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HIV-1 nucleocapsid protein induces "maturation" of dimeric retroviral RNA *in vitro*

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ABSTRACT **After a retrovirus particle is released from the cell, the dimeric genomic RNA undergoes a change in conformation. We have previously proposed that this change, termed maturation of the dimer, is due to the action of nucleocapsid (NC) protein on the RNA within the virus particle. We now report that treatment of a 345-base synthetic fragment of Harvey sarcoma virus RNA with recombinant or synthetic HIV-1 NC protein converts a less stable form of dimeric RNA to a more stable form. This phenomenon thus appears to reproduce the maturation of dimeric retroviral RNA in a completely defined system *in vitro*. To our knowledge, maturation of dimeric RNA within a retrovirus particle is the first example of action of an "RNA chaperone" protein *in vivo*. Studies with mutant NC proteins suggest that the activity depends upon basic amino acid residues flanking the N-terminal zinc finger and upon residues within the N-terminal finger, including an aromatic amino acid, but do not require the zinc finger structures themselves.**

After a retrovirus particle is released from the cell, the dimeric genomic RNA within the particle undergoes a change in conformation (1–3). This change, which renders the dimer more thermostable, has been referred to as "maturation" of the dimer; maturation requires the activity of the viral protease (PR) (2, 3).

The requirement for PR activity suggested that RNA maturation results from interaction of one or more of the Gag proteins with the RNA after PR-mediated cleavage releases these proteins from the Gag polyprotein precursor. We have specifically suggested (2) that one of these cleavage products, the viral nucleocapsid (NC) protein, is responsible for maturation of the RNA, because (*i*) NC is known to be associated with the RNA in the nucleoprotein core of the mature particle (4–6), and (*ii*) NC is known to facilitate the formation of the optimal, most thermodynamically stable structures in nucleic acids (7, 8).

The nature of the difference between the immature and mature dimeric forms of viral RNA is not known; indeed, the structure of the dimeric RNA in retrovirus particles is very poorly understood. The two monomers are believed to be joined near their 5′ ends (9). One important step in the analysis of these structures was the finding that relatively short RNA transcripts containing sequences from near the 5′ end of retroviral genomes can dimerize spontaneously *in vitro* under conditions of high ionic strength (4, 5, 10).

In the course of experiments designed to identify sequences from the Harvey sarcoma virus (HaSV) genome capable of dimerization *in vitro*, we noted (11) that a 345-base transcript could give rise to two distinct dimeric forms differing in thermostability: dimerization at 37°C produces a population of dimeric molecules of which some can be dissociated into monomers at temperatures near 45°C, whereas dimerization at 55°C produces dimers that are all stable to temperatures of 55°C or higher.

We now report that incubation of the HaSV dimers formed at 37°C with recombinant or synthetic HIV-1 NC protein converts them to the more thermostable form. This phenomenon appears to represent an *in vitro* assay for the RNA "maturation" previously described *in vivo*; the activity of NC in this assay provides strong support for the hypothesis that NC performs this function within the maturing virion. Further, we report studies with mutant NC proteins that shed some light on the mechanism of this effect.

As noted above, NC is able to promote the formation of the most stable structures when it interacts with nucleic acids *in vitro*; this property of NC protein has been termed "nucleic acid chaperone" activity (for review, see ref. 8). The maturation of RNA within the retrovirus particle may be the first known example of RNA chaperone activity *in vivo*.

MATERIALS AND METHODS

RNA. Transcription of HaSV nucleotides 34–378 (12), dimerization, microdialysis, and analysis of thermostability were all performed as previously described (11). Thus, RNA was synthesized by SP6 RNA polymerase in the presence of [α^{32}P]CTP. Dimers were formed by incubation of 0.5–1.0 μg RNA for 2 h at 37°C in 10 μl dimerization buffer (0.25 M NaCl/0.01 M Tris, pH 7.0/1 mM $MgCl_2$) containing 3.9 units /μl RNasin (Promega). The RNA was then microdialyzed for 45 min at 4°C against 0.05 M NaCl/0.01 M Tris, pH 7.0/1 mM EDTA, using a type V6 filter, 0.025 μm pore size (Millipore). Thermostabilities were determined by removing aliquots of the microdialyzed RNA and incubating them for 10 min at the indicated temperatures. The proportions of dimer and monomer were measured by electrophoresis at room temperature in 2.5% Metaphor (FME) in 50 mM Tris·borate (pH 8.0)/1 mM EDTA. In many experiments, the melting profiles were analyzed using a Molecular Dynamics PhosphorImager.

NC Protein. Recombinant wild-type HIV-1 NC protein was produced as follows. The 55-aa NC coding sequence was amplified from the MN HIV-1 molecular clone (a gift of M. Reitz, National Cancer Institute) (13) and was placed into either the pMal (Protein Fusion and Purification Kit, New England Biolabs) or the pGEX (Pharmacia) expression vector. In either case, proteins were expressed, purified, and cleaved using conditions recommended by the manufacturer. A cleavage site was introduced immediately adjacent to the N-terminal methionine of NC so that the final protein product contained no nonviral amino acid residues. The NC protein was further purified using high pressure liquid chromatogra-

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Abbreviations: PR, protease; NC, nucleocapsid; HaSV, Harvey sarcoma virus.

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phy (HPLC) (14). The product was lyophilized and redissolved in water containing 2 moles $ZnCl_2$ per mole of NC. Aliquots of the products were tested and quantitated by sequencing or total amino acid analysis; all of these tests gave the expected results. The 1:1, 2:2, and 2:1 "finger switch" mutants, in which the zinc fingers within NC have been exchanged with each other (15), were also produced using these techniques.

Wild-type and all mutant proteins other than the finger switch mutants were obtained by chemical synthesis. Proteins were assembled in an Applied Biosystems model 430A peptide synthesizer using fluorenylmethyloxycarbonyl chemistry. After deprotection and cleavage from the solid support, proteins were purified by HPLC, lyophilized, redissolved, and tested and quantitated as described above. In general, synthetic and recombinant wild-type NC preparations gave identical results.

Treatment with NC. Dimeric RNAs were treated with NC proteins in dimerization buffer containing 0.02% bovine serum albumin. Control treatments were identical except that no NC protein was added to the solution. Unless indicated otherwise, treatments were for 30 min at 37°C. The treated and control RNAs were then deproteinized by proteinase K digestion and phenol-chloroform extraction, and were analyzed for thermostability after microdialysis as described above.

RESULTS

Stabilization of Dimeric RNA by Treatment with NC. As noted above, a portion of the dimers formed by incubation of HaSV 34-378 RNA at 37°C are significantly less thermostable than those formed by incubation of the same RNA at 55°C (11). To determine whether HIV-1 NC was capable of converting the less thermostable dimers to more stable forms, we treated dimers formed at 37°C with NC and then removed the protein. The thermostabilities of these RNA dimers were then compared with those of control dimers formed at 37°C. As shown in Fig. 1, dimers formed at 37°C were significantly more stable after treatment with NC than after incubation in the absence of NC. Thus, the control dimers showed significant dissociation after heating to 37°C, whereas the treated dimers remained almost exclusively dimeric until they were incubated at 55°C or above.

We also tested the ability of NC protein to stabilize dimers of HaSV 34-378 RNA that had been formed by incubation at 55°C rather than at 37°C; however, treatment of these dimers had no effect on their melting profile (data not shown). Similarly, a smaller fragment of HaSV 34-378 RNA, i.e., HaSV 271-378 RNA, was allowed to dimerize at either 37°C or 55°C; these dimers exhibited identical thermostabilities regardless of the temperature at which they had formed (data not shown). NC did not induce a detectable stabilization of the HaSV 271-378 dimers formed at 37°C (data not shown). Thus, the stabilization by NC is only evident when an RNA molecule is capable of forming alternative dimeric structures and only occurs when the less stable form is treated. These observations lend support to the hypothesis that the stabilization is due to a change in the conformation of the RNA, rather than the presence of residual NC on the RNA after treatment.

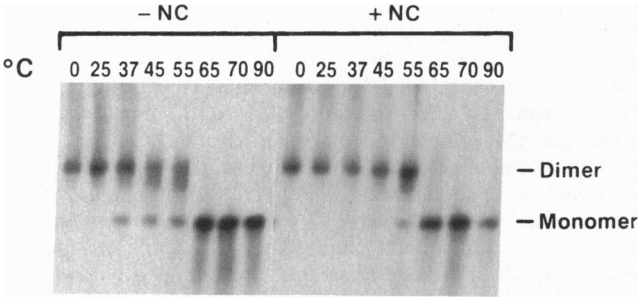

FIG. 1. Effect of NC on thermostability of HaSV 34-378 dimers formed at 37°C. ^{32}P-labeled RNA was allowed to dimerize at 37°C. One aliquot (0.5 μg) was treated with 1.0 μg NC while a control aliquot was incubated without NC. The two samples were then deproteinized and their thermostabilities were determined as described.

Quantitative Properties of the Maturation Reaction. The results presented above were obtained by treatment of 0.5 μg of RNA with 1.5 μg of NC for 30 min at 37°C. This represents one NC molecule per 6.6 nucleotides. In an attempt to obtain some insight into the mechanism of the effect, we examined the dose-response and time-course of the reaction in a variety of situations. The melting profiles were analyzed using a PhosphorImager in these experiments.

Initial experiments showed that stabilization is complete after only 5 min of treatment at the protein/RNA ratio used above (data not shown). We therefore examined the time-course of the reaction using a lower protein/RNA ratio. Dimeric HaSV 34-378 RNA (0.5 μg) (formed by incubation at 37°C) was treated with 0.5 μg of NC (representing one NC molecule per 20 nucleotides) for times ranging between 30 min and 300 min, and the thermostabilities of the dimers were tested in each case. As shown in Fig. 2*A*, the profiles exhibited a gradual, progressive shift to greater stability. Complete stabilization [i.e., stability of the dimer at temperatures as high as 55°C, as in the dimers formed at 55°C (11)] was not attained even in 300 min at this protein/RNA ratio. (Fig. 2*A* also shows that the % dimer is higher after the 55°C treatment than after the 45°C treatment. This is a reproducible feature of these experiments; presumably, some stabilization and/or dimerization occurs during the 55°C treatments.) The gradual increase in stability depicted in Fig. 2*A* was also displayed (Fig. 2*B*) by plotting the percentage of dimers surviving treatment at 45°C as a function of the time of incubation with NC.

We also tested the ability of different doses of NC to stabilize the dimers in a prolonged (10 h) incubation period. The doses ranged from 0.2 μg (1 NC per 50 nucleotides) to 1.5 μg (1 NC per 6.6 nucleotides). As shown in Fig. 2 *C* and *D*, the degree of stabilization varies directly with the dose of NC. It is notable that the stabilization induced by 0.2 μg in 10 h (Fig. 2 *C* and *D*) is significantly less than that induced by 1.5 μg in 0.5 h (Fig. 1; data not shown).

Stabilization of Dimeric RNAs by Mutant NC Molecules. Examination of the amino acid sequence of HIV-1 NC (13) reveals the following features, which are highlighted in Fig. 3: there are two zinc-finger motifs of the form $C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_4\text{-}C$ (underlined); the N-terminal zinc-finger is surrounded on both sides by clusters of basic residues; and there is an aromatic residue (F16 and W37) between the two N-terminal cysteines in each finger. Each of these properties is, in fact, highly conserved among lentiretroviruses and oncoretroviruses (16). To determine the role of each of these conserved features in the stabilization reaction, we tested a series of mutant HIV-1 NC proteins for their ability to induce RNA maturation. Results of some of these tests are shown in Fig. 4 *A* and *B*, and the entire series of experiments is summarized in Table 1.

As shown in Fig. 4*A*, a mutant NC in which eight lysine or arginine residues flanking the N-terminal finger have all been changed to alanine was apparently incapable of inducing the stabilization of the dimers. However, changing all six cysteine residues in the two zinc-fingers to alanine did not inactivate the NC protein in this assay. Fig. 4*B* shows that replacement of F16 with alanine eliminated the ability of NC to stabilize the dimers, whereas the W37A mutant was still able to induce the stabilization. In other experiments, treatment of the unstable dimers with F16A mutant NC protein for 2 h, rather than the standard 30-min treatment, yielded no detectable change in the thermostability of the dimers (data not shown).

As summarized in Table 1, replacement of the N-terminal zinc-finger with two glycines inactivated the protein, whereas

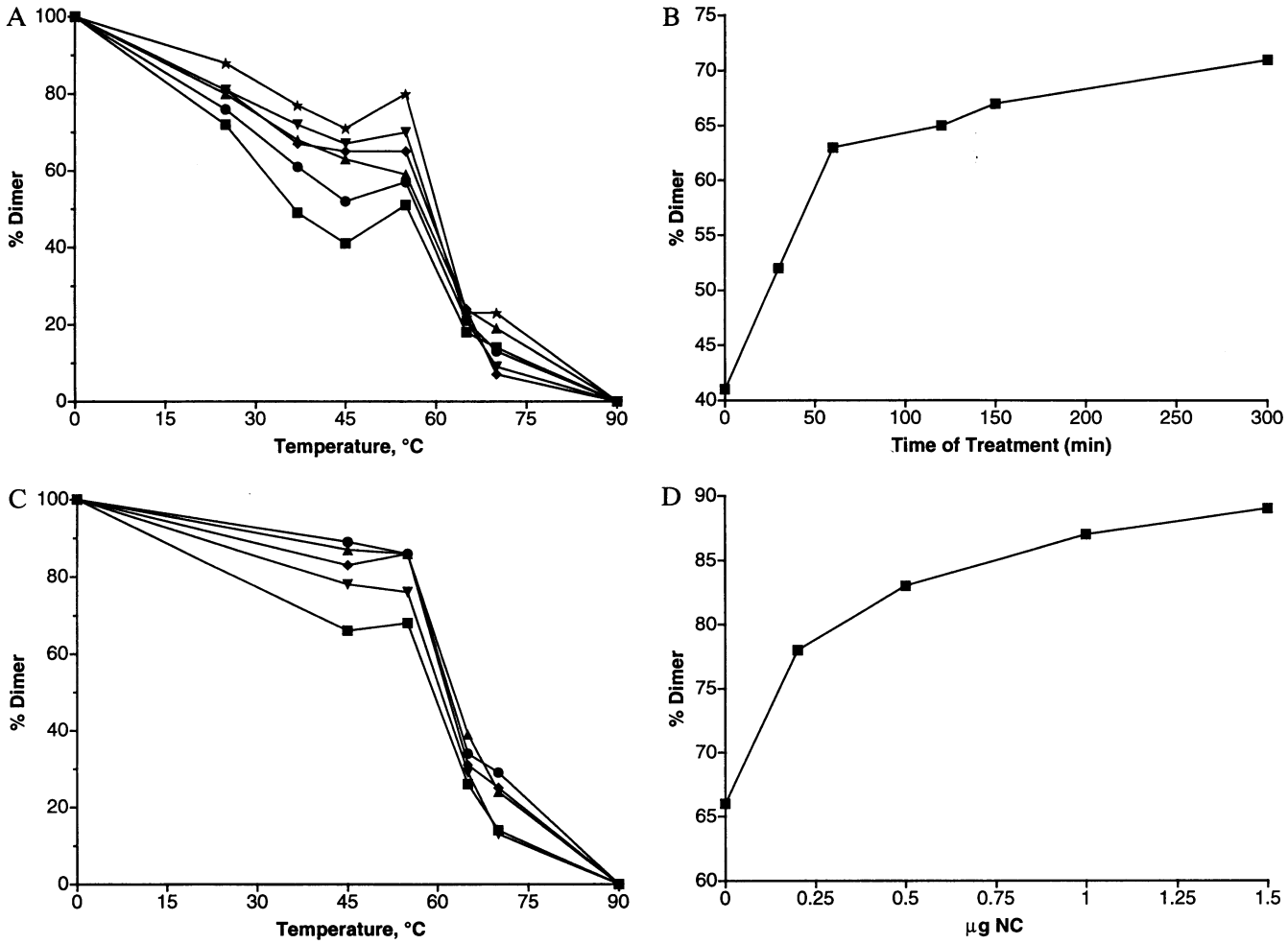

FIG. 2. Quantitative assays of RNA maturation. ^{32}P-labeled HaSV 34-378 RNA was allowed to dimerize at 37°C. After incubation with NC, the RNAs were deproteinized and their thermostabilities were determined as described. (*A*) Time-course of dimer stabilization. Dimeric RNA (0.5 μg) was incubated with 0.5 μg NC for times ranging between 30 min and 300 min. ■, no NC; ●, 30 min; ▲, 60 min; ◆, 120 min; ▼, 150 min; ⋆, 300 min. (*B*) Data from *A*: plot of % dimer at 45°C versus time of incubation. (*C*) Dose-response of dimer stabilization. Dimeric RNA (0.5 μg) was incubated for 10 h in 10 μl with amounts of NC ranging from 0.2 μg to 1.5 μg. ■, no NC; ▼, 0.2 μg; ◆, 0.5 μg; ▲, 1.0 μg; ●, 1.5 μg. (*D*) Data from *C*: plot of % dimer at 45°C versus amount of NC.

similar replacement of the C-terminal finger did not. Further, 1:1, 2:2, and 2:1, i.e., mutants in which one finger has been replaced by a duplicate of the other finger or in which the positions of the two fingers are reversed, are also active in the stabilization assay. In all cases, treatments for 5 or 15 min showed that the mutants induce stabilization less rapidly than the wild-type protein (data not shown).

DISCUSSION

The dimeric RNA in a retrovirus particle undergoes a stabilization event (called "maturation" of the dimer) after the particle is released from the cell (1–3). We have previously proposed (2) that binding of NC to the RNA in the maturing virion is responsible for the stabilization. We show here that treatment with pure (i.e., recombinant or synthetic) NC protein can convert a less stable dimeric form of a small, synthetic retroviral RNA to a more stable form. This system appears to represent a simple, well-defined model for the maturation phenomenon *in vitro*.

The fact that maturation of the dimer can be induced by NC protein in a completely defined system is strong support for the hypothesis that NC is solely responsible for RNA maturation within the virion. Further, this simple system can be used for manipulation and analysis *in vitro* of this step in virus maturation.

It seems significant that the maturation described in virions depends on PR activity (2, 3); thus, the *in vivo* and *in vitro* data are all consistent with the hypothesis that NC protein is responsible for maturation of dimeric RNA. In contrast, others have shown that NC protein (or partial cleavage products of Gag that contain NC) can accelerate the dimerization of synthetic retroviral RNAs and the placement of the tRNA primer on the primer-binding site *in vitro* (4, 5, 10, 17, 30). However, RNA dimerization and primer placement are both independent of PR activity *in vivo* (2, 3, 6, 18); thus, it is quite unlikely that NC protein *per se* has a role in these events.

FIG. 3. Amino acid sequence of HIV-1 NC protein. The two zinc fingers are indicated by underlining, while the basic residues surrounding the N-terminal finger and the aromatic residues within each finger are shown in white letters on a black background.

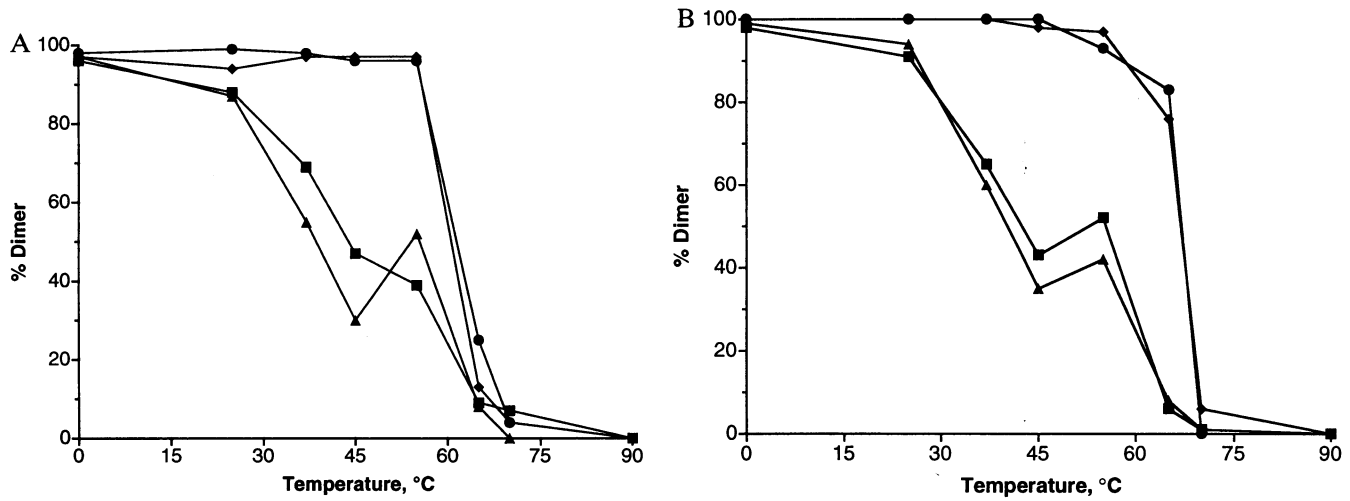

FIG. 4. Effect of mutations in NC protein on its activity in RNA maturation. ^{32}P-labeled HaSV 34-378 RNA was allowed to dimerize at 37°C. RNA (0.5 μg) was then incubated with 1.5 μg wild-type or mutant NC protein for 30 min at 37°C, and was then deproteinized and its thermostability analyzed as described. (*A*) Effect of changing basic residues or cysteines to alanine. ■, no NC; ●, wild-type NC; ▲, 8(R,K) → A; ♦, 6(C) → A. (*B*) Effect of changing aromatic residues to alanine. ■, no NC; ●, wild-type NC; ▲, F16A; ♦, W37A.

The molecular mechanism responsible for the maturation reaction is not understood at present. However, NC proteins have been shown to induce conformational changes in nucleic acids in a wide variety of experimental systems *in vitro* (4, 5, 7, 10, 17, 19–24). In some of these systems, as in the present work, NC treatment results in formation of a more stable structure (7). It would appear that NC can catalyze the breakage and reformation of base pairs in nucleic acids, thus lowering the energy barrier for conversion of a less stable structure (with fewer base pairs than the optimum structure) to the optimum, thermodynamically favored structure. Because of this property, NC has been called a "nucleic acid chaperone" (see ref. 8).

In our previous study on dimerization of HaSV 34-378 RNA (11), we suggested that dimers formed at 55°C are more thermostable than those formed at 37°C because some intramolecular bonds present in monomers at 37°C are broken at 55°C, rendering more bases available for intermolecular interaction at the higher temperature. According to this hypothesis, NC stabilizes the dimers by virtue of its chaperone activity, transiently breaking intramolecular bonds in the less thermostable dimeric form so that it can be converted to the more stable form. While a number of proteins have been shown to possess nucleic acid chaperone activity *in vitro*, the maturation of RNA within a retrovirus particle appears to be the first known manifestation of chaperone activity *in vivo*.

Table 1. Activity of NC proteins in RNA maturation assay

Wild-type	++
6(C) → A	+
8(R,K) → A	−
F16A	−
W37A	+
F16A, W37A	−
Δfinger 1*	−
Δfinger 2†	+
1:1‡	+
2:2§	+
2:1¶	+

++, Stabilization complete in 5 min, +, stabilization incomplete in 5 min, complete in 30 min; −, no detectable stabilization in 30 min. RNA (0.5 μg) that had dimerized at 37°C was treated with 1.5 μg of wild-type or mutant NC protein. The thermostability of the dimer was then tested as described.
*Residues 15–28 replaced by GG.
†Residues 36–49 replaced by GG.
‡Residues 36–49 replaced by residues 15–28.
§Residues 15–28 replaced by residues 36–49.
¶Residues 15–28 and 36–49 exchanged.

Two respects in which the present *in vitro* model system differs from RNA maturation within the virion should be noted. First, the present experiments have used HIV-1 NC and HaSV-derived dimeric RNAs. The activity of HIV-1 NC in this heterologous system is consistent with the hypothesis that maturation of dimeric RNAs is a result of nonspecific interactions between NC and the RNA. Indeed, many of the studies demonstrating chaperone activity of NC proteins have used completely nonviral nucleic acid sequences (7, 19–24). However, Laughrea and Jetté (25) have recently reported that transcripts of HIV-1 sequences, like those of HaSV (11), can form two types of dimers *in vitro* and that these dimers differ in their stability. It would be of interest to know whether HIV-1 NC protein can convert the less stable HIV-1 dimers to the more stable form.

Second, we previously found that the thermostability of HaSV genomic RNA dimers was not detectably affected by the presence of an active PR in the virion (11). Thus, the behavior of HaSV genomic RNA differs in this respect from that of Moloney murine leukemia virus and HIV-1, despite that fact that its conformation is altered as a result of PR activity [as detected by a change in electrophoretic mobility (11)]. It would appear that the 345-base fragment of HaSV RNA used in the present work can be "trapped" in a relatively unstable conformation during incubation at 37°C; in contrast, full-length HaSV RNA evidently does not assume this structure *in vivo*, even in the absence of PR-catalyzed production of NC protein within the virion.

A number of studies of NC–nucleic acid interactions have indicated that binding is complete with approximately one NC molecule per seven nucleotides (23, 26, 27); this is the NC/RNA ratio in a retrovirus particle (14). We have analyzed the maturation phenomenon *in vitro* in some simple quantitative experiments. Our standard conditions represent one NC molecule per 6.6 nucleotides, and thus should be just sufficient for saturation of the RNA with NC molecules. The results (Fig. 2; data not shown) show that the reaction is very rapid under these conditions, but occurs slowly and gradually in lower amounts of NC. It is striking that complete stabilization is not induced by 0.2 μg of NC (representing 1/7.5 of the standard amount), even when the incubation period is increased to 10 h, i.e., 20-fold over the normal time. This might suggest that when NC binds to RNA, it is limited in its ability to migrate from one

site to another, either intra- or intermolecularly; however, we cannot exclude the alternative possibility that NC is gradually denatured during an extended incubation.

We have also approached the question of the mechanism of maturation by testing a series of mutant NC proteins in the assay. In a prior study, Müller *et al.* (24) analyzed a series of mutant HIV-1 NC proteins for chaperone activity in a nonviral assay system. They found that the basic residues flanking the N-terminal zinc finger were required in their assay, whereas proteins with deletions of either of the zinc fingers were active. We found (Fig. 4, Table 1) that the basic residues are absolutely required for activity in the RNA maturation assay. Further, residues within the N-terminal finger, including the aromatic residue between the first two cysteines, are evidently essential as well. However, the zinc-finger structures themselves are unnecessary, because replacement of the six zinc-coordinating cysteines with alanine did not destroy the activity. Residues within the C-terminal finger also appear to be unnecessary.

Taken together, these findings suggest the hypothesis that the maturation effect involves electrostatic interaction between basic amino acids and phosphate groups in the RNA, and in addition a stacking interaction between an aromatic residue within the N-terminal finger and bases of the RNA. This residue is normally phenylalanine, but it can be replaced by tryptophan, as in the 2:1 and 2:2 mutants (Table 1). Several reports have provided evidence for intercalation of aromatic residues during NC–nucleic acid interactions (26, 28, 29). It is striking that in all lentiretroviruses and oncoretroviruses, the N-terminal zinc finger (or sole finger in the mammalian type C viruses) is flanked on both sides by basic residues and contains an aromatic residue between the two N-terminal cysteines (16). The results presented here suggest that these conserved features are precisely those that are essential in the maturation reaction: an aromatic residue in the context of a highly basic environment. It is surprising that another conserved aspect of the protein, i.e., the zinc-finger structure within which the aromatic residue is normally embedded, is apparently not required.

A more detailed molecular understanding of maturation of dimeric RNAs may depend on a variety of empirical studies, e.g., the definition of RNA sequences necessary for formation of alternative dimer structures; structural studies on these alternative structures and on NC itself; and a detailed analysis of the binding of wild-type and mutant NC to specific DNAs and RNAs.

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