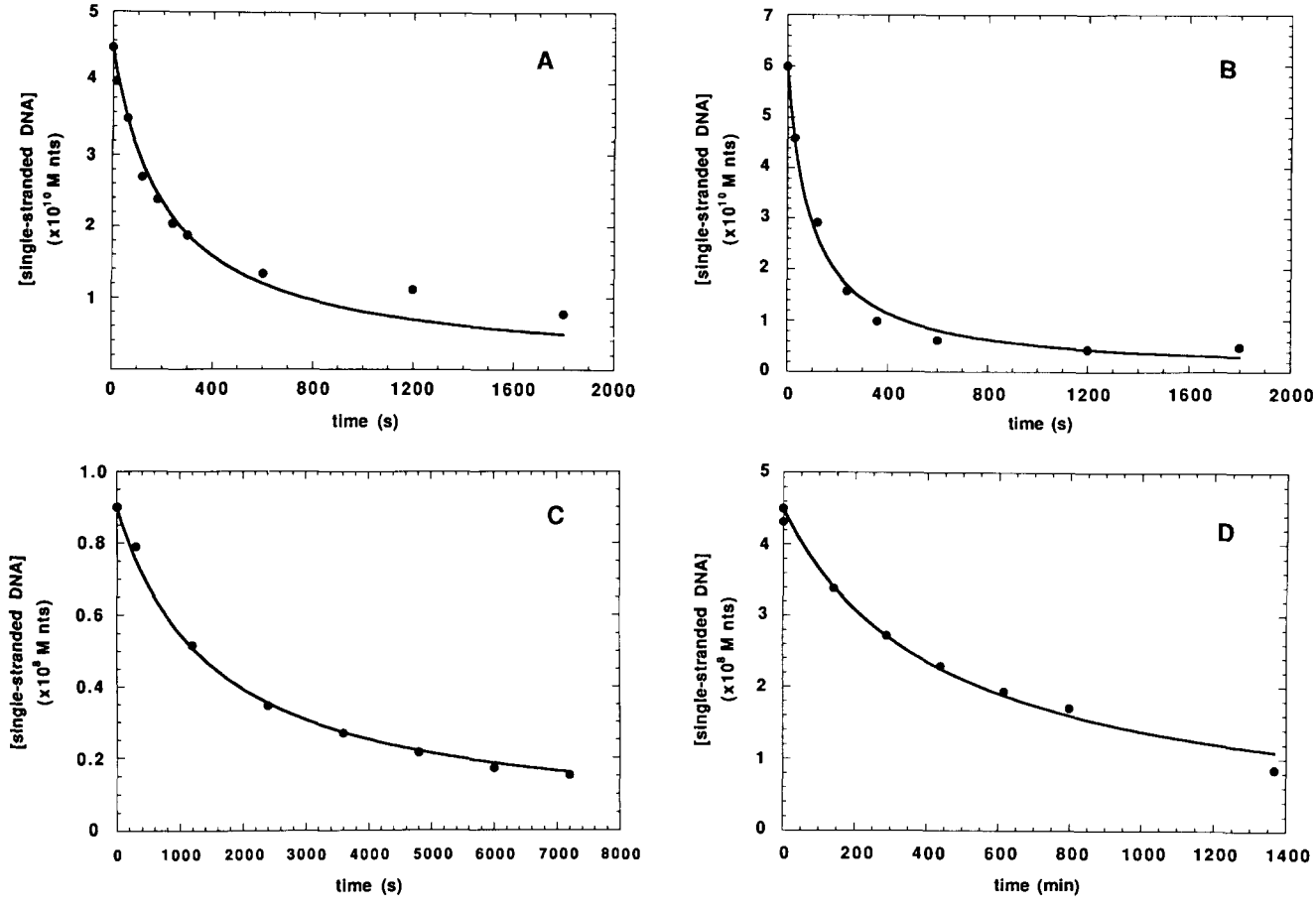

Fig. 4. Representative kinetic plots of the rate of renaturation of a 149-bp DNA duplex in the presence of 30 nM NC71, 37 °C (**A**); 90 nM MPMV NC96, 37 °C (**B**); no added protein, 68 °C, 1 M NaCl (**C**); and no added protein, 37 °C (**D**). Conditions: 10 mM sodium phosphate, pH 7.0, 1 mM Na_3EDTA, 80 mM NaCl, 37 °C (unless otherwise noted). Each solid curve shown in each panel represents a nonlinear least-squares nonweighted fit to a simple second-order decay of ss to duplex DNA characterized by the following second-order rate constants (k_2, M nt^{-1} · s^{-1}): A, 1.0×10^7; B, 8.9×10^6; C, 7.1×10^4; D, 845.

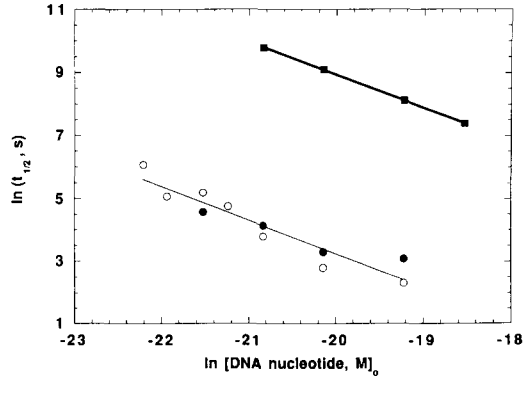

Fig. 5. Kinetics of the DNA renaturation expressed as a plot of the natural logarithm of the half-time for DNA strand renaturation, $t_{1/2}$ (s), of the indicated concentration of ssDNA (c_0) vs. the natural logarithm of the initial ssDNA concentration (c_0, in M nt) in the absence of added protein, 1 M NaCl, 68 °C (■), or in the presence of 30 nM NC71 (○) or 180 nM MPMV NC96 (●), 80 mM NaCl, 37 °C. The solid lines represent linear least-squares fits of each set of data and give the following slopes: 68 °C, 1 M NaCl, −1.04; NC-assisted, −1.07 (±0.20).

acceleration by NC is a general one (Table 1). We have noted that the renaturation activity of NC proteins appears to decrease upon extended periods of storage or repeated freezing and thawing (up to ca. fivefold), apparently without any detectable change in the equilibrium binding properties of the protein (F.D.-H. & D.P.G., unpubl.). Similar findings have been reported for the T4 gene 32 protein (Alberts & Frey, 1970). MPMV NC96 appears as active as HIV-1 NC71 under the same conditions (Table 1), although it seems to take slightly higher [NC96] to drive the reaction at maximal rates. To illustrate, the NC concentration dependence at a constant amount of ssDNA is shown in Figure 6 for HIV-1 NC (Fig. 6A) and MPMV NC96 (Fig. 6B). Here, the extent of renaturation is plotted as the mole fraction of duplex nucleotides formed at a single incubation time (5 min) as a function of [NC]. As expected for a reaction that functions stoichiometrically, there is a relatively narrow range over which the reaction is either greatly facilitated by NC or it is not (Alberts & Frey, 1970; Pontius & Berg, 1990). An NC71 concentra-

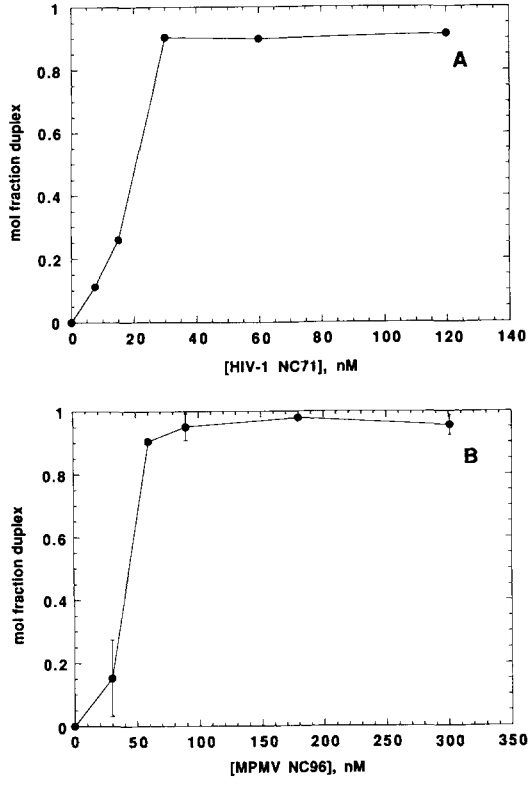

Fig. 6. The extent of renaturation of a 149-bp duplex DNA as a function of NC71 concentration (**A**) or MPMV NC96 concentration (**B**). The extent of renaturation following a 5-min incubation in standard conditions, 37 °C in the presence of the indicated concentration of protein was determined as described in Figure 5 and Materials and methods and expressed as mole fraction of duplex nucleotides.

tion of 30 nM appears to be about the level of protein required under these conditions, below which the reaction is very slow and above which the reaction appears largely indistinguishable from the 30 nM rate. For MPMV NC96, the sharp breakpoint occurs at a concentration greater than 30 nM but less than 60 nM. Quantitation of the rate data supports these conclusions (Table 1).

NC71 catalyzes renaturation of a 149-bp duplex via formation of the n = 8 saturated complex even in the presence of a large excess of nonhomologous DNA

We next compared the efficiency of renaturation of the denatured 149-bp duplex at two high concentrations of NC71 (260 and 800 nM) in the presence of increasing amounts of nonhomologous ϕX174 ssDNA. These are conditions under which essentially all of the input NC71 and ssDNA will be bound at subsaturating amounts of protein (Khan & Giedroc, 1992). The ssDNA concentration was held at 4.5 nM. Figure 7 shows the extent of renaturation at a single incubation time expressed as the mole fraction of duplex nucleotides as a function of the

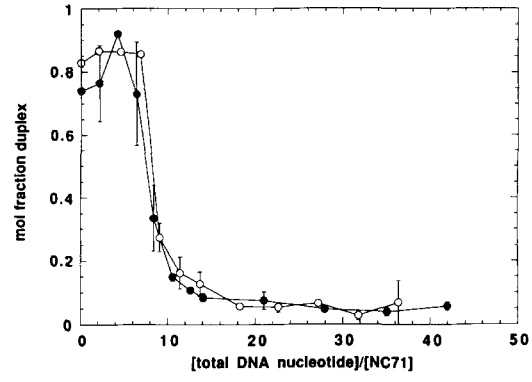

Fig. 7. NC71-facilitated renaturation of 4.5 nM ssDNA as a function of an increasing concentration of nonhomologous ϕX174 ssDNA, with the total concentration of DNA expressed as a [DNA nucleotide]/[NC71] ratio. Five-minute renaturation reactions were performed in duplicate in the presence of 260 nM NC71 (●) or 800 nM NC71 (○), processed, and subjected to electrophoresis as described in Figure 3 and Materials and methods. The error bars represent the range in the determinations made in two independent experiments.

total DNA nucleotide/NC71 ratio. As can be seen, the extent of renaturation appears relatively unaffected until a ratio in excess of 7–8 nucleotides per NC71 monomer is present. At higher ratios the extent of renaturation essentially returns to background or uncatalyzed levels. These data strongly suggest that NC71-catalyzed renaturation requires an NC71-coated strand bound in the $n = 8$ site-size mode. Note also that in the 800 nM NC71 reaction mixtures at molar ratios just under seven, even a 1,000-fold excess of nonhomologous DNA does not greatly influence the kinetics of renaturation of the 149-bp fragment. NC71 will also catalyze the base-pairing of one strand of the denatured 149-bp duplex to a complementary region contained within a 3-kb ss circle (data not shown).

Discussion

Retroviral nucleocapsid proteins have been shown to bind to both DNA and RNA and appear to preferentially interact with ss sequences (Davis et al., 1976; Nissen-Meyer & Abraham, 1980). These proteins appear kinetically competent to destabilize or unwind a variety of double-stranded nucleic acids and can therefore be considered helix-destabilizing proteins (Khan & Giedroc, 1992; Summers et al., 1992). High-resolution solution structural studies of synthetic peptides and virally produced NC from HIV-1 seem to indicate that whereas the two Zn(II) coordination domains adopt well-defined and folded structures that are structurally very similar, the other regions of the protein, including the interfinger and N-terminal domains, do *not* appear to adopt a defined secondary or tertiary structure in solution. Comparatively little is known about how Zn(II)-complexed retroviral NC proteins interact with nucleic acids of any kind, although

much attention has focused on the Zn(II) domains as providing at least a subset of important protein–RNA interactions (Delahunty et al., 1992; Summers et al., 1992).

The binding of both Zn_2 NC proteins to the model polynucleotide poly(A) appears complex, apparently exhibiting binding site-size heterogeneity under these conditions. If polynucleotide binding by NC results in formation of a single nucleoprotein complex under tight binding conditions, then the apparent site-sizes determined with the same optical probe (NC fluorescence quenching) would be *identical* regardless of whether the binding isotherm proceeds in a forward or reverse direction. The forward (Fig. 1) and reverse (Fig. 2) titration data collected for NC96 under tight binding conditions suggest that as many as two putative binding modes on polynucleotides are available to NC, which differ in apparent site-size by a factor of ≈1.8. One binding mode would predominate under conditions of limiting protein and excess nucleic acid (i.e., at the beginning of a forward titration and the end of a reverse titration) and is characterized by a larger apparent saturation point ($n_{app} \approx 14$). The other putative binding mode must predominate under conditions of excess protein (i.e., at the end of a forward titration and the beginning of a reverse titration) and is characterized by a smaller apparent site size ($n_{app} \approx 8$). If these two binding modes interconvert in such a way that there is no or little detectable change in the optical signal beyond the apparent saturation point, then the transition will go unnoticed in *both* forward and reverse titrations. Alternatively, the complex interconversion may simply be slow kinetically and thus will not occur to an appreciable extent under these conditions. Interestingly, a previously reported single titration of the RNA homopolymer poly(ethenoadenylic) acid with NC isolated from simian immunodeficiency virus was suggestive of a biphasic isotherm with clear deviations at ratios of ≈15 and 8 nucleotides per NC monomer (Henderson et al., 1988), potentially consistent with two types of complexes that change the fluorescence of the polynucleotide to differing degrees.

At low salt concentration, the apparent site-sizes of the two proteins on poly(A) and tRNA (Khan & Giedroc, 1992; D.P.G., unpubl.) appear comparable (Figs. 1, 2). This suggests that the N-terminal, C-terminal, and interfinger extensions of NC96 relative to NC71 do not greatly influence the site-size. This is particularly surprising because NMR structural studies of HIV-1 NC (Omichinski et al., 1991; Summers et al., 1992) and molecular modeling of NC96 (D.P.G., unpubl.) suggests that the interfinger domain separation can be as much as ≈25 Å in Zn_2 NC71 but ≈45 Å in NC96, in the event that the linker regions adopt a relatively extended polypeptide chain conformation. For example, assuming an extended polynucleotide chain (5.5 Å between bases), this corresponds to as few as five bases for NC71, and as many as nine bases for NC96. The finding of similar apparent site-sizes for the two proteins perhaps suggests that the path of the RNA across each NC monomer may be quite different.[1]

At least some of the physicochemical properties of NC–ss nucleic acid complexes are not unprecedented when one considers the binding of other ss binding proteins to ss homopolymers. For example, *E. coli* SSB is known to bind to ssDNA in two different binding modes, a highly cooperative nonequilibrium low site-size mode ($n_{app} = 33$) and a weakly cooperative high site-size binding mode ($n_{app} = 65$) (Griffith et al., 1984; Lohman & Overman, 1985; Bujalowski et al., 1988). The low-site form is only formed under conditions of excess SSB and low [NaCl] (Lohman et al., 1986), analogous to what we see with NC proteins (Fig. 2). In addition, the interconversion between binding modes in SSB appears to be extremely slow kinetically (Lohman et al., 1986). With NC, we have preliminary evidence that additional NC can be readily incorporated into a saturated $n = 14$ complex to form the $n = 8$ complex on poly(A). In contrast, additional poly(A) apparently cannot be incorporated into the saturated $n = 8$ complex under the same conditions, even over the course of several hours at 22 °C.[2]

It is well established that single-strand specific binding proteins, like T4 g32P, can accelerate the forward reac-

[1] On the other hand, the net charges of NC71 and NC96 at pH 8.3 are +14 and +11, respectively (the latter assuming normal pK_a's for four non-zinc-finger His in NC96), quite close to the number of nucleotides occluded in the putative high site-size (≈14). Given the extreme conformational flexibility of HIV-1 NC and the fact that all charged side chains are exposed to solvent (Summers et al., 1992), NC might present itself to a nucleic acid lattice largely as an unstructured polycation, not unlike poly-L-lysine or poly-L-arginine. Polycations such as poly-L-lysine have been shown to stimulate the rate of renaturation of complementary DNA fragments (Cox & Lehman, 1981). It is widely known that oligo- and polypeptides rich in or exclusively composed of arginine and lysine residues can collapse nucleic acid lattices and form precipitable complexes due to extensive charge neutralization (Olins et al., 1967; Pörschke, 1979). We have found that all polynucleotide solutions titrated with NC become noticeably opalescent during the course of a titration. Turbidity measurements carried out using fluorescence (both excitation and emission monochromators set at 350 nm) and absorption spectroscopies indicate an incremental increase in the extent of light scattering in a manner that closely coincides with the reported changes observed in the protein fluorescence or poly(A) CD spectrum upon complex formation (see Figs. 1, 2). The turbidity changes cease at approximately the point that corresponds to apparent saturation in both forward (Fig. 1) and reverse (Fig. 2) directions. This indicates that both $n = 8$ and $n = 14$ NC–polynucleotide complexes scatter light and are ultimately precipitable due to extensive aggregation of NC–RNA complexes (Sykora & Moelling, 1981) and/or simple NC-induced collapse or compaction of the poly(A) lattice (Olins et al., 1967). Interestingly, each complex seems to scatter light with different scattering coefficients, suggestive of different particle dimensions (Gaskin et al., 1974; R.K. & D.P.G., unpubl.). The same qualitative conclusions concerning apparent site-sizes have been obtained with oligo$(A)_n$ (where $n > 15$) complexes that are formed in the absence of appreciable light scattering. It is unknown whether saturated NC–polynucleotide complexes are characterized by a regular structural morphology.

[2] Taking advantage of the fact that the $n = 8$ and $n = 14$ complexes scatter light to different degrees, we have found that once the saturated $n = 8$ complex is formed, additional polynucleotide is not incorporated into the complex over a period of hours, i.e., the $n = 14$ complex is not readily formed via redistribution of the $n = 8$ complex under low salt conditions (R.K. & D.P.G., unpubl.).

tion or the *inter*molecular DNA duplex formation (annealing) by destabilizing *intra*molecular duplex regions due to stoichiometric binding and unfolding of the strands (Alberts & Frey, 1970; Karpel et al., 1982; Kumar & Wilson, 1990). As the duplex forms, the single-strand binding protein is displaced because its affinity for duplex DNA is far lower than that for ssDNA. In contrast, a direct annealing activity, like that of a recombinase (e.g., *E. coli* recA and T4 uvsX), for example (Morrical & Alberts, 1990), requires that the local concentration of homologous sequences be greatly increased. These two mechanisms can be distinguished kinetically by quantifying the rate and the order (first or second) of the intermolecular base-pairing reaction.

NC71 accelerates the intermolecular base-pairing of two perfectly complementary single strands with an overall kinetic order of second order in ssDNA concentration (Table 1; Figs. 4, 5), as expected for a helix-destabilizing protein. Thus, a molecular aggregate does not appear to function under these conditions. Furthermore, the maximum rate enhancement due to protein binding requires sufficient NC to saturate both strands. As a consequence, there will be an extremely sharp protein concentration dependence (Fig. 6), consistent with that previously found for *E. coli* SSB (Christiansen & Baldwin, 1977), T4 g32P (Alberts & Frey, 1970), and hnRNP A1 (Pontius & Berg, 1990). Because the data summarized in Figures 1 and 2 are consistent with two types of NC–polynucleotide complexes that differ in apparent site-size, it seemed possible that one or the other putative complex might specifically accelerate the renaturation reaction. The data in Figure 7 strongly suggest that it is the low site-size ($n = 8$) binding mode that accelerates this process, rather than the $n = 14$ complex.

Examination of the kinetic acceleration factor by NC71 reveals that it is substantial, being greater than four orders of magnitude larger than the uncatalyzed rate and about 200-fold greater than renaturation at 68 °C and 1 M NaCl. These latter conditions are thought to be optimal, greatly reducing the electrostatic repulsion of two strands and formation of intramolecular secondary structures (Wetmur & Davidson, 1968). For comparison, the g32P-assisted rate at 37 °C appears negligible in the absence of Mg^{2+} and is only 10–15-fold enhanced in the presence of high Mg^{2+} concentration (Table 1), consistent with previous findings (Alberts & Frey, 1970). In NC-facilitated reactions, Mg^{2+} has relatively little effect, reducing k_2 by only ca. twofold (Table 1). The rate enhancements of denatured phage λ DNA reported for saturating *E. coli* SSB (Christiansen & Baldwin, 1977) under optimized conditions of low pH (5.5) and 0.02 M Mg^{2+} or Ca^{2+} or 2 mM spermidine and neutral pH appeared comparable to that obtained in the 68 °C/1 M NaCl solution conditions (about 500–1,000-fold over the uncatalyzed reaction). With much smaller DNA molecules of a length comparable to the one used in this study, the rate enhancement by SSB is much more modest (sixfold) (Christiansen & Baldwin, 1977).

Because the rate enhancements observed for other helix-destabilizing proteins seem generally to be smaller and apparently restricted to a defined set of conditions relative to retroviral NC proteins, it seems plausible that favorable, but relatively short-lived, NC–NC or NC–RNA interactions between saturated strands provide additional stimulation of the observed rate (Pontius & Berg, 1990). To mediate the renaturation reaction via the electrostatic interactions between ssDNA-bound NC and the phosphate backbone on a complementary strand, more than one nucleic acid binding subsite would have to be available on each NC71 molecule. Although this has not yet been directly demonstrated, one way to visualize the putative high site-size binding mode of ≈14 nt is that it consists of two subsites of approximately 8 nt. In the low-site size $n = 8$ complex then, one subsite would be bound to ssDNA and the other would be free, available to mediate the reaction via this mechanism. This also predicts that the kinetic acceleration of renaturation would be specific to the low site-size complex. This is apparently the case (Fig. 7). Heterogeneous nuclear RNP A1 is known to have at least two RNA-binding sites (Kumar & Wilson, 1990). The N-terminal fragment of A1 contains just one such site and fails to appreciably accelerate the renaturation of complementary strands, although the C-terminal fragment appears to function almost as effectively as intact A1 (Kumar & Wilson, 1990; Pontius & Berg, 1990). With NC proteins, this of course predicts that single zinc-finger or other domain deletion fragments would be inactive in this renaturation activity, even while maintaining high binding affinities for single-stranded sequences at equilibrium. This is currently under investigation. In any case, the acceleration of strand renaturation by NC does not require Zn(II) bound to the protein (Table 1). In other studies, introduction of amino acid substitutions or extensive alkylation of the cysteine ligands of HIV-1 NC that inactivate metal binding by the N-terminal, C-terminal, or both zinc-finger domains of HIV-1 NC has little or no effect on this activity (F.D.-H. & D.P.G., unpubl.). These findings in this model strand renaturation system correlate well with existing data, which suggests that RNA genome dimerization and tRNA positioning by HIV-1 NC involve domains outside of the zinc-fingers themselves (de Rocquigny et al., 1992).

The observation that the presence of a large excess of noncomplementary DNA sequences has relatively little influence on the renaturation kinetics requires that the associations between saturated strands be either much greater in number in a given time period or a larger fraction of collisions gives rise to productive nucleation events. This type of activity may well have significant consequences for NC protein function in vivo. For example, in the first strand transfer step in reverse transcription, the tRNA-primed minus strand strong-stop cDNA syn-

thesized from the 5′ end of one genomic RNA subunit is, following RNase H degradation of the RNA template, transferred to the far 3′ end of the RNA genome, forming a cDNA–RNA duplex through the complementarity between R regions (Varmus & Brown, 1989). We anticipate that provided enough NC is available to cover all RNA nucleotides in vivo, which is likely to be the case (Karpel et al., 1987), the substantial rate accelerations that we observe in vitro may be achievable in vivo (Pontius & Berg, 1991) for this and other reactions. NC has already been shown to be a major component of reverse transcriptionally active nucleoprotein complexes (Chen et al., 1980).

We speculate that fundamental aspects of genomic RNA dimerization and tRNA annealing derive from the potent intrinsic nucleic acid strand renaturation activity of NC proteins reported here. The mechanistic details may well be different to some extent, because, for example, previous findings published by other laboratories suggest that NC-saturated viral RNA (assuming n_{app} ranging from 8 to 15) does *not* appear to be required in genomic dimer formation (Darlix et al., 1990) or tRNA positioning in vitro (Prats et al., 1988). However, in most in vitro genomic RNA dimerization assays, there is some evidence in support of a rapid reaction that appears to require a critical concentration of NC or molar ratio of NC/RNA nucleotide, below which the reaction seems inefficient (cf. Darlix et al., 1990). These characteristics are precisely those that we observe in our model strand renaturation assays, consistent with NC functioning in a stoichiometric rather than catalytic fashion in genomic RNA dimer formation (Darlix et al., 1990). It is important to bear in mind that a saturated complex in the context of a complex RNA molecule may consist of only one or a small number of occupied high-affinity NC binding sites where local unfolding (Khan & Giedroc, 1992) strongly facilitates the reaction at maximal rates. That is, rather than the number of potential nucleation sites being large, as it must be on the DNA molecules used in this study, one or only a small number of interstrand nucleation sites may function in these specialized cases. Firm knowledge of the reaction stoichiometry and experiments that address whether NC remains bound to these complex RNA structures would seem to be required in order to shed additional mechanistic light on these important aspects of NC function.

Materials and methods

Materials

All buffers were prepared with doubly distilled and deionized Milli-Q water. Single-stranded DNA-cellulose was prepared as described (Giedroc et al., 1992). DTNB, isopropyl β-D-thiogalactopyranoside, and chromatographically purified DNase were obtained from Sigma. Poly(A) was purchased from the Midland Certified Reagent Company (Midland, Texas) and used following chromatography on Sephacryl S-400 and exhaustive dialysis into 10 mM Tris-HCl, 0.1 mM Na_3EDTA, 0.1 M NaCl, pH 8.1. Poly(A) concentrations were determined using ϵ_{260} = 10,300 M $nt^{-1} \cdot cm^{-1}$. Phage φX174 ssDNA was obtained from New England Biolabs and was used without further purification. The concentration of ssDNA nucleotides was calculated using 40 μg/mL for 1 AU at 260 nm. Double-stranded plasmid DNA concentrations were determined spectrophotometrically using 50 μg/mL for 1 AU at 260 nm. T4 g32P was purified from a recombinant overexpression plasmid and purified according to Giedroc et al. (1992).

Methods

Plasmid constructions

All molecular biological methods were carried out according to standard methods. The plasmid pMPMV/$6A_3$ (Sonigo et al., 1986) (kindly provided by Eric Hunter, University of Alabama at Birmingham) was the source of the coding region for MPMV NC. This plasmid was used as a template for 20 cycles of PCR with mutagenic primers ST1 (5′-ACACATATGGCCGCCGCCTTTAGCGGG-3′) and ST2 (5′-TGGATCCTTA**CTA**ATAAGCTTGTTTC GGG-3′). The 5′ ST1 primer introduces an *Nde*I restriction site (underlined) that places an initiator methionine codon next to Ala^{526} in the gag polyprotein $Pr78^{gag}$ precursor (Sonigo et al., 1986). ST2 introduces a TAG stop codon following the codon for Tyr^{621} (boldface), as well as a *Bam*HI restriction site further to the 3′ side of the stop codon (underlined). These manipulations produce a translational reading frame that should encode the mature 96 amino NC protein (Henderson et al., 1985) plus an N-terminal Met. The crude PCR product was then treated with Klenow fragment and dNTPs to form blunt ends and cloned into the *Sma*I site of pIBI24 in the opposite orientation relative to the *lac* and T7 promoters to make pIBInc96.mpmv (3.2 kb). The 300-bp *Nde*I–*Bam*HI fragment was then excised from pIBInc96.mpmv and subcloned into the same sites of expression vector pET-3b (Studier et al., 1990) behind the phage T7 promoter to create pT7nc96.mpmv (4.8 kb). Then, pT7nc96.mpmv directs the expression of NC96 from the inducible T7 RNA polymerase promoter.

Protein purification

HIV-1 NC71 was purified from *E. coli* BL21(DE3)/pLysS cells (Studier et al., 1990) harboring pT7nc71.hiv1 exactly as described (Khan & Giedroc, 1992). MPMV NC96 was purified essentially as described for NC71 (Khan & Giedroc, 1992), except that the low-speed supernatant fraction of *E. coli* BL21(DE3)/pLysS/pT7nc96.mpmv was the source of this NC. This supernatant fraction was brought to 20% (v/v) TFA and 6 M guanidine hydrochlo-

ride in 10 mM Tris-HCl, pH 8. This fraction was loaded directly onto a 2.5 × 10-cm C18 column (Waters preparative C18, 125 Å pore, 55–105 μm particle size) and washed with 0.05% TFA, and then with 0.05% TFA, 10% acetonitrile. The NC96 fraction was eluted by developing the column with a 10–40% (v/v) acetonitrile gradient. The pooled fraction was made 0.1 mM in protease inhibitor PMSF and lyophilized to dryness. This acidified NC96 fraction was dissolved and reneutralized in 20 mL 40 mM Tris-HCl, pH 8, 0.1 M β-mercaptoethanol, 0.2 mM $ZnCl_2$, 50 mM NaCl and loaded onto an ssDNA-cellulose column (1.5 × 15 cm) equilibrated with 40 mM Tris-HCl, pH 8, 0.1 mM DTT, 0.2 mM $ZnCl_2$, 50 mM NaCl. After washing, the column was developed with a 100-mL linear gradient from 50 mM to 1 M NaCl in the same buffer. This material was then chromatographed on a TSK-Gel CM-650M carboxymethyl-Sephadex (1 × 9 mL) (Supelco) column equilibrated with 10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.5 mM DTT. Following a wash with equilibration buffer, the column was developed with a step to 0.1 M NaCl, and a 35 mL/35 mL linear 0.1/0.5 M NaCl gradient in the same buffer. The major pool fraction was pooled, exhaustively dialyzed under N_2 against 12.5 mM sodium phosphate, pH 6.8, 0.05 M NaCl and stored under N_2 atmosphere at −80 °C. The 280/260 ratio exceeded 1.4 for all preparations. The molar extinction coefficient of NC96 was estimated to be ϵ_{280} = 13,000 $M^{-1} \cdot cm^{-1}$, calculated from the net Trp (ϵ_{280} = 5,600 $M^{-1} \cdot cm^{-1} \times 2$) and Tyr ($\epsilon_{280} = 1{,}800\ M^{-1} \cdot cm^{-1} \times 1$) contribution. Protein concentration quantitated by amino acid analysis gave similar results (within 10% of expected from absorption).

Characterization of MPMV NC96

MPMV NC96 purified from a bacterial overexpression system is greater than 95% homogeneous by SDS-PAGE and migrates with an apparent molecular mass of ≈14 kDa, as observed for the virally isolated form (Henderson et al., 1985). NC96 purified as described above contains 2 g·at Zn(II) by atomic absorption and 7.2 ± 0.4 reactive thiols as determined by titration with DTNB, consistent with the deduced amino acid sequence (Henderson et al., 1985; Sonigo et al., 1986). Results from amino acid analysis and N-terminal sequencing reveal that the N-terminal Met is processed by bacteria leaving as the N-terminal sequence Ala-Ala-Ala ..., which corresponds to that found in MPMV virions (Henderson et al., 1985). In the experiments reported here, a fraction (about 20%) of the NC96 protein lacked the N-terminal Ala residue. The C-terminal Tyr^{96} of MPMV NC96 is clearly present in one- and two-dimensional NMR spectra of metallated NC96 (D.P.G., unpubl.).

Circular dichroism spectroscopy

Near UV-CD spectra of poly(A) were recorded as a function of added NC protein essentially as described in (Khan & Giedroc, 1992) unless otherwise noted. Tight binding conditions (i.e., conditions under which all of the input protein is bound) were operationally defined by showing that the apparent saturation point (n) did not vary significantly with input polynucleotide concentration and/or a reduction of the [NaCl].

Fluorescence binding experiments

The intrinsic Trp fluorescence of MPMV NC96 or HIV-1 NC71 was monitored with an SLM 8000C spectrofluorometer with excitation at 292 nm (0.5–1 nm bandpass) and emission at 347 nm (4 nm bandpass). The temperature was maintained in a thermostatted sample compartment at 25 ± 0.1 °C. Normal or "forward" mode fluorescence titrations were carried out in 1.7-mL 10-mm-pathlength quartz cuvettes in 10 mM Tris-HCl, pH 8.3, 20 mM NaCl, and 0.1 mM DTT as follows. Identical small aliquots of Zn_2 NC96 were added to each of two cuvettes. Cuvette 1 contained poly(A) at the indicated concentration (typically 10–25 μM nucleotide), and cuvette 2 contained buffer alone. After addition of protein to each cuvette, the solutions were stirred for 30 s to ensure complete mixing, then incubated another 2–3 min in the dark. After this time the fluorescence intensity was measured by averaging six or seven 10-s integrations of the signal. Under these conditions there is essentially no photobleaching and any inner filter correction is negligible. The fluorescence of the polynucleotide-containing cuvette (corrected for dilution) is presented in arbitrary units as a function of total added NC96. Under tight-binding conditions (determined as described above), the fluorescence of the first phase of the titration will be linear with protein concentration and reflects the fluorescence yield of the bound NC96. After saturation, a second slope will be obtained that should equal the slope obtained with the uncomplexed protein (cuvette 2). This was found to be the case. The intersection of these two lines represents the apparent saturation point, n_{app}. For reverse titrations, the fluorescence of a given concentration of NC in 2.0 mL 10 mM Tris-HCl, pH 8.3, 0.1 M NaCl and 0.1 mM DTT was determined as above during the course of titration with small aliquots of a concentrated stock of poly(A). The fluorescence obtained was corrected for inner filter contribution, photobleaching, and dilution, and the extent of fluorescence quenching (Q_{obs}) was determined essentially as described (Giedroc et al., 1990).

DNA duplex strand renaturation experiments

A plasmid containing the gene encoding $tRNA^{his}$ from *Saccharomyces cerevisiae* cloned behind the T7 RNA polymerase promoter into *Sma*I-digested pUC18 was the generous gift of Dr. Gary Kunkel. A 149-bp duplex was obtained from *Hind*III–*Eco*RI digestion of this pUC18-$tRNA^{his}$ plasmid and purified by anion exchange chromatography carried out on a Waters Powerline HPLC system and a Waters ProteinPak DEAE-5PW column. A

136-bp *Ban*I fragment was also obtained from pIBI24 (IBI, New Haven, Connecticut) in the same way. The purified fragments were end-labeled by using [α-^{32}P]dATP (NEN Dupont) and the Klenow fragment of DNA polymerase I, phenol extracted, desalted on a Sephadex G-25 (Pharmacia) spun-column, and electrophoresed on a 1.5% agarose gel. The concentration of the duplex was estimated from ethidium bromide staining of increasing amounts of a *Hind*III–*Eco*RI-digested pUC18-tRNA^{his} plasmid or *Ban*I-digested pIBI24 quantitated by UV spectroscopy and was estimated to be accurate to within 30%.

The renaturation experiments were carried out essentially as described by Pontius and Berg (1990). Briefly, the indicated concentration of duplex fragment (expressed in nucleotides) was prepared in 120–180 μL of 10 mM potassium phosphate, pH 7.0, 1 mM EDTA, 80 mM NaCl, heated at 95 °C for 5 min, and quick cooled on ice. For protein-containing or control reactions, the mixture was then incubated at 37 °C for 2 min to establish the temperature, and the indicated concentration of NC protein or final NC dialysis buffer (control) was added at t_0. For 1 M NaCl reactions, the reaction mixture was heated to 68 °C for 1 min and NaCl added to 1 M at t_0. At the indicated times, 20-μL aliquots were withdrawn and added to 3 μL of a solution containing *E. coli* tRNA at 0.3 mg/mL, 0.3 mg/mL proteinase K, 0.67% SDS, 30% glycerol, and 0.3% bromophenol blue and incubated for another 5 min at 37 °C. The reactions were stored on ice until all were ready and electrophoresed on a 10% nondenaturing polyacrylamide gel in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 200 V for 2.5 h at room temperature. Gels were dried and subjected to autoradiography. The radioactivity associated with the duplex and monomer bands was quantitated using the Betascope 603 (Betagen). The yield of total radioactivity recovered in each gel lane did not vary by more than 15%, and we observed no systematic trend in the amount of radioactivity recovered in individual time course experiments. This permits normalization of the data in each lane to a fraction of total radioactivity in the single-strand (cpm^{ss}) and duplex (cpm^{ds}) bands. The mole fraction of single-stranded nucleotides (X^{ss}) was calculated from the following equation: $X^{ss} = cpm^{ss}/(cpm^{ss} + 2 * cpm^{ds})$, with the concentration of ss nucleotides (c^{ss}) given by $c^{ss} = c_0 * X^{ss}$. When kinetic data are presented, they are in the form of concentration of ssDNA (in nucleotides), c^{ss}, remaining at time, t, with an initial concentration, c_0. These data were then fit to a simple second-order reaction scheme, $c^{ss}(t) = c_0/(t * k_2 * c_0 + 1)$ to estimate the second-order rate constant, k_2 ($M\ nt^{-1} \cdot s^{-1}$) by nonlinear least-squares methods. In this analysis, all data points were weighted equally. When renaturation reactions were carried out in the presence of nonhomologous or homologous ssDNA circles, the probe strands and the added DNA were mixed at the indicated concentrations in 20 μL, denatured with heating as described above, incubated at 37 °C for 2 min, and the NC added to initiate the renaturation reaction. The mole fraction of duplex was determined at a single time point (5 min) as described.

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