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Determinants of the Human Immunodeficiency Virus Type 1 p15NC-RNA Interaction That Affect Enhanced Cleavage by the Viral Protease

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During human immunodeficiency virus type 1 (HIV-1) virion assembly, cleavage of the Gag precursor by the viral protease results in the transient appearance of a nucleocapsid-p1-p6 intermediate product designated p15NC. Utilizing the p15NC precursor protein produced with an in vitro transcription-translation system or purified after expression in *Escherichia coli*, we have demonstrated that RNA is required for efficient cleavage of HIV p15NC. Gel mobility shift and nitrocellulose filter binding experiments indicate that purified p15NC protein specifically binds its corresponding mRNA with an estimated K_d of 1.5 nM. Binding was not affected by the presence or absence of zinc or EDTA. Moreover, mutagenesis of the cysteine residues within either of the two Cys-His arrays had no effect on RNA binding or on RNA-dependent cleavage by the viral protease. In contrast, decreased binding of RNA and diminished susceptibility to cleavage in vitro were observed with p15NC-containing mutations in one or more residues within the triplet of basic amino acids present in the region between the two zinc fingers. In addition, we found that 21- to 24-base DNA and RNA oligonucleotides of a particular sequence and secondary structure could substitute for p15 RNA in the enhancement of p15NC cleavage. Virus particles carrying a mutation in the triplet of NC basic residues (P3BE) show delayed cleavage of p15NC and a defect in core formation despite the eventual appearance of fully processed virion protein. These results define determinants of the p15NC-RNA interaction that lead to enhanced protease-mediated cleavage and demonstrate the importance of the triplet of basic residues in formation of the virus core.

The human immunodeficiency virus type 1 (HIV-1) viral protease (PR) functions during virion assembly and maturation in the cleavage of the Gag and Gag-Pro-Pol precursors (47, 58). Without PR activity, virions are released but are not infectious and retain an immature, spherical morphology (2, 36). Upon cleavage of the precursors, the virion undergoes maturation with the formation of an electron-dense core, and particle becomes infectious (19, 30, 32, 56, 58). Little is known about the process of virion morphogenesis, but it is likely to be a controlled event given the complexity of virion structure and its importance in producing an infectious particle.

It has been recognized for some time that the Gag processing sites are cleaved in an ordered manner by PR in the virion and in vitro (17, 23, 43, 49, 64). Initial cleavage of Gag occurs at the amino terminus of the NC domain, producing a 15-kDa intermediate, p15NC (17, 43, 67), consisting of nucleocapsid-p1-p6 and a 39-kDa intermediate consisting of matrix-capsid-p2 (23, 43, 49). Later in the processing cascade, these intermediates are further cleaved to yield the mature products (24, 43, 49, 64). Thus, during virion morphogenesis, there appear at least four different forms of nucleocapsid (NC): as Gag, as p15NC, as NC-p1, and as mature NC. The exact role of each NC form is not known, but the presence of functional differences between them suggests that each has a particular role during virion morphogenesis.

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Studies of Gag processing in infected cells and in vitro have provided some indication that the timing of Gag cleavages is regulated and important for proper virion morphogenesis. Immature HIV-1 particles formed in the presence of PR inhibitors are unable to obtain maturity and infectivity upon removal of inhibitor (28, 36, 52). Mutation at the CA-p2 region produces particles with lowered infectivity despite the presence of the proper final products (33, 49). In similar studies, mutagenic alteration of the rate at which the matrix-capsid junction is cleaved results in particles with lowered infectivity (26a). In addition, RNA enhances cleavage of the p15NC intermediate in vitro (60), suggesting that external factors have the potential to influence Gag processing.

Mature NC is composed of 55 amino acids and contains two zinc fingers of a characteristic C-C-H-C motif separated by a seven-residue linker (22, 24, 45). Basic residues are frequent in the areas flanking both fingers and in the linker domain. NC carries out a number of essential functions, including RNA dimerization (18, 68) and primer tRNA positioning (4, 42); NC functions have been recently reviewed (12). NC also modulates reverse transcriptase (RT) activity through formation of a tripartite complex with the primer tRNA (4, 42, 68) and interacts with the Vpr protein (37). NC as a component of Gag functions in the selection of the specific RNA to be encapsidated through interaction with the PSI packaging site of the viral RNA (6, 71). Binding of mature NC, p15NC, or Gag Pr55 to viral and synthetic RNAs and DNAs has been demonstrated in a number of in vitro studies (5, 10, 11, 13, 27, 31, 38, 39, 59, 61, 62, 70). Differences in assay conditions preclude direct comparisons of such studies, but in general NC-containing proteins

bind with a K_d in the nanomolar range under conditions of low salt concentration and in the presence of zinc (10, 11, 31, 59, 62, 70). The binding site size or footprint appears to be smaller for mature NC than for its larger precursors (14, 31, 38, 39, 70), and multiple stem-loop structures within the PSI region of the viral RNA are crucial for this binding (10, 41, 57).

Mutagenic studies of NC peptides and precursors have identified areas necessary for function. Initial studies recognized the zinc fingers (1, 7, 11, 15, 20, 21) and basic residues flanking the first zinc finger (13, 18, 25, 48, 50, 59, 62) as necessary for viral RNA encapsidation. Others used chimeras from multiple retroviruses to establish a role for the zinc finger regions of NC in the selection of the proper RNA for encapsidation (6, 71). The two zinc fingers appear to have separate and essential roles in the interaction with viral RNA (7, 20), and the spatial distance between the fingers is critical (15, 45, 46, 48). An understanding of the necessity of intact zinc fingers for production of infectious virions has led to the recent identification of a class of zinc finger modification agents designated 2,2′-dithiobisbenzamides which show anti-HIV activity (54, 66).

Mutations of NC have diverse effects on virion morphogenesis. Zinc finger mutants have been described as being similar to the wild type (7, 20, 22) or largely immature (1); differences in the infectious clones and cell types used may account for these opposing findings. Where examined, mutagenesis of the linker between the fingers resulted in aberrant morphogenesis, in some cases resulting in immature particles (48, 50). Taken together, these results suggest an important role for NC in RNA encapsidation and assembly, maturation, and infectivity of particles.

We previously reported initial results that suggested that viral protease-mediated cleavage of the p15NC intermediate was RNA dependent (60). In this study, we investigated the nature of the interaction between p15NC and RNA in a model system. We explored the minimal size and structural requirements for enhancement of p15 cleavage upon interaction with nucleic acids. We also investigated by mutagenesis the individual contribution of a triplet basic of residues located between the zinc fingers of p15NC. Finally, we determined the role of this triplet of residues on viral p15NC cleavage, global proteolytic processing, viral RNA content, infectivity, and morphogenesis. These results characterize the determinants that influence p15NC-RNA interaction and demonstrate the importance of the basic residues at NC positions 32 to 34 in timely cleavage of p15NC and in core formation during virion morphogenesis.

MATERIALS AND METHODS

Preparation of RNA. RNA corresponding to the coding sequence of p15NC (nucleotides 1132 to 1534 of the BH10 isolate [44]) was synthesized by in vitro transcription from plasmid pDAB15 as previously described (60). To prepare ^{35}S-labeled RNA, 0.63 μM [α-^{35}S]UTP (1,000 to 1,500 Ci/mmol) or [α-^{35}S]ATP (1,100 Ci/mmol) was included in the transcription reaction. Labeled RNA was recovered from in vitro transcription reactions by extraction with Pro-Cipitate (Affinity Technology, Inc.) or phenol-chloroform followed by ethanol precipitation. The specific activity of RNA prepared in this way was typically 20 to 30 μCi/nmol. Synthetic RNA oligonucleotides were purchased from the Biochemistry Department, University of Pennsylvania, or from Oligos Etc., Inc. (Wilsonville, Oreg.). Synthetic oligodeoxynucleotides were synthesized by DuPont Merck Pharmaceutical Co. or purchased from Oligos Etc. To prepare the ^{35}S-labeled RNA oligonucleotide designated RNA24, or to prepare ^{35}S-labeled bovine tRNA, purchased oligonucleotides were labeled at the 5′ end, using T4 polynucleotide kinase and [γ-^{35}S]ATP at 37°C for 30 min, and purified by Sephadex G-25 chromatography (40). The specific activity for RNA24 prepared in this manner was typically 30 μCi/nmol. The specific activity of bovine tRNA prepared in this manner was typically 4 μCi/nmol.

Expression and purification of recombinant p15NC. The coding sequence of HIV-1 p15NC protein (*Nde*I-*Bam*HI fragment of the BH10 isolate) or of p15NC containing desired mutations was derived from the in vitro transcription plasmids previously described (60). Recombinant p15 was expressed by a T7 expression system in vector pET3A (Novagen) in *Escherichia coli* BL21(DE3) cells (55). Three hours postinduction, cells were harvested by centrifugation, broken in a French press in 50 mM morpholineethanesulfonic acid (MES)–4 M urea (pH 6.5), and clarified by centrifugation. The soluble fraction was enriched for p15NC protein by using batch chromatography with S-Superose resin (Pharmacia) in 50 mM MES–4 M urea (pH 6.5) and eluting the resin stepwise at 0.25, 0.5, and 1 M NaCl in the same buffer. The bulk of p15NC was recovered in the 0.5 M NaCl elution, and this material was dialyzed against 50 mM MES–4 M urea (pH 7.0) and applied to a Mono S column (Pharmacia). Proteins were eluted with a linear gradient of 0 to 1 M NaCl in 20 min (HR 5/5 column) or 50 min (HR 10/10 column). Recombinant p15NC eluted at 0.5 to 0.7 M NaCl. Fractions were pooled, and material from several columns was combined, concentrated, and applied to a Zorbax Protein Plus reverse-phase high-pressure liquid chromatography column in 0.1% trifluoroacetic acid. Proteins were eluted with a gradient of 0 to 63% CH_3CN in 40 min at a flow rate of 2.5 ml/min. The p15NC protein eluted at 42% CH_3CN as assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The pooled fractions were lyophilized, and the dried residue was brought up in phosphate-buffered saline (PBS; pH 6.5) containing 10% (vol/vol) glycerol. The presence of bound zinc in the preparation was not determined, but the effect of adding or removing zinc on subsequent processing was later assessed in vitro. The amino-terminal amino acid sequence of Met-Gln-Arg-Gly-Asn corresponding to native p15NC was confirmed by automated sequencing (Beckman Instruments). Thus, recombinant p15NC protein includes only authentic sequences starting from the initiator methionine residue that is the amino terminus of viral p15NC (44).

Mutagenesis of p15 coding sequences. For generation of p15NC containing mutations, mutagenesis of both the p15NC transcription-translation vector and *E. coli* expression vector was performed as described previously (60). Mutations were p15NC with first zinc finger cysteine residues 15 and 18 mutated to tyrosine (CCYY-1); p15NC with second zinc finger cysteine residues 36 and 39 mutated to tyrosine (CCYY-2); and p15NC with the intra-array basic residues ^{32}Arg-Lys-Lys^{34} mutated to glutamic acids (3BE), glutamines (3BQ), or leucines (3BL). Single-codon substitution mutations in p15 were arginine 32 mutated to glutamic acid (R32E); lysine 33 mutated to glutamic acid (K33E), and lysine 34 mutated to glutamic acid (K34E). For generation of p15NC mutations in virus, a subclone of the HIV-1 HXB2 isolate containing the p15NC coding domain was mutated by using a commercially available kit. Mutations were p15NC with second zinc finger cysteine residues 36 and 39 mutated to serine (CS2) and p15NC with the intrafingers basic residues ^{32}Arg-Lys-Lys^{34} mutated to glutamic acids (P3BE).

Cleavage of p15NC by HIV-1 protease. Cleavage of p15NC generated by in vitro transcription-translation reactions was conducted as previously described (60). Cleavage of purified recombinant p15NC by the HIV-1 protease was carried out in 20 mM sodium phosphate buffer (pH 6.5)–150 mM NaCl (PBS), using 2 to 5 μg of p15NC protein and 0.055 μg of HIV protease (9) in a total volume of 15 μl. Reactions were carried out in the presence or absence of RNA or DNA oligonucleotides as indicated in the figure legends. After incubation at 30°C for 60 min, reactions were terminated by addition of an equal volume of SDS-PAGE sample buffer (34) and boiling for 2 min. Samples from protease digestion of translation products or recombinant p15NC protein were analyzed on SDS–4 to 20% polyacrylamide gels (Novex) and visualized by Coomassie blue staining. Recombinant p15NC migrates at an apparent molecular mass of about 17 kDa as previously noted (17, 49, 60).

Filter binding assay for RNA-p15NC interactions. Filter binding assays were modified from Draper et al. (16) and similar to assays described previously for NC-RNA interactions (10, 11, 59, 62, 70). Because of the dependence of filter retention of complexes on salt concentration, reaction volume, incubation time, temperature, and filter handling (16), the assay procedure described below was strictly adhered to. Under these conditions, 50 to 70% of input counts were reproducibly retained with saturating protein concentrations, well within the range of retentions described for idealized systems (16). The binding reactions contained a constant concentration of ^{35}S-labeled HIV-1 RNA (0.5 nM) and increasing concentrations of p15NC protein (0.1 to 18 nM) in a final volume of 200 μl of PBS. After 60 min of incubation at room temperature, 190 μl of the reaction was filtered through prewetted HAWP nitrocellulose filters (Waters-Millipore) under vacuum, washed once with 500 μl of PBS, air dried, and counted in 10 ml of scintillation cocktail. The filtration and washing steps were timed (total of 45 s) to ensure consistency of retention percentage. Background or nonspecific retention of labeled RNA to the filters was determined in parallel reactions lacking the p15NC protein. The equilibrium dissociation constant, K_d, was estimated by using SigmaPlot software from the equation

$$\mathrm{PR\ (cpm)} = \frac{\mathrm{PR}_m\ \mathrm{(cpm)}}{2R_t}\{K_d + P_t + R_t - \sqrt{(K_d + P_t + R_t)^2 - 4P_tR_t}\}$$

where PR_m is the maximum retention of the protein-RNA complex at saturating protein concentration, P_t and R_t are the total p15NC and RNA concentrations present in the reaction, and K_d is the dissociation constant for the binding of RNA by p15NC protein. For competition experiments, unlabeled oligonucleotides, oligodeoxynucleotides, or RNA was diluted in PBS immediately before use and added to reactions prior to addition of labeled RNA.

Construction of HIV-1 infectious clones, cell transfection, and viral characterization. Subclones of HIV-1 isolate HXB2 containing either the second zinc finger (CS2) or inter-zinc finger basic amino acid (P3BE) p15NC mutation were

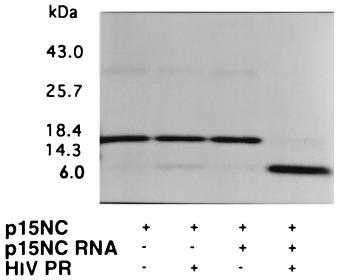

FIG. 1. Enhanced cleavage of purified p15NC by HIV-1 protease in the presence of RNA. Purified recombinant p15NC (final concentration, 15 μM), recombinant HIV protease (18 nM), and p15NC RNA (final concentration, 2 μM) were combined as indicated and incubated in PBS (pH 6.5) for 60 min at 30°C prior to electrophoresis on SDS–8 to 16% polyacrylamide gels and Coomassie blue staining. In this gel system, the cleavage products, p6 and p7NC, comigrate near the dye front.

used to regenerate full-length HXB2 viral clones (51). Purified DNA was isolated (Qiagen), and the presence of the desired mutations was confirmed by DNA sequencing. For pulse-chase experiments, HeLa cells (8) were transfected in six-well plates. At 48 h posttransfection, cells were starved in RPMI medium minus cysteine for 3 h, followed by a pulse for 45 min in 1 ml of medium lacking cysteine supplemented with 300 μCi of [^{35}S]cysteine (1,000 Ci/mmol). Cells were washed twice with medium and chased with 1 ml of complete medium for chase periods of 1 to 5 h. At times indicated in the figure legends, medium (containing released virus) was removed, clarified by centrifugation, and mixed with radioimmunoprecipitation assay buffer (29). The remaining cells were washed and then lysed in radioimmunoprecipitation assay buffer. Immunoprecipitation of labeled virion proteins was performed with rabbit polyclonal serum raised to recombinant p15NC or with HIV-1-infected patient serum as described previously (29).

For electron microscopy (EM), HeLa cells in 100-mm-diameter plates were transfected with 20 μg of DNA of the purified infectious clone HXB2 or a clone containing the P3BE or CS2 mutation as described previously (8). At 48 h posttransfection, cells were scraped and fixed overnight in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After washing, the cells were postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer for 45 min, dehydrated with ethanol, and embedded in araldite 502/EMbed 812 as instructed by the manufacturer (Electron Microscopy Sciences). Thin sections were cut and stained with uranyl acetate and lead citrate prior to examination in a Philips EM400 electron microscope at a setting of 60 kV.

RESULTS

p15NC-RNA interaction and enhanced proteolytic cleavage in vitro. Our previous studies with in vitro translation products corresponding to p15NC and the ~400-nucleotide RNA encoding it allowed a sensitive means to measure the RNA-enhanced processing of p15NC by the viral protease (60). We found that within this system, the 400-bp p15 transcript was functionally equal to the full-length Gag transcript in its ability to enhance protease-mediated cleavage of p15NC. The complex nature of the rabbit reticulocyte expression system, however, precluded detailed investigation of the mechanism of RNA-enhanced cleavage. We thus prepared purified p15NC protein to define the nature of interactions between p15NC and RNA, which results in increased susceptibility to the HIV protease. Figure 1 shows the cleavage of purified p15NC (estimated purity >95% by densitometry) in the reconstituted system containing p15NC, purified HIV protease, and p15NC RNA and also shows the lack of processing when any of the components is omitted. These results confirm our previous observation of enhanced cleavage of p15NC in the presence of RNA (60) and show that in our reconstituted system, the p15 RNA alone is sufficient to act as a positive modulator of protease-mediated cleavage of p15.

We next examined the binding of purified p15NC protein to its corresponding RNA transcript, using filter binding and gel shift assays. Figure 2 shows the saturable binding of recombinant p15NC with labeled RNA transcript, using a filter binding assay under conditions of fixed RNA concentration and moderate salt concentration (0.15 M NaCl). Saturation occurred when the level of recombinant protein was about 20- to 30-fold

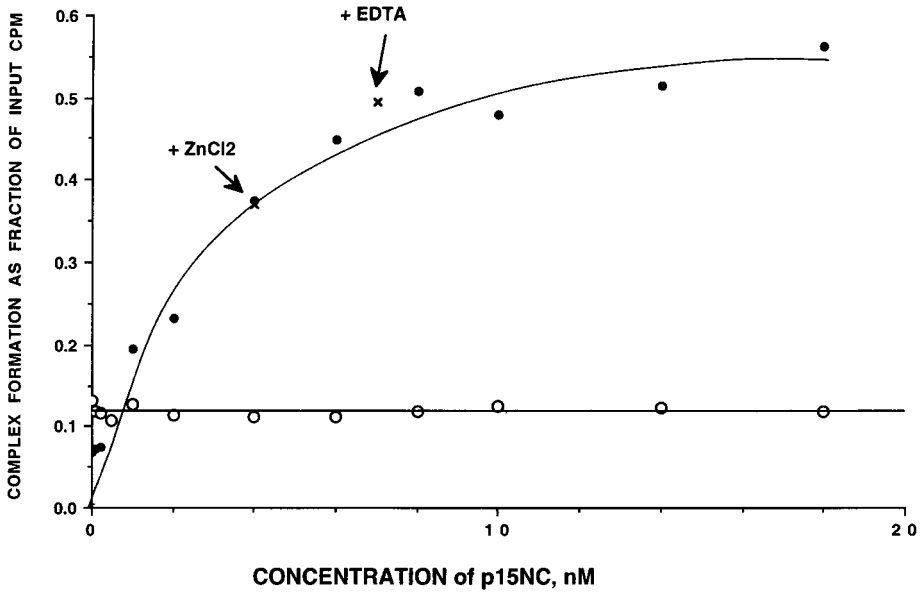

FIG. 2. Binding of p15NC mRNA to p15NC. Filter binding assays contained a constant concentration of ^{35}S-labeled p15NC RNA (0.5 nM) (closed symbols) or ^{35}S-labeled bovine liver tRNA (1.8 nM) (open symbols). The p15NC concentration was varied from 0 to 18 nM. Samples were filtered after a 60-min equilibration period at 25°C. Where indicated, 20 nM $ZnCl_2$ or 45 μM EDTA was included to evaluate the role of the NC zinc fingers. The data are shown as fractional binding relative to input counts per minute. Because the tRNA and p15NC RNA are present at different concentrations, there are two relevant comparisons. If the data are considered as the molar ratio of p15NC to RNA, then the 18 nM p15NC point of the tRNA binding curve should be compared to a position at 5.5 nM p15NC RNA (70% saturation). If the ratio is based on total nucleotide concentration, then the 18 nM point should be compared to approximately 12 nM p15NC RNA. In both cases, the binding to p15NC is approaching saturation providing the sensitivity to distinguish between the specific and nonspecific component interactions.

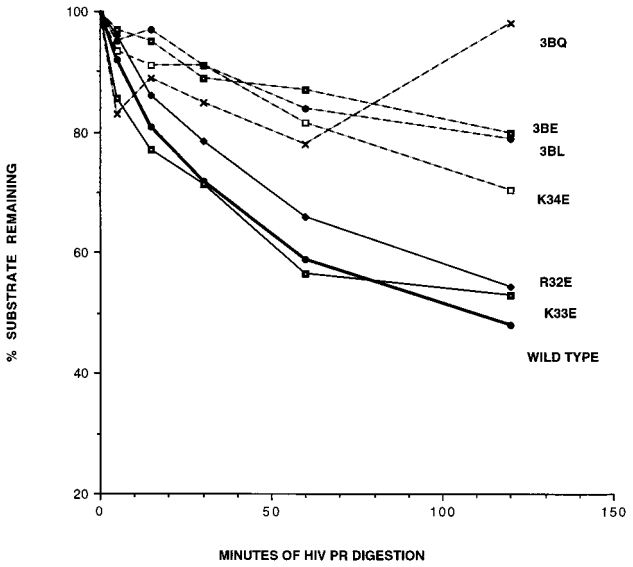

FIG. 3. Cleavage of wild-type and mutant forms of p15NC in vitro. Mutant and wild-type p15NC species were generated via in vitro transcription-translation as described in Materials and Methods. All samples were digested with purified HIV protease at 30°C; aliquots were removed at the times indicated and analyzed by SDS-PAGE. An Ambis Radioanalytic Scanner was used for quantitation. Curves represent the averages from two to five time course experiments. The courses of proteolysis with wild-type p15NC, 3B mutants, and single mutants at positions 32, 33, and 34 are indicated.

that of RNA. In contrast, no saturable, concentration-dependent (specific) binding of labeled tRNA was detected under these binding assay conditions. RNA does not bind directly to the HIV protease dimer as measured in filter binding assays. In the reverse titration, that of fixed p15NC protein, and variable amounts of labeled RNA, saturation also occurred at a ratio of protein to RNA species of approximately 30:1 (data not shown). In gel shift experiments, addition of 0.4 to 7 equivalents of recombinant p15NC protein caused the migration of RNA to be increasingly retarded in an agarose gel (data not shown). Formation of the p15NC-RNA complex was not affected by addition of 20 nM $ZnCl_2$ (a fivefold excess relative to Gag p15NC concentration) or 45 μM EDTA (Fig. 2), suggesting that binding did not involve the Cys-His zinc-binding domains or that the function of the zinc-binding domains was not affected under these conditions. This result contrasts with the well-documented role that these domains play in binding to PSI site regions of HIV RNA (10, 11, 41, 57, 70). The K_d value estimated from the protein concentration required for half-maximal binding under these conditions was 1.5 nM. This K_d estimate, though not rigorously defined, allowed the relative affinities of various oligonucleotide competitors to be subsequently compared.

Basic amino acids located between the zinc fingers are required for RNA binding and enhanced protease-mediated cleavage in vitro. The p15NC protein of HIV contains two conserved zinc-binding, Cys-His finger motifs within the NC domain that are separated by a flexible linker of seven residues. The linker contains a triplet of basic amino acids at positions 32 to 34 (44). Using p15NC produced by in vitro transcription and translation, we previously showed impaired cleavage of p15NC when the basic triplet had been mutated to glutamic acid (3BE) (60). The p15NC mutant 3BE is also defective for binding RNA under conditions where two mutations that disrupt either Cys-His array (CCYY-1 and CCYY-2) have no effect on RNA binding or cleavage. Analysis of the cleavage of recombinant p15NC and p15NC containing the various mutations confirmed that the zinc finger mutants bound RNA and displayed cleavage enhancement equal to that found for the wild type. Purified 3BE p15NC, however, bound 20% as much labeled p15NC RNA and showed reduced cleavage by protease (data not shown).

The individual contribution of basic residues 32 to 34 to RNA-enhanced cleavage was examined by mutagenesis. Figure 3 shows the relative time course for viral protease-mediated cleavage in the presence of RNA of wild-type and mutant forms of p15NC in which the basic triplet residues have been altered. Cleavage was three- to fourfold slower when the three basic amino acids were changed to glutamic acid, as previously documented (60), but also when these amino acids were changed to leucine or glutamine, which are isosteres for lysine in terms of chain length and hydrogen-bonding capabilities, respectively. When the three basic amino acids were individually mutated to glutamic acid, K34E was cleaved 50% more slowly than wild-type p15NC in the presence of RNA. R32E and K33E were cleaved at rates similar to wild-type rates. These results suggested that multiple electrostatic interactions involving the triplet of basic amino acids play a role in the protein-nucleic acid interaction that confers protease susceptibility with the terminal lysine (K34) as the key component.

Identification of viral RNA-derived sequences that confer enhanced protease susceptibility to p15NC. We wished to define a minimum RNA sequence sufficient to confer enhanced protease-mediated cleavage by annealing to specific regions of the p15 RNA. We took two different approaches to determining the RNA requirement. First, we prepared a library of contiguous 21-mer oligodeoxynucleotides complementary to p15NC RNA in an attempt to block enhancement of p15NC cleavage through heteroduplex formation and assessed the result in the reconstituted system containing recombinant protein and added p15NC RNA. Despite several attempts using a variety of ratios of synthetic oligodeoxynucleotide to p15NC RNA and different annealing conditions, no inhibition of protease-mediated cleavage of p15NC was observed (data not shown). However, we noticed that one synthetic oligodeoxynucleotide, designated N-5, actually enhanced the cleavage of in vitro-translated p15NC when added to this system. This result was surprising, as previous experiments had suggested that small oligodeoxynucleotides were poor modulators of p15NC cleavage (60).

We then examined directly the ability of this set of oligodeoxynucleotides to potentiate the cleavage of purified p15NC by the HIV protease in the reconstituted system. As shown in Fig. 4 (lane 5), only oligodeoxynucleotide N-5 showed ability to confer susceptibility to the viral protease comparable to the result for the control cleavage reaction (lane A). Three other oligodeoxynucleotides, N-12, N-14, and N-16 (Fig. 4, lanes 12,

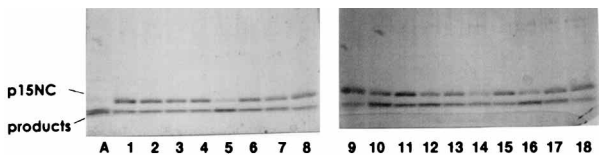

FIG. 4. Oligodeoxynucleotides of particular sequence enhance cleavage of purified p15NC in vitro by protease. All reaction mixtures contained purified p15NC (final concentration, 15 μM), HIV protease (18 nM), and 2 μM synthetic 21-mer oligonucleotide in PBS. Reaction mixtures were incubated at 30°C for 60 min, and products were visualized via SDS-PAGE and Coomassie blue staining. Lane A, 2 μM p15NC RNA; lanes 1 to 18, contiguous 21-mer oligodeoxynucleotides.

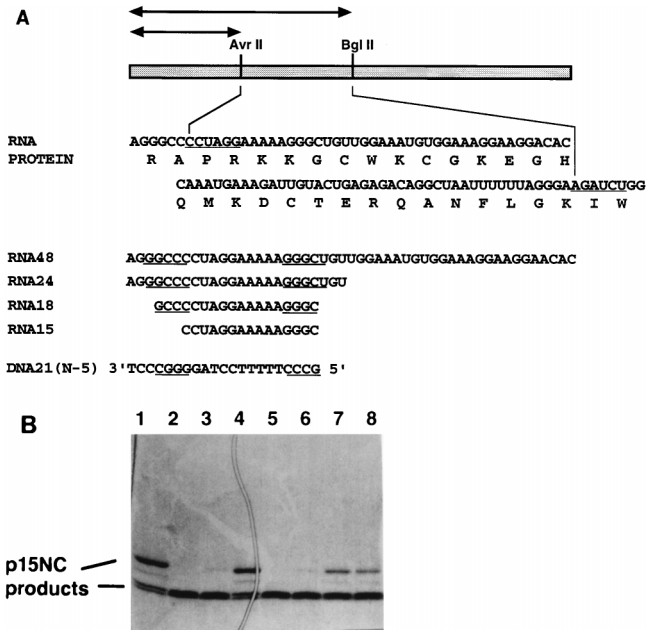

FIG. 5. Truncated RNAs support HIV protease-mediated cleavage of p15NC. (A) Diagram showing locations of *Avr*II and *Bgl*II restriction endonuclease sites within the p15NC region and sequence of synthetic RNA species used to probe RNA requirement for HIV protease-mediated cleavage of p15NC protein. Below are the sequences of DNA and RNA oligonucleotides tested for the ability to enhance cleavage of p15 in vitro. The complementary bases with the potential to form a stem-loop structure are underlined. (B) Protease digestion of purified p15NC protein. Purified HIV protease was added to p15NC in PBS (pH 6.5) in the presence of the following: lane 1, no added RNA; lane 2, 60 pmol of full-length p15NC RNA; lane 3, 60 pmol of *Bgl*II runoff transcript; lane 4, 60 pmol of *Avr*II runoff transcript; lane 5, 60 pmol of RNA48; lane 6, 60 pmol of RNA24; lane 7, 60 pmol of RNA18; lane 8, 60 pmol of RNA15.

14, and 16), also conferred susceptibility to protease cleavage but to a much lower degree. A contiguous set of 21-base oligodeoxynucleotides prepared in the sense orientation (designated S-1 to S-18) failed to enhance cleavage significantly, although there was a discernible loss of substrate in the reaction corresponding to oligodeoxynucleotide S-5 (data not shown). S-5 and its complement N-5 correspond to the region that encodes NC residues 29 to 34 between the Cys-His fingers and contain internal complementary sequences (see Fig. 5A for their sequences).

In the second approach, we mapped the area of p15NC RNA responsible for enhanced cleavage of p15NC. We used two restriction sites within the p15NC portion of the in vitro transcription-translation plasmid, pDAB15, to generate truncated RNA species (Fig. 5A). Transcription of plasmid linearized with *Bgl*II produces a truncated transcript corresponding to nucleotides 1 to 200 of the p15NC sequence, a region that encodes NC through both zinc fingers. Transcription of the plasmid linearized with *Avr*II produces a transcript of ~100 nucleotides, representing coding sequence through the region corresponding to the first zinc finger of NC and ending just short of the region to which oligonucleotide N-5 maps. When we compared *Bgl*II and *Avr*II RNAs to the full-length p15 transcript with respect to the ability to confer enhanced susceptibility to viral protease cleavage, we observed that *Bgl*II RNA but not *Avr*II RNA enhanced cleavage of p15NC by the viral protease (Fig. 5B). The shorter *Avr*II RNA was quite inefficient compared to the longer RNA species even with eightfold more RNA present in the reaction (data not shown). This result suggested either than an RNA species of ~100 nucleotides was not sufficient to support HIV protease-mediated cleavage or that sequences and/or secondary structures found in nucleotides 100 to 200 were necessary for the RNA-enhanced cleavage of p15. Considering the findings from the oligonucleotide mapping experiments, we further explored the latter possibility.

We mapped the RNA sequences required for RNA-enhanced cleavage of p15NC by determining the ability of a synthetic RNA species corresponding to the first 48 bases of the region defined by *Avr*II and *Bgl*II (designated RNA48) and correspondingly smaller fragments (RNA24, RNA18, and RNA15) to enhance HIV PR-mediated cleavage of recombinant p15NC (Fig. 5B). We observed that RNA48 and RNA24 were comparable to full-length p15NC RNA (on a molar basis) in enhancing protease-mediated cleavage of p15NC, whereas RNA18 and RNA15 were less efficient, in that visible substrate remained after a 30-min digestion period. An RNA species of 9 nucleotides, corresponding to the middle portion of RNA15, did not measurably enhance cleavage of p15NC (data not shown). Use of the GCG program MFOLD (65, 72) which considers both AU, GC, and GU potential base pairs, predicted a thermodynamically favorable folding pattern of a 5-bp stem-loop for both RNA24 and oligodeoxynucleotide N-5.

We prepared 5′-end ^{35}S-labeled RNA24, in order to directly compare its binding to p15NC to that of full-length p15NC RNA. The apparent K_d for ^{35}S-labeled RNA24 binding by purified p15NC protein was about threefold higher than for binding of full-length p15NC RNA (K_d = 5.2 nM). A reduced interaction was further inferred by the lessened ability of unlabeled RNA24 to compete with labeled p15 RNA compared to unlabeled p15 RNA (not shown). We also investigated the ability of synthetic oligodeoxynucleotide analogs of N-5 to compete with labeled p15NC RNA for p15NC binding. Oligodeoxynucleotide N-5 was able to compete with labeled p15NC RNA for binding, but S-5 or two oligodeoxynucleotides identical in base composition to N-5 and S-5 but with random sequence were not (Fig. 6). Oligodeoxynucleotide N-5GT, containing a G-to-T substitution to disrupt the stem-loop, was also less effective over the concentration range examined. Taken together, these data suggest that binding of p15NC to highly localized regions of RNA secondary structure confers the increase in HIV protease susceptibility. Structural studies to elucidate the precise location of the interaction(s) suggested from the binding and processing studies described here are in progress.

Mutagenesis of NC basic residues 32 to 34 affects HIV-1 viral morphogenesis. Our results indicated the necessity of the basic residues located at positions 32 to 34 of the NC domain in the recognition of nucleic acids and the upregulation of p15NC in vitro. To evaluate the importance of these residues to virion assembly, we placed the 3BE mutation (designated P3BE) in an infectious clone of HIV-1 and transfected the clone into HeLa cells to produce virus. As a control, we constructed a second viral clone containing the CS2 mutation (substitution of second zinc fingers cysteine residues 36 and 39 with serines). The CS2 mutation is actually located closer to the affected protease cleavage sites in the p15 precursor than the P3BE mutation. The virus morphology of the CS2 mutation has been previously characterized in the pNL4-3 background by Gorelick et al. (22). Two days posttransfection, the effect of the mutations was determined. Western blot analysis showed no defect in global proteolytic processing, as similar extents of processing of Gag to matrix (p17), p24-capsid, and p15NC were seen (not shown).

Pulse-chase analysis of Gag processing, however, indicated an altered rate of processing of p15NC with the P3BE muta-

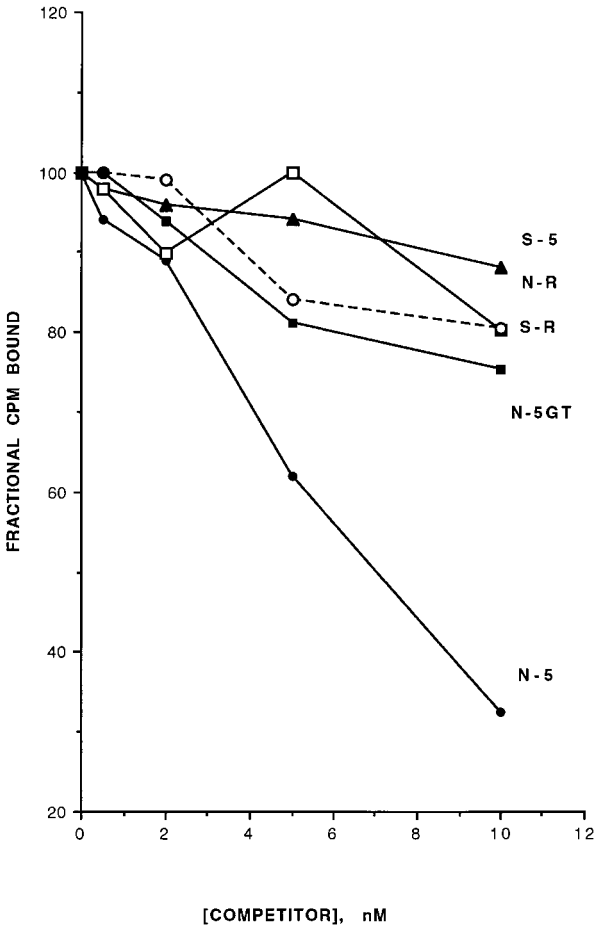

FIG. 6. Ability of synthetic oligodeoxynucleotides to displace ^{35}S-p15NC RNA in the filter binding assay. Reaction mixtures contained 4 nM Gag p15NC, 0.5 nM ^{35}S-labeled RNA, and the indicated concentration of unlabeled oligodeoxynucleotide in PBS. Closed circles, oligodeoxynucleotide N-5 (GCCCTTTTT CCTAGGGGCCCT); closed triangles, oligodeoxynucleotide S-5 (AGGGCCC CTAGGAAAAAGGGC); open squares, oligodeoxynucleotide N-R (GCTCGT TTATTCCCTGGGCCC); open circles, oligodeoxynucleotide S-R (GCAGCAA ATAAGGGACCCGGG); closed squares, oligodeoxynucleotide N-5GT (GCC CTTTTTCCTAGGTGCCCT).

tion compared to wild-type p15NC. P3BE p15NC was cleaved more slowly than the wild type in cells and in released virions, and precursor was evident even after a 5-h chase (Fig. 7). The CS2 mutation resulted in a p15NC intermediate that was actually less stable than the wild type. In contrast to the differential phenotypes of the P3BE and CS2 mutations on the rate of cleavage of p15NC, both mutations resulted in a small delay in cleavage at the CA-p2 site (not shown). We previously noted that the rate of cleavage of the CA-p2 site is downregulated by downstream conformational changes induced through the p2 domain (49, 64). Neither mutation had a significant effect on overall protease activation, as the half-life of the full-length Gag precursor and the generation of the CA-p2 intermediate were similar to those for the wild type during the chase period. Furthermore, the mutations did not affect packaging of Gag-Pol products into virions. Amounts of processed RT were similar to wild-type amounts as determined by Western blot analysis (data not shown). While the mutations had little effect on particle release as judged by medium-associated RT activity or p24 levels, the infectivity of the released virions was severely impaired. P3BE and CS2 virions were reduced at least 100-fold in infectivity as determined by MAGI cell assay (29), and these particles were unable to establish a productive infection of CEM cells. These infectivity results and the lack of a gross processing defect are similar to those reported by others for zinc finger and inter-zinc finger array mutants (1, 15, 20–22, 48). The RNA level in released virions from the 48-h CS2 transfection was 37% of that for the wild type (adjusting for the small difference in p24 measured), whereas the RNA level in P3BE virions was less than 12% of that for the wild type, as measured by a sensitive RNA hybridization-based assay (3). Gorelick et al. previously reported that in the CS2 mutant, virion RNA levels were 20% of the wild-type level and that the virions had a wild-type morphology (22).

When released virions were examined by thin-section EM, a unique morphology was observed for the P3BE virus (Fig. 8). Upon cursory examination, the P3BE virions were somewhat similar in morphology to immature or protease-deficient virions in lacking a condensed core (28, 52). However, P3BE virions are clearly different from immature virions in that the center contains diffuse material. In addition, the electron-dense material juxtaposed to the membrane lacks the typical banding pattern observed in immature wild-type HXB2 virions. Thus, the P3BE virions are defective for core formation despite the eventual cleavage of Gag and Gag-Pro-Pol to the final products. No P3BE virions were observed with the wild-type morphology of a condensed core in any field examined. Wild-type and CS2 fields contained both immature and fully mature virions, consistent with earlier observations (1, 20, 22).

DISCUSSION

Previously we reported that cleavage of the p15NC processing intermediate was upregulated by the presence of Gag mRNA during the processing of the full-length Gag in vitro (60). In this report, we refined the components of the in vitro system in order to obtain a more detailed examination of the determinants required for upregulation of p15NC cleavage upon interaction with viral RNA. With purified components, we determined that p15-encoding RNA is the only additional component necessary for enhanced cleavage of the p15NC in vitro. Through a systematic approach, we have defined several determinants on both the RNA and p15NC necessary for maximal upregulation of p15NC cleavage in vitro. Additionally, we

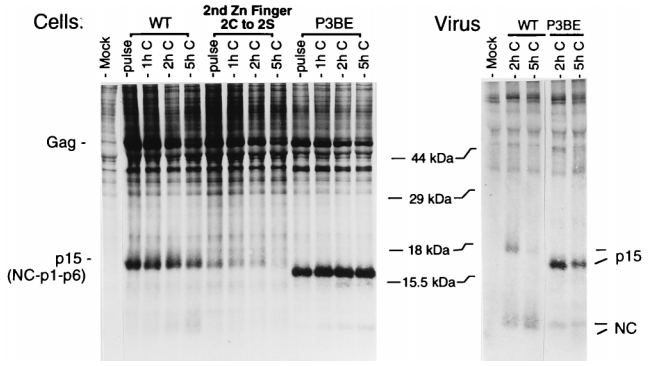

FIG. 7. Pulse-chase analysis of p15NC processing in infected cells and released virions. HeLa cells transfected with the wild-type HXB2 full-length viral clone or a clone containing mutations in the NC coding domain were prepared as described in Materials and Methods. Following [^{35}S]cysteine pulse-labeling and chase, released virions and cell extracts were immunoprecipitated with antibodies to purified p15NC and separated by SDS-PAGE. Wild-type HXB2 isolate (WT) and the CS2 and P3BE mutants were compared. The positions of molecular mass markers and of p15NC and mature NC are shown.

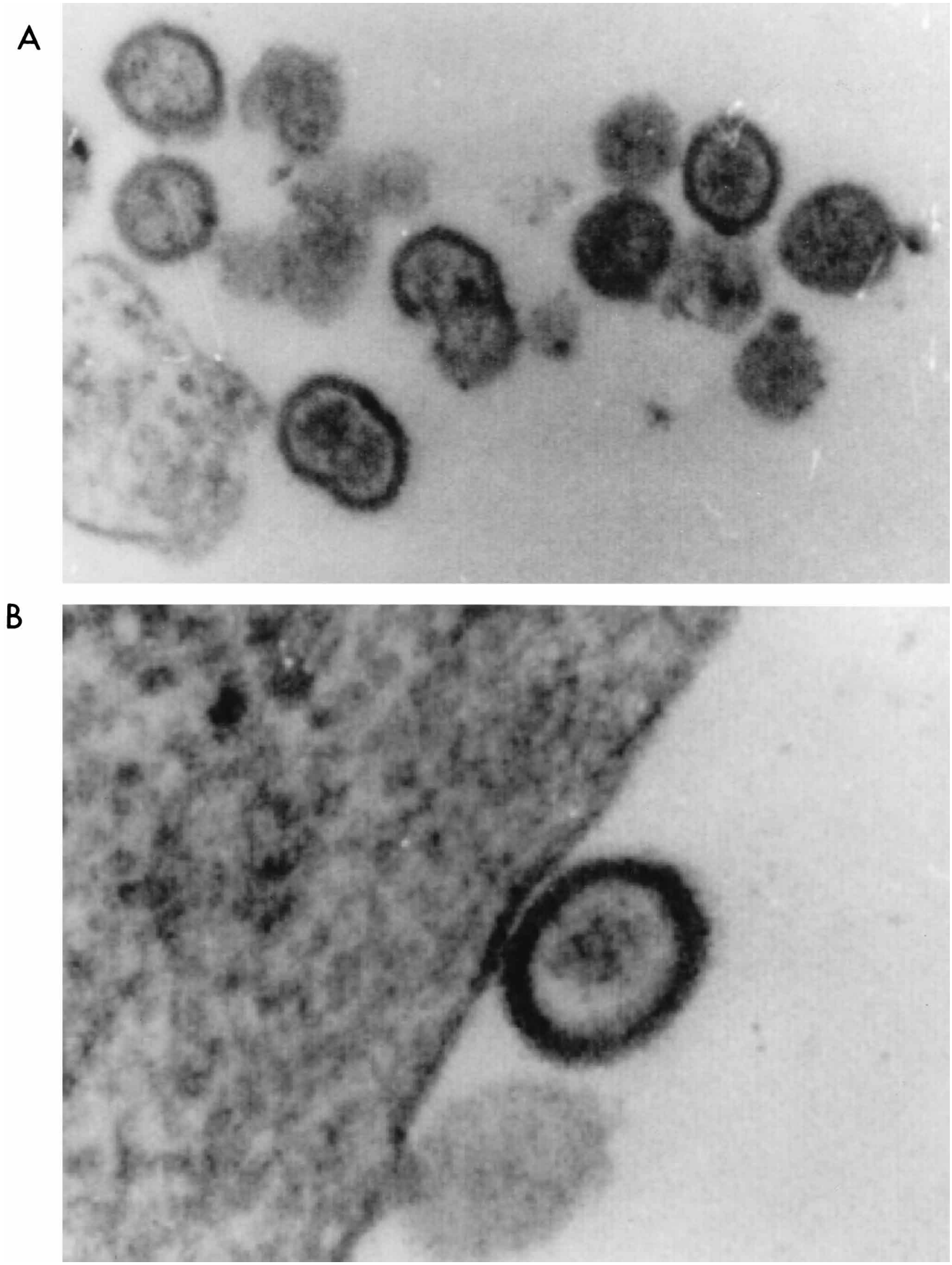

FIG. 8. Electron micrographs of virions containing the P3BE mutation. HeLa cells were transfected with the P3BE-HXB2 infectious clone and prepared for EM as described in Materials and Methods. The picture of released P3BE mutant virions was taken at a magnification of 18,000. The inset of a single P3BE particle at higher magnification shows in detail the unique morphology of the mutant. No virions of wild-type morphology were observed.

investigated mutations in the residues in p15NC necessary for RNA enhanced cleavage in vitro for their effects on the assembly and morphogenesis of virions.

The p15NC-p15NC RNA interactions that we observed in the enhanced cleavage by the viral protease involve a specific region of this portion of the viral RNA and NC-precursor protein. Using a combination of runoff transcripts and synthetic oligonucleotides, we found a minimum-sized oligonucleotide fragment capable of interacting with the recombinant p15NC and positively modulating cleavage by HIV protease. An RNA species of 24 nucleotides, RNA24, contained within the p15NC RNA sequence was fully competent for binding to p15NC and imparting enhanced susceptibility to HIV protease-mediated cleavage (Fig. 5B). The binding interaction(s) leading to protease-mediated cleavage was not limited to RNA. Oligodeoxynucleotide N-5, predicted to form a stem loop ($\Delta G = -6.9$ kcal/mol), was able to function, albeit less efficiently than p15NC RNA (N-5 bound with 5- to 10-fold lower affinity), in the enhancement of p15NC cleavage. The complement of this sequence (S-5), which forms a less stable stem-loop $\Delta G = -4.9$ kcal/mol), and random or stem-loop-disrupted oligodeoxynucleotides of identical composition failed to interact with p15NC as detected by binding and cleavage assays. This result strongly suggests that a particular secondary structure is an important determinant of the interaction that permits enhanced cleavage of p15NC by the viral protease. The possible mechanisms of cleavage upregulation include polynucleotide-mediated conformational effects to expose buried cleavage sites, stabilization of substrate-enzyme binding, and transition state stabilization. Although beyond the scope of this study, such possibilities may be testable by using more sophisticated binding assay methodologies.

The requirement for stem-loop-forming ability explains, in part, the apparent discrepancy with our earlier observation that oligodeoxynucleotides showed no enhancement of p15NC cleavage in the in vitro transcription system (60). The oligodeoxynucleotides tested previously contained no significant inverted repeats. However, full-length single-stranded p15NC DNA was also ineffective in conferring protease susceptibility (60). Perhaps other secondary structures were favored with this DNA species that were unfavorable for p15NC binding. The *Avr*II transcript, which was also inefficient in enhancing p15NC cleavage (Fig. 5) and predicted to have a complicated secondary structure by MFOLD (65, 72), supports the idea that particular secondary structural elements are involved in the RNA-p15NC-protease interaction. Sequence requirements may add further complexity to the stem-loop requirement; preliminary studies suggest that partial cleavage enhancement (at the p1-p6 junction) results with stable stem-loops regardless of sequence, whereas completion of the p7NC-p1 processing step is sequence dependent (48a).

The RNA and DNA oligonucleotides in this model system represent only a fraction of the potential secondary structure found in virion RNA. We found by MFOLD at least four other regions of the viral genome, corresponding to nucleotides 2483 to 2498 (within *pol*), 6929 to 6951 and 7103 to 7117 (within *env*), and 4666 to 4680 (within a splice junction), that have small stem-loop-forming regions similar to that defined by RNA24. Wu et al. recently described the interaction of a stem-loop region of murine leukemia virus RNA located near the polypurine tract with HIV-1 NC which resulted in an improved efficiency in DNA synthesis (69). These authors suggested there might be a similar role for NC and stem-loop regions within HIV-1. Further studies to elucidate whether the homologous regions that our search has identified bind to p15NC are in progress.

Decreased cleavage of the P3BE p15NC mutant in cells and virus particles (Fig. 7) suggests that the interaction between p15NC and RNA is an important feature of virion morphogenesis, although we have not determined the effect of this mutation on p15NC folding. If appropriately timed cleavage of the p15NC in virions does require RNA, then our experiments raise several issues. First, if the cleavage is promoted by binding to a small number of specific features of RNA secondary structure, as suggested by the productive interaction with a small region of p15NC RNA, then each RNA structure must bind many p15NC molecules sequentially, with the cleaved, mature p7NC dissociating to bind elsewhere on the RNA. The relative dissociation constants of 1.5 nM for p15NC and ~20 nM for p7NC (59) and the determination of high- and low-affinity binding states for p7NC that vary with p7NC/RNA ratio (14, 31, 70) are consistent with such sequential binding interactions. Previously, a model for RNA-dependent maturation of Rauscher leukemia virus which involved multiple, sequential binding interactions was proposed based on inferences derived from actinomycin D effects (26). Alternatively, since we have tested only a small proportion of the genome, we do not know the density of RNA binding sites that could contribute to accelerated p15NC cleavage in vivo. Finally, the generation of some mature p7NC may be sufficient to allow cleavage of the remaining p15NC intermediates through complex protein-protein interactions. Such a model would predict rate enhancements late in the course of p15NC cleavage. The time-dependent decrease in rate that we typically observe in vitro, which we have reasoned reflects the dissociation of protease dimers at the low concentrations used, may have obscured such effects. These models suggest several experiments, including a need to know the fate of p15NC in virions made without RNA.

The structure of the NC domain within the p15NC precursor intermediate is not known. Several lines of evidence suggest that the domain within the p15NC precursor has a conformation different from that of fully processed p7NC. Tanchou et al. (63) noted differences in the binding of conformationally sensitive monoclonal antibodies to mature NC versus p15NC. We have also identified apparent conformation-dependent monoclonal antibodies within a panel raised to p15NC (63a). Others have suggested a difference in the specific recognition of viral RNA and in the size of the binding site or footprint on RNA (14, 31, 38, 70). Thus, the different forms of NC present during the maturation phase of the viral life cycle may have distinct functions during virion morphogenesis. Controlling the appearance of the various forms through regulation of p15NC cleavage may be an important aspect of viral maturation.

In terms of the specific regions of the p15NC involved, the triplet of basic amino acids located between the zinc fingers are crucial as determined for both RNA binding and protease cleavage upregulation (Fig. 3). The residues of the fingers and the fingers themselves have been extensively studied via nuclear magnetic resonance analysis and mutagenesis of mature p7NC (12, 13, 35, 38, 45, 46, 48, 50, 61). Nuclear magnetic resonance structures of HIV-1 NC suggest the area between the fingers plays a role in the coordination of the zinc fingers. Pro-31 causes a kink in the linker that brings the fingers in closer proximity (46). Mutation of Pro-31 to the bulkier leucine affects both viral assembly and maturation (46, 48). Phe-16, Trp-37, and Met-46 interact through van der Waals contacts with the viral RNA (35, 45, 46). The linker region has thus been described as being independently flexible from the rigid fingers, with Lys-33 and Lys-34 solvent exposed and available for interactions (45, 46). Our mutational analysis studies suggest Lys-34 is the most critical residue involved, but additional interactions involving Arg-32 and Lys-33 are required for full

enhancement of protease cleavage (Fig. 3). This interpretation is similar to the conclusions by Poon and Aldovini (50), who found that multiple mutations had a more significant effect on NC function. Arg-32 has been strongly linked to PSI site RNA binding and infectivity (11, 48, 50). In contrast to the importance of the triplet of basic residues, mutation of the second zinc finger had little effect on binding and protease-mediated cleavage in vitro (Fig. 7) or on virion morphology (22) despite the fact that this region is closer to the scissile bond. Thus, the specificity that we have determined for p15NC cleavage enhancement by RNA overlaps, but is not identical to, that described for PSI site binding-mediated effects (5, 10, 11, 13, 25, 27, 31, 38, 39, 59, 61, 62, 70).

An obvious prediction of our proposed role for the intra-Cys-His basic array is that viruses with mutations in this region should show specific defects in processing, namely, in p15NC maturation. Studies that have been carried out with packaging-defective mutant virus have concluded that protease-mediated processing is independent of RNA packaging or particle assembly (1, 11, 13–15, 20, 22, 50, 53, 59). In general, the probes and methods used to assess processing were focused on p24-containing regions of Gag. We have shown that virions containing the P3BE mutation are delayed selectively in p15NC cleavage and are defective for core formation despite the eventual appearance of the final processed products. Other viral mutants that disrupt the zinc finger motifs and result in defective virion RNA packaging have wild-type morphology similar to that of the CS2 mutant presented here (20, 22). Thus, the altered morphology that we observed appears not to be due to a lack of viral RNA per se. Others have reported NC mutations that result in virion morphology similar to that of the P3BE mutation, although most of these other mutations appear to have more of a global effect on protease activity and virion release (15, 50, 53). Our results with the P3BE mutation demonstrate the importance of the triplet of basic residues in the core formation and suggest that this mutation is in a separate phenotypic class.

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