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Elimination of Protease Activity Restores Efficient Virion Production to a Human Immunodeficiency Virus Type 1 Nucleocapsid Deletion Mutant

David E. Ott,[1]* Lori V. Coren,[1] Elena N. Chertova,[1] Tracy D. Gagliardi,[1] Kunio Nagashima,[2] Raymond C. Sowder II,[1] Dexter T. K. Poon,[1] and Robert J. Gorelick[1]

AIDS Vaccine Program[1] and Research Technology Program,[2] SAIC-Frederick Inc., National Cancer Institute at Frederick, Frederick, Maryland 21702-1201

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The nucleocapsid (NC) region of human immunodeficiency virus type 1 (HIV-1) Gag is required for specific genomic RNA packaging. To determine if NC is absolutely required for virion formation, we deleted all but seven amino acids from NC in a full-length NL4-3 proviral clone. This construct, DelNC, produced approximately four- to sixfold fewer virions than did the wild type, and these virions were noninfectious (less than 10^{-6} relative to the wild type) and severely genomic RNA deficient. Immunoblot and high-pressure liquid chromatography analyses showed that all of the mature Gag proteins except NC were present in the mutant virion preparations, although there was a modest decrease in Gag processing. DelNC virions had lower densities and were more heterogeneous than wild-type particles, consistent with a defect in the interaction assembly or I domain. Electron microscopy showed that the DelNC virions displayed a variety of aberrant morphological forms. Inactivating the protease activity of DelNC by mutation or protease inhibitor treatment restored virion production to wild-type levels. DelNC-protease mutants formed immature-appearing particles that were as dense as wild-type virions without incorporating genomic RNA. Therefore, protease activity combined with the absence of NC causes the defect in DelNC virion production, suggesting that premature processing of Gag during assembly causes this effect. These results show that HIV-1 can form particles efficiently without NC.

Orthoretroviruses assemble into virions from Gag and Gag-Pol polyproteins, Env protein complexes, and two genomic RNAs (24, 68). Gag is solely sufficient for particle production and contains the mature structural proteins of the virus. The human immunodeficiency virus type 1 (HIV-1) Gag polyprotein, $Pr55^{Gag}$, contains the primary mature structural proteins linked in a matrix (MA), capsid (CA), spacer peptide 1 (SP1, also called $p2^{Gag}$), nucleocapsid (NC), SP2 (also called $p1^{Gag}$), $p6^{Gag}$ configuration (Fig. 1). In HIV-1, the Gag-Pol polyprotein is expressed by a −1 frameshift just after NC that occurs in 5% of Gag translations. In addition to most of Gag, this polyprotein contains the protease, reverse transcriptase, and integrase enzymes that carry out the required virus-specific enzymatic steps required in the replication cycle. HIV-1 assembles on and buds from the plasma membrane.

The Gag and Gag-Pol polyproteins are cleaved by the viral protease into their mature structural proteins and enzymes, respectively. This process is required for infectivity of orthoretroviruses, converting Gag and Gag-Pol from polyproteins devoted to assembly into several mature proteins that function in the infection process. The cleavage of HIV-1 Gag induces a reorganization of the newly budded, doughnut-shaped, immature virion into a mature form with a dense conical core (68, 69, 72). While protease processing of Gag occurs after budding in most retroviruses, HIV-1 is an exception, since processing initiates during assembly and is completed after virus release (44, 45). HIV-1 processing appears to be required for efficient budding since virus production is significantly slowed when processing is reduced or blocked (44, 62). Conversely, decreases in HIV-1 budding caused by certain mutations in $p6^{Gag}$ or proteasome inhibitors are accompanied by decreases in Gag processing (40, 63) and mutational inactivation of HIV-1 protease can rescue the budding of one of these $p6^{Gag}$ mutants (40). Together, these findings suggest a functional link between HIV-1 polyprotein processing and budding.

The assembly function of Gag can be characterized by three different assembly domains (26, 68, 74): a membrane-binding domain (M) present in the N terminus of MA, which is important for Gag association with the plasma membrane; the interaction domain (I) present in the C-terminal half of CA and the zinc fingers of the NC protein, which is important for tight interactions between the Gag proteins in the assembling virus; and the late domain (L) found in different locations in Gag, which is required for efficient release of virions from the cell (reviewed in reference 25). The HIV-1 L domain is found in the $p6^{Gag}$ protein of $Pr55^{Gag}$, centered around a PTAP sequence. In most cases, these domains can be interchanged among retroviruses and in some cases other unrelated viruses.

The NC protein acts in many diverse steps of retroviral assembly and infection (reviewed in references 6, 60, and 68). The most prominent feature of this positively charged protein is the presence of one or two Zn^{2+}-coordinating fingers that have an uncommon C-X_2-C-X_4-H-X_4-C motif (5). HIV-1 $Pr55^{Gag}$ contains two Zn^{2+} fingers. In the Gag precursor, the NC Zn^{2+} fingers are critical for specific genomic RNA packaging as well as efficient reverse transcription and integration (10, 17, 30, 31, 64, 75). Despite this, the Zn^{2+} fingers do not

* Corresponding author. Mailing address: AIDS Vaccine Program, SAIC-Frederick Inc., National Cancer Institute at Frederick, Frederick, MD 21702-1201. Phone: (301) 846-5723. Fax: (301) 846-5588. E-mail: ott@ncifcrf.gov.

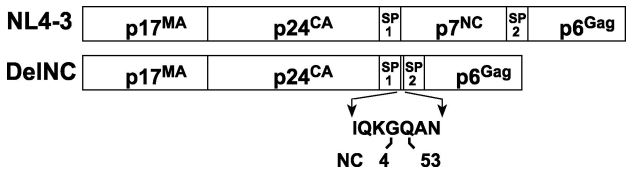

FIG. 1. DelNC mutant Gag. Diagrams of the Gag regions of the wild-type and DelNC constructs are presented. The NC sequences remaining after deletion of the majority of NC are displayed in single-amino-acid code just below the diagram, with the amino acid positions relative to NC at the fusion point indicated.

appear to be of primary importance for particle formation; rather, it is the basic residues that appear to be required for RNA binding in general and for proper Gag-Gag interaction (9, 19, 20, 57). Many observations link NC-RNA interactions with virus assembly. In vitro, RNA can be required for the formation of particles from Gag or portions of Gag (12–15, 37). Also, NC is required for Gag-Gag interactions in yeast two-hybrid experiments (23, 76) and the production of virus-like particles from Gag expression systems (16, 27, 42, 61). Based on these observations, it has been proposed that Gag assembles on an RNA scaffold when NC within Gag interacts with RNA, either small RNAs or genomic RNA, to promote the Gag-to-Gag interactions necessary for particle formation (12, 13, 15, 19, 20, 42, 53, 61). However, the assembly function of NC can be replaced by protein domains that dimerize (1, 41, 50, 78). This has generated an alternate proposal that NC-NC protein interactions are themselves the initial driving force of assembly (41, 50, 78). This idea is supported by chemical cross-linking studies of NC in virions (51). Also, RNA packaging has been linked to virion stability rather than assembly or release (71).

In this study we have investigated the requirement for NC in virus assembly by removing all but seven amino acids of NC from Gag in an otherwise intact HIV-1 proviral clone. The results showed that this mutant construct produced noninfectious particles, although at a lower efficiency than the wild type did, and packaged negligible amounts of genomic RNA. The efficiency of particle production could be rescued by a mutation that inactivates protease. These results show that NC is not absolutely required for particle production.

MATERIALS AND METHODS

DNA mutagenesis. The pNL4-3 infectious molecular clone of HIV-1 (2) (GenBank accession no. AF324493) was altered by site-directed mutagenesis using the PCR-based overlap extension procedure (39). Briefly, *Spe*I-*Bcl*I or *Apa*I-*Bcl*I fragments containing the desired mutations were generated by the PCR procedure and cloned into pNL4-3 as previously described (55). The deletion of NC by this method fused nucleotide (nt) 1932 (the third position C of the glycine 4 codon in NC) to nt 2077 (the first position C of the glutamine 53 codon in NC). The protease-deficient mutant, PR_{R57G}, which contained an arginine-to-glycine change at protease residue 57, was produced in pNL4-3 by an A-to-G change at nt 2421. The Pol-deficient mutant, PR_{R4X}, was produced by inserting a stop codon in the *pol* frame at arginine 4 of protease by introducing a C→T mutation at nt 2274. After construction, the PCR-amplified regions of the various mutants were DNA sequenced to confirm the mutation and the integrity of the sequences exposed to the mutagenesis process.

Cell culture methods. The 293T transformed human kidney and HeLa-CD4-LTR-*lacZ* (HCLZ) (a gift of David Waters, AIDS Vaccine Program) cell lines were cultured in Dulbecco's modified Eagle's medium; the H9 T-cell leukemia line was cultured in RPMI 1640 medium. All media were supplemented with 10% (vol/vol) fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. All cell culture products were obtained from Invitrogen (Carlsbad, Calif.). Transient transfections of 293T cells were carried out using the calcium phosphate method (35) or with 293T TransIT reagent (Mirus Corp., Madison, Wis.) as recommended by the manufacturer. Virion production was measured by the reverse transcriptase assay ([^{3}H]TMP incorporation using an exogenous template) on cell culture supernatants as previously described (33), and CA levels were measured by an HIV-1 p24 enzyme-linked immunosorben assay (Perkin-Elmer Life Sciences, Boston, Mass.) The HIV-1 infection assays using HCLZ cells as a *lacZ* transcomplementation reporter assay for HIV-1 infection were carried out as previously described (33). Virus replication assays were carried out as follows. Various dilutions of virus from a transfection (approximately 6,000 cpm of reverse transcriptase [RT] activity) were used to infect 10^6 H9 cells in a 24-well plate (Costar Corp., Cambridge, Mass.), and clarified supernatant samples were taken periodically and monitored for RT activity. All HIV-1 infections were carried out in the presence of 2 μg of hexadimethiene bromide per ml (Polybrene; Sigma, St. Louis, Mo.).

RNA analysis. Northern blot analysis was performed as described previously (29), except that an 8.1-kbp *Ava*I fragment from pNL4-3 was used for preparing the random-primed ^{32}P-labeled probe. The blot was washed in 0.3 M NaCl–30 mM sodium citrate–0.5% (wt/vol) sodium dodecyl sulfate for 30 min at 65°C. Analysis of viral RNA by metabolic labeling was carried out by transfecting 293T cells at 30% confluency in 150-cm^2 flasks with TransIt reagent as above. Prior to addition of the DNA complexes, the medium in the culture flasks was replaced with phosphate-free Dulbecco's modified Eagle's medium (Specialty Media, Phillipsburg, N.J.) containing dialyzed fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. One millicurie of [^{32}P]orthophosphoric acid (300 Ci/mg; Perkin-Elmer Life Sciences) was then added to each flask. Viruses were collected at 72 h posttransfection by centrifugation for 1 h at 120,000 × *g* in an SW28.1 Ti rotor (Beckman-Coulter, Fullerton, Calif.) at 4°C. The pellets were resuspended in lysing buffer (29), and RNA was isolated as described previously (29). RNA samples, equal to 18% of the transfection supernatant, were fractionated by formaldehyde-denaturing agarose gel electrophoresis as described previously (29). The gel was dried via a gel drier onto a 3MM filter (Whatman, Clifton, N.J.) and then exposed to a phosphorimager screen (Kodak Life Sciences, Rochester, N.Y.). Images were examined using a Molecular Imager FX fluorescent imager (Bio-Rad, Hercules, Calif.) with Quantity One software for the Apple Macintosh. Real-time reverse RT-PCR was carried out essentially as previously described (67) using HIV-1-specific primers and probe combinations (31) that span pNL4-3 nt 1367 to 1535.

Virion density analysis. Sucrose density gradient centrifugation was performed essentially as described previously (32), except that pelleted virus from four 150-cm^2 flasks, produced by the calcium phosphate transfection method, was applied to the gradient and 0.25-ml fractions were collected. The amounts of NL4-3 and DelNC virions in the gradient fractions were monitored by the RT assay. For gradients analyzing DelNC/PR_{R57G}, virions in fractions were detected by $p6^{Gag}$ immunoblot analysis and relative levels of virions were quantitated by scanning densitometry of the resulting bands with Scion Image for Windows version 4.0.2 (Scion Corp., Frederick, Md.). Densities of the fractions were determined by measuring their refractive indices.

Transmission electron microscopy. Micrographs of positively stained virions were obtained as previously described (28).

Protein analysis. Virions were isolated by centrifugation through a 20% sucrose pad in an SW28.1 Ti rotor at 120,000 × *g* at 4°C for 1 h. Immunoblot analyses of virion preparations (the equivalent of 10% of the transfection supernatant) were performed as described above, using peroxidase-conjugated secondary antibodies, and the blots were developed with enhanced chemiluminescence reagent (Amersham Life Science, Arlington Heights, Ill.). Rabbit antiserum against $p6^{Gag}$ (DJ-30552) and goat antiserum against $p7^{NC}$ (serum 77), $p17^{MA}$ (serum 83), or $p24^{CA}$ (serum 81) were obtained from the AIDS Vaccine Program. Monoclonal antibody against reverse transcriptase was obtained from Perkin-Elmer/NEN Life Science. N-terminal protein sequence analysis was carried out on a Procise model 494 microsequencer (Applied Biosystems, Foster City, Calif.) as previously described (18). Microscale high-pressure liquid chromatography (HPLC) was performed as previously described (18). Matrix-assisted laser desorption/ionization-time-of-flight (MALDI) mass spectrometry was carried out as previously described (55), using a Kratos Kompact Probe (Kratos Analytical Inc., Chestnut Ridge, N.Y.).

RESULTS

The importance of NC in HIV-1 $Pr55^{Gag}$ was tested by removing all of the NC residues in Gag except for the first four

amino acids and last three amino acids from the pNL4-3 full-length molecular clone to produce the DelNC construct (Fig. 1). These seven NC residues were included so that the SP1-NC and NC-SP2 protease cleavage sites could potentially be cleaved by HIV-1 protease. The *pol* frameshift site and *pol* coding sequences were also maintained. The DelNC construct reproducibly released sixfold less RT activity and fourfold less CA into the medium on transfection of 293T cells (data not shown). The infectivity of the DelNC virions produced from 293T transfections was determined by a single round assay using a Tat-transactivated *lacZ* reporter cell line. While transfection of the pNL4-3 construct produced a titer of 10^6 blue-cell-forming units (BCFU), the DelNC construct produced a essentially no titer (4 BCFU, similar to the 3 BCFU titer from the mock transfection). Similarly, the DelNC virus did not replicate on H9 cells while wild-type virus was detected at the 10^7 dilution. Together, these data show that this mutant was profoundly defective, being 10^{-6} to 10^{-7} as infectious as the wild type.

Analysis of DelNC virions. To examine the composition of the virions, equal percentages of the virus preparations produced by transfection were analyzed by immunoblotting (Fig. 2). The results with $p24^{CA}$ antiserum showed that the DelNC construct produced a band at 25 kDa, CA, and a less intense band at 48 kDa, Gag minus NC. Compared to wild-type virions, there was somewhat more unprocessed Gag polyprotein in the DelNC particles. However, there was a considerable amount of CA present in the mutant virions relative to Gag, revealing only a subtle decrease in processing. Unlike most processing defects, there was no detectable increase in Gag cleavage intermediates (Fig. 2), suggesting that DelNC Gag was either mostly processed or not cleaved at all. Immunoblotting with $p6^{Gag}$ or $p17^{MA}$ antiserum produced a similar result to that found for the wild type: the DelNC sample contained somewhat less protein than did the wild type and an increased proportion of unprocessed precursor. Stripping and reacting this blot with $p7^{NC}$ antiserum led to the detection of NC in only the wild-type lane; no signal was detected in the DelNC sample, as expected. Blotting also showed that RT was present in the virion samples (Fig. 2), demonstrating that Pol proteins were produced and packaged into DelNC. The presence of Env in DelNC was demonstrated by immunoblot analysis with $gp41^{TM}$ antibody (Fig. 2).

To examine the DelNC virions further, we added them to a microscale reversed-phase HPLC column to separate and isolate the different proteins in the virions (Fig. 3A). The analysis detected all of the major mature Gag products except NC; however, protein sequence analysis of fraction 15 yielded an N-terminal sequence of a protein that matched the seven remaining residues of NC and the first three of SP2 (Fig. 3B). Mass spectroscopy showed that this protein had a molecular mass of 2,581.1 kDa, consistent with the 2,580.98-kDa theoretical mass for a deleted NC-SP2 protein (differing by 0.18%). Thus, DelNC virions contained this partial cleavage product. Additionally, protein sequencing of DelNC fraction 14 (the fraction containing SP2 in the NL4-3 chromatogram) produced the expected SP2 N-terminal sequence: FLGXI (single amino acid code). Thus, while processing of the deleted NC-SP2 partial Gag cleavage product was not complete, it did occur. This result, along with the finding that the majority of the mutant Gag protein is cleaved (Fig. 2 and 3), shows that Gag processing is only slightly decreased.

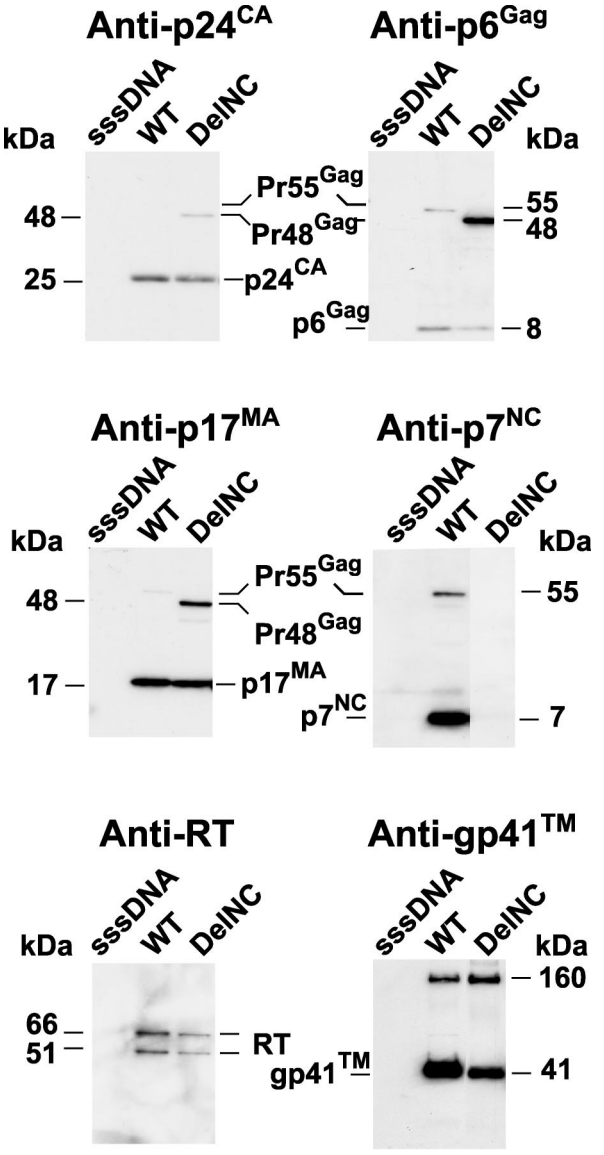

FIG. 2. Immunoblots of DelNC virions. Immunoblots of virion preparations produced by transfection are presented. The antiserum or antibody used is indicated above the respective blots, and samples are identified above the respective lanes. Molecular masses, as calculated by relative mobility, and identities of bands are indicated at the margins of the blots. WT, wild type.

DelNC virions lack genomic RNA. The amount of intact genomic RNA in DelNC particles was examined by denaturing Northern blot analysis. RNA was isolated from equal amounts of virions as measured by the RT activity. Samples from wild-type virions produced a band at 9.3 kb when probed with an 8.1-kb *Ava*I HIV-1 probe, consistent with the expected size of full-length genomic RNA (Fig. 4A). However, the DelNC RNA sample did not contain any detectable genomic RNA signal. Lanes containing dilutions of the NL4-3 RNA preparation showed that a signal from a 1/125 dilution could still be detected in this blot (Fig. 4A). Therefore, the packaging of

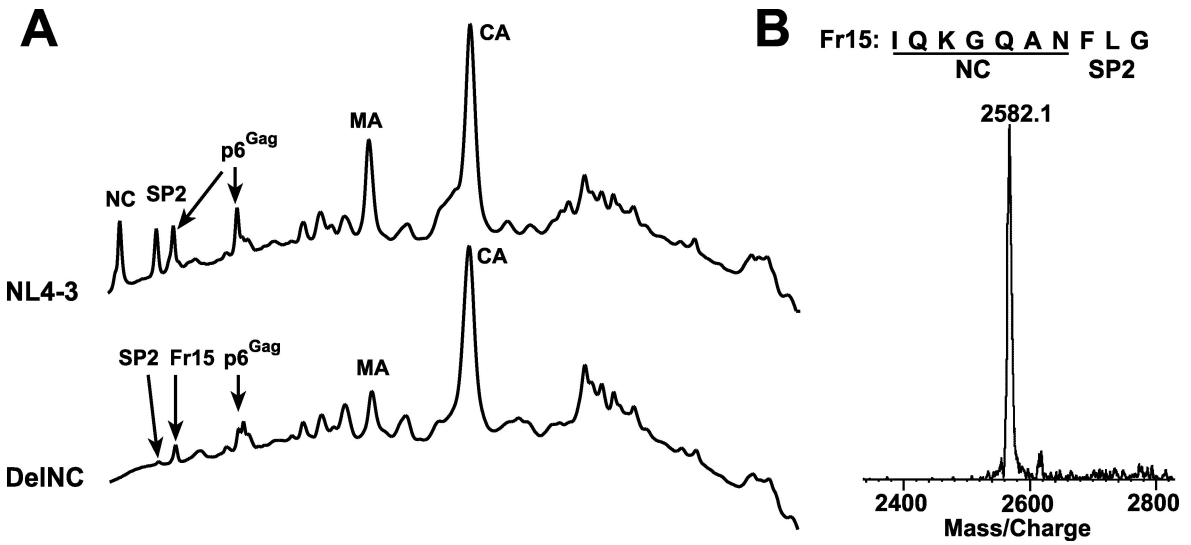

FIG. 3. HPLC analysis of DelNC. (A) Chromatograms of equal percentages of NL4-3 and DelNC virus preparations are presented. Peaks containing Gag proteins are identified above the respective peaks. (B) Protein sequence analysis of fraction 15 is displayed at the top, with the DelNC amino acids underlined. MALDI mass spectrometry results for fraction 15 are presented at the bottom.

DelNC appears to be less than 0.8% of that the wild type. For comparison, we also analyzed samples from three well-characterized Zn^{2+} finger mutants on this same blot. The results agreed with those we have previously published: NC_{H23C}, a change in the first Zn^{2+} finger histidine to a cysteine (residue 23), packaged near wild type (31); NC_{C36S}, a change in a second Zn^{2+} finger cysteine to a serine (residue 36), packaged between 1/5 and 1/25 of the wild type (33); while $NC_{SSHS/SSHS}$, which has the complete replacement of cysteines for serines in both Zn^{2+} fingers, failed to package detectable amounts of viral RNA (38). To examine viral RNA content another way, we metabolically ^{32}P-labeled RNA in virus-producing cells, isolated RNA from virion preparations, and examined the samples by denaturing gel electrophoresis (Fig. 4B). Phosphorimaging analysis of the dried gel showed that genomic RNA was present in the wild-type NL4-3 preparation and absent in the DelNC samples, consistent with the Northern blot data. Thus, DelNC does not contain detectable amounts of intact genomic or spliced viral RNA. The presence of both 28S and 18S rRNA is most probably due to the presence of microvesicles, membrane vesicles which can contain rRNA and contaminate virion preparations (7). However, we cannot rule out rRNA being packaged into virions: ribosomes are packaged into some murine leukemia virus virions carrying certain NC mutations (54).

The amount of HIV-1 RNA containing *gag* in virus preparations was quantitated by real-time RT-PCR. The results showed that the levels of *gag* RNA in the DelNC sample were drastically reduced from the NL4-3 sample, being approximately 10,000-fold lower than in the wild-type sample (Table 1). To date, this is the most severe genomic RNA packaging defect for an NC mutant examined by this procedure (29, 31). The amount of *gag* detected in the DelNC sample was 1,000-fold larger than in a negative control (Table 1), a preparation isolated from a sheared salmon sperm DNA (sssDNA) transfection supernatant. This residual presence of *gag* RNA may be due to RNA present in the virion preparations within microvesicles or simply adhered to particles. It is important to note that the *gag* mRNA does not need to be intact for this assay. The number of particles in the DelNC sample examined was somewhat smaller than in the wild type (typically four- to sixfold lower as estimated by immunoblotting) due to the assembly defect. Nonetheless, these results and those above show that DelNC has a profound defect in viral RNA packaging.

DelNC virions exhibit an I-domain defect. Other NC deletion mutants have a mutant I-domain phenotype: their virions are less dense than wild-type virions, apparently due to a defect in Gag-Gag packing (19, 21, 61, 77). To check for an I-domain defect, DelNC virions were examined by sucrose density gradient centrifugation. Fractions were collected and then assayed for the presence of virions by the RT activity. The results revealed that DelNC virions were lighter than those from the wild type: the mutant virions banded around an average of 1.127 g per ml, while the wild-type virions were distributed around 1.141 g/ml (Fig. 5). The density distribution of the mutant virions was wider than that of the wild-type virions, ranging from 1.11 to 1.16 and 1.13 to 1.15 g/ml, respectively. The observed density shift of 0.013 g/ml from the mutant to the wild type was somewhat smaller than the 0.02-g/ml differential measured for other I-domain mutations (4, 19, 21, 61, 77). However, this result strongly suggests a defect in the I domain of this mutant. Immunoblotting of the fractions containing peak activity confirmed the relative presence of virus in the peaks (data not shown). Thus, DelNC particles are relatively lighter and more heterogeneous than those of NL4-3, characteristic of an I-domain defect.

Heterogeneous morphology of DelNC virions. Preparations of NL4-3 and DelNC virions were examined by transmission electron microscopy (Fig. 6). Unlike wild-type samples, the electron-dense staining features found in the DelNC particles were amorphous, cylindrical, or similar to the classic immature retroviral forms. In some cases, a small core-like object in an

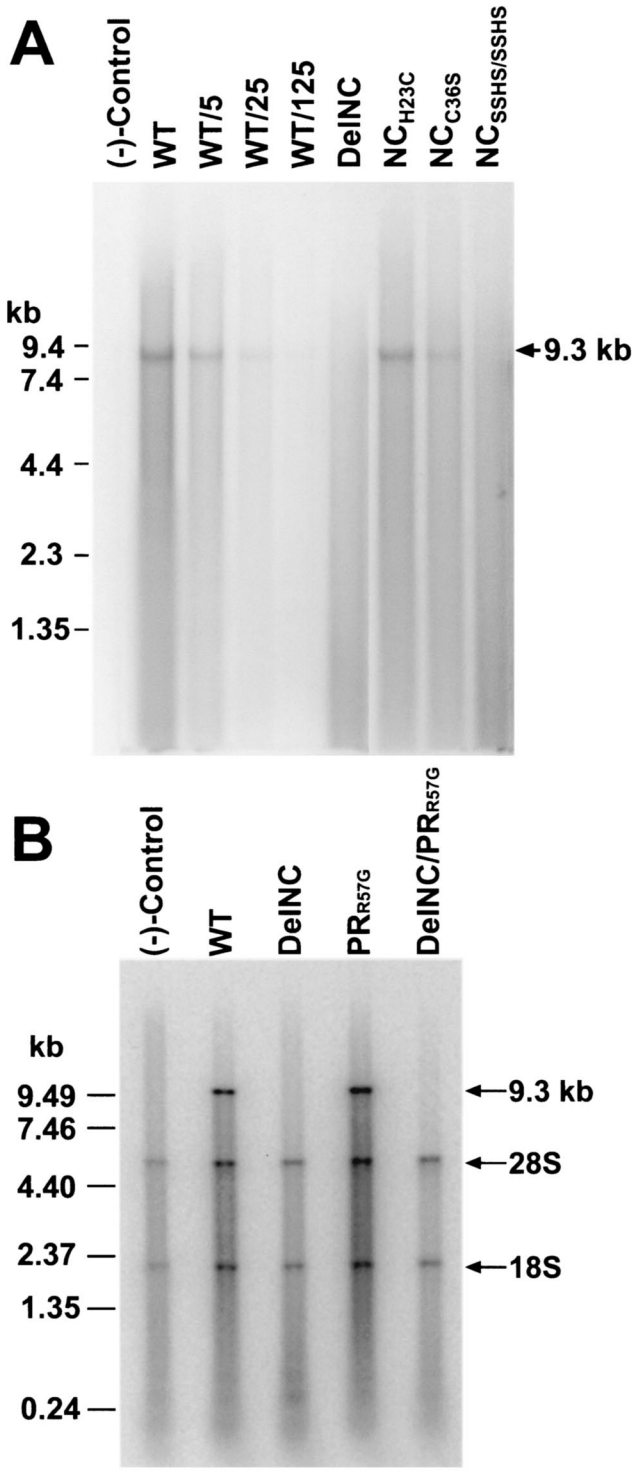

FIG. 4. Analysis of particles for intact genomic RNA. (A) Northern blot analysis of virion RNA preparations. Samples contained 5.2×10^6 cpm of RT activity from each mutant and wild-type (WT) virus preparation. The blot was probed with a ^{32}P-labeled 8.1-kbp *Ava*I fragment from pNL4-3. In addition to the wild-type NL4-3 sample, 1/5, 1/25, and 1/125 dilutions of this sample were tested. Samples are identified above their respective lanes, with RNA markers indicated on the left and the size of the full-length genome indicated on the right. (B) Autoradiogram of metabolically ^{32}P-labeled virions analyzed by RNA denaturing agarose gel electrophoresis. Virion preparations isolated from equal amounts of transfection culture supernatants were examined. Samples are indicated at the top of the gel, RNA marker sizes are indicated on the left, and the positions of the 9.3-kb viral RNA and 28S and 18S ribosomal bands are indicated on the right. For both panels, "(−)-Control" denotes a virus preparation produced from transfected sssDNA.

TABLE 1. Real-time RT-PCR analysis of virion samples

Virus	No. of HIV-1 RNA/ml[a]	No. of copies relative to NL4-3
sssDNA	$(3.49 \pm 4.93) \times 10^2$	2.0×10^{-7}
NL4-3	$(1.41 \pm 0.13) \times 10^9$	1
DelNC	$(1.98 \pm 0.29) \times 10^5$	1.4×10^{-4}
PR$_{R57G}$	$(6.84 \pm 0.20) \times 10^8$	0.49
DelNC/PR$_{R57G}$	$(2.48 \pm 0.05) \times 10^5$	1.7×10^{-4}
PR$_{R4X}$	$(2.52 \pm 0.00) \times 10^8$	0.18
DelNC/PR$_{R4X}$	$(5.55 \pm 1.57) \times 10^5$	3.9×10^{-4}

[a] The highest level of contaminating DNA in the virion samples was 4×10^{-8} that of the number of RNA copies (data not shown).

otherwise immature virion morphology was seen (Fig. 6). These results show that the DelNC virions were quite different from wild-type particles, possessing defects in their Gag organization. This is consistent with the density measurements presented above and confirms a defect in assembly.

DelNC can be rescued in *cis* by protease inactivation. DelNC buds inefficiently. Interestingly, the budding defect of an L-domain mutant appears to be able to be complemented by a second mutation that inactivates protease (40). To test if this holds true for our mutant, the DelNC mutation was combined with a protease-inactivating mutation, PR$_{R57G}$ (an arginine-to-glycine mutation at protease residue 57), to produce the DelNC/PR$_{R57G}$ construct. Virions expressed by transfection of this construct into 293T cells were examined by p24^{CA} immunoblot analysis. The results revealed that, unlike the reduced amount of CA in the DelNC versus wild-type samples, the DelNC/PR$_{R57G}$ construct produced particles at a level similar to that of the protease mutant with wild-type NC, PR$_{R57G}$ (Fig. 7A). Stripping and reacting the blot with protease antiserum readily detected a difference in the amount of protease in the wild-type and DelNC lanes that was similar to the CA blot result. The lane containing DelNC/PR$_{R57G}$ produced a faint yet detectable Gag-Pol, while the lane containing PR$_{R57G}$ did not (Fig. 7A). The ability to detect protease in the DelNC/PR$_{R57G}$ sample and not in that of the wild-type protease mutant demonstrates that this double mutant produces particles at least as well as the NC-containing single mutant does.

To examine protease activity and mutant budding in another way, 293T cells were transfected with NL4-3 or DelNC and then treated with the protease inhibitor saquinavir (10 nM) for 24 h or left untreated. The results showed that the protease inhibitor essentially eliminated DelNC Gag processing while causing only a slight reduction in the cleavage of NL4-3 Gag at this concentration (Fig. 7B). Furthermore, the DelNC virion sample produced from the protease inhibitor-treated cells contained more Gag than did the corresponding material from the untreated cells. Therefore, inhibition of Gag processing increased the production of DelNC particles. In contrast, the amount of NL4-3 virions found in the medium was not largely

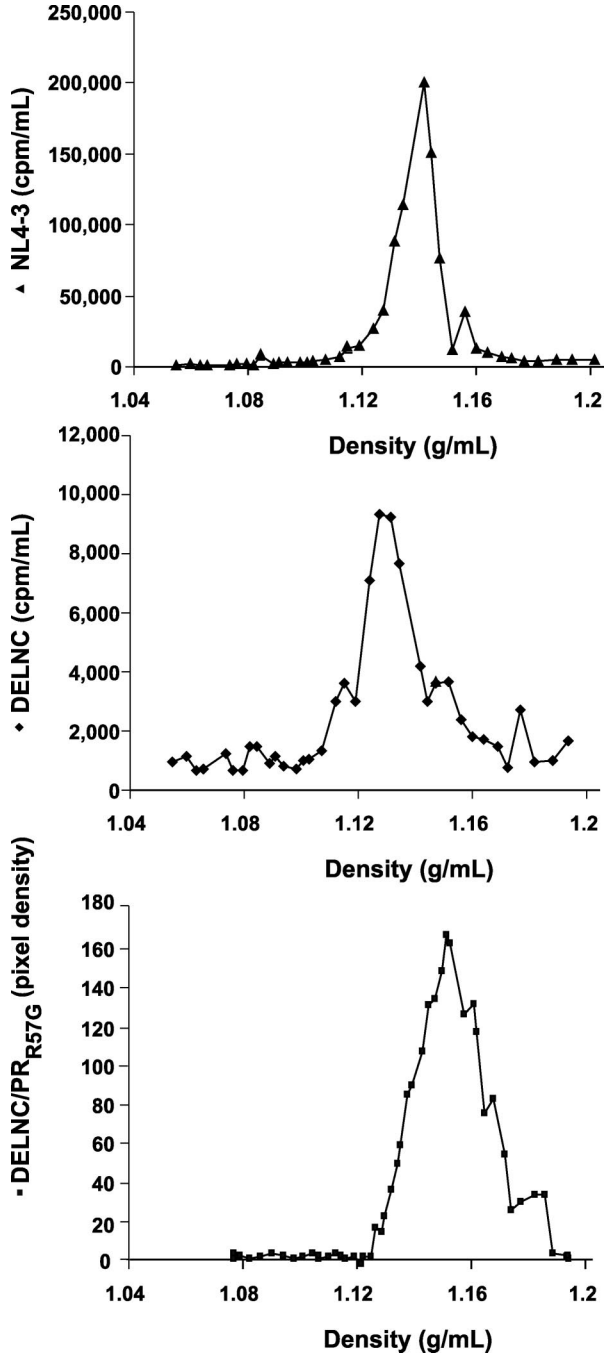

FIG. 5. Equilibrium density gradient centrifugation of viruses. Profiles of wild-type NL4-3, DelNC, and DelNC/PR_{R57G} viruses subjected to centrifugation through 10 to 50% (wt/vol) sucrose gradients are presented. Amounts of virions detected as measured by RT activity ([^{3}H]TMP incorporation) or by scanning densitometry (pixel gray-scale density) from a $p6^{Gag}$ immunoblot of selected fractions are reported on the *y* axis versus density of sucrose on the *x* axis. Virions analyzed are identified to the left of the respective graphs.

increased by the protease inhibitor (Fig. 7B), even at concentrations that blocked processing (data not shown). Thus, the elimination of protease activity, either by mutation or by protease inhibitor, allows the DelNC mutant to produce particles efficiently. To examine any potential role of the Pol precursor itself in this result, we used a mutant with a stop codon at position 4 of NL4-3 protease, PR_{R4X} (arginine to nonsense at position 4) that eliminates nearly all of Pol (data not shown). A DelNC/PR_{R4X} double mutant produced similar levels of particles to the protease-deficient NC mutant as demonstrated by immunoblotting (Fig. 7A). Therefore, the absence of protease activity is sufficient to rescue the DelNC assembly defect as opposed to simply the presence of uncleaved Pol.

Rescue does not restore genomic RNA packaging. Examination of the RNA levels in DelNC/PR_{R57G} by metabolic ^{32}P labeling of RNA showed that, similar to our results with DelNC, DelNC/PR_{R57G} did not contain detectable levels of RNA (Fig. 4). Analysis of both DelNC/PR_{R57G} and DelNC/PR_{R4X} by real-time RT-PCR found that both of these double mutants had severe defects in viral RNA packaging (Table 1), approximately 10,000-fold and 1,000-fold reduced from their NC-containing counterparts (i.e., PR_{R57G} and PR_{R4X}, respectively). These differences are comparable to the difference between DelNC and the wild type. Unlike the DelNC preparations, virus preparations from the protease and protease-DelNC mutants used in this analysis contained nearly equal numbers of virions (as determined by immunoblotting and HPLC [data not shown]). Thus, the rescue of assembly did not involve the restoration of genomic RNA packaging.

Morphology of DelNC/PR_{R57G} particles. The particles produced by DelNC/PR_{R57G} and PR_{R57G} were examined by electron microscopy. The particles produced by PR_{R57G} showed the typical distended circle or teardrop shape for protease-deficient virions (Fig. 6): the virions had no central core but contained a ring of electron-dense material that covered ~270° of the virion with a notable thinning to absence of electron-dense material through the remaining portion of the virion. The inner border of the ring also contained a thin black band. These teardrop shapes are seen with HIV-1 Gag- and Gag-Pol-containing virions and not with particles formed by Gag alone (34, 42, 43, 62), which display the doughnut-like concentric ring morphology displayed by some other immature orthoretroviruses (47). The DelNC/PR_{R57G} particles were essentially uniform in radial density even though they contained Pol proteins. Thus, they were closer in morphology to virus-like particles that are produced by Gag expression (42). The electron-dense region just under the viral membrane in the DelNC/PR_{R57G} particles was somewhat thinner than the similar region in the PR_{R57G} particles (Fig. 6) or those produced from Gag alone (data not shown). Additionally, the inner black band was absent in these virions. Despite these differences, the protease mutation in DelNC/PR_{R57G} reversed the morphologically observed assembly defect of the DelNC mutation, allowing for the production of uniform particles.

DelNC/PR_{R57G} forms dense virions. The density of DelNC/PR_{R57G} was measured by sucrose gradient centrifugation, and the fractions were examined for the presence of $Pr48^{Gag}$ by immunoblotting. The relative amounts of Gag signal on the blot were measured by pixel gray-scale density and used to quantitate the relative amount of DelNC/PR_{R57G} in the gradient fractions (Fig. 5). The results showed that the peak of virus recovery was at approximately 1.151 g/ml, considerably denser than the DelNC particles and slightly heavier than wild-type ones. Therefore, the absence of protease activity

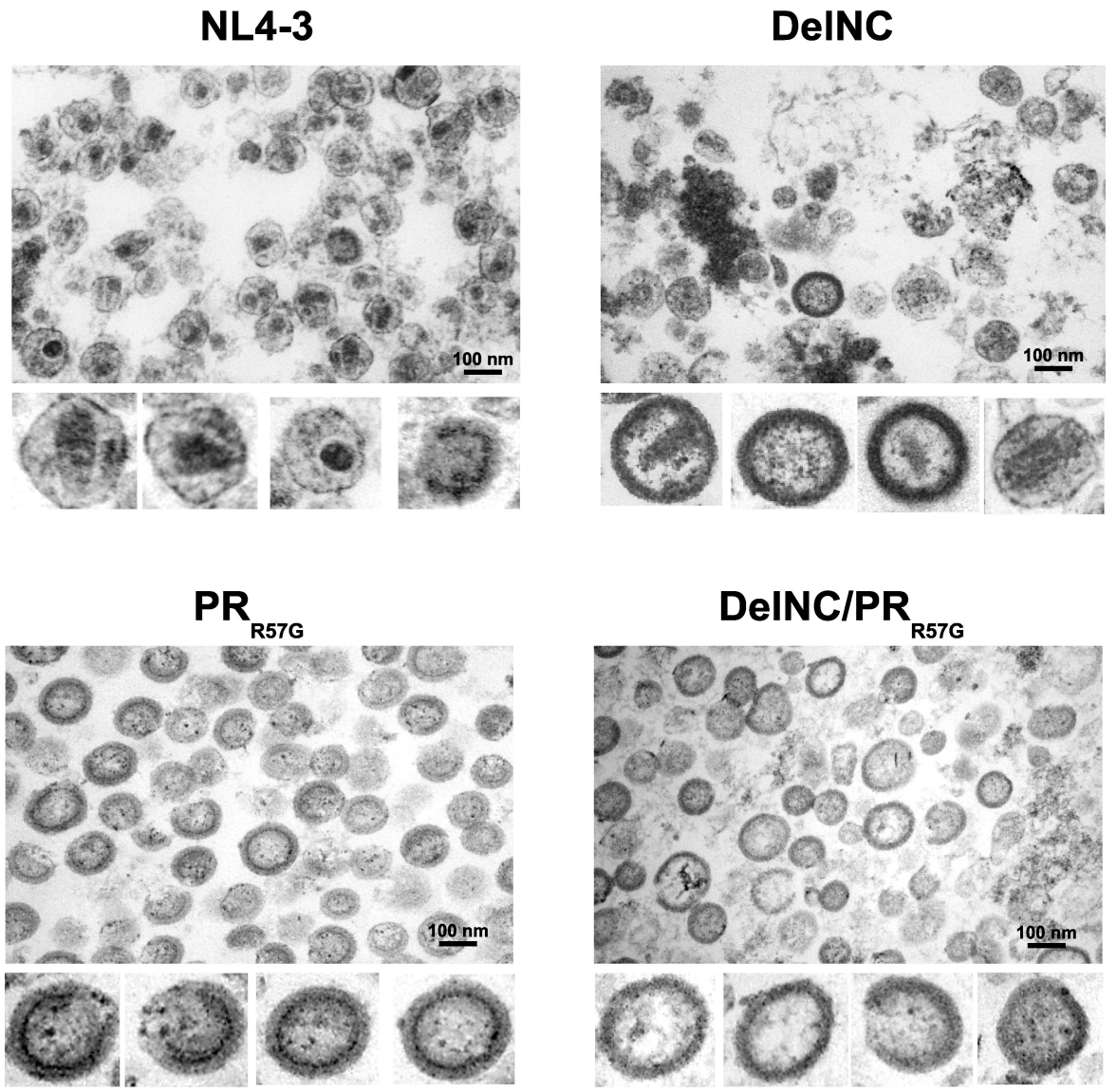

FIG. 6. Electron micrographs of virions. Transmission electron micrographs of positively stained wild-type, DelNC, PR_{R57G}, and DelNC/PR_{R57G} virion preparations are presented at ×40,000 magnification. Enlarged images of representative virions present in this field and others are displayed underneath the field micrographs.

appears to have restored the I-domain function to the DelNC/PR_{R57G} mutant.

DISCUSSION

Our results demonstrate that the NC region of $Pr55^{Gag}$ is not absolutely required for particle production. Other groups have produced NC deletion mutants and analyzed them to different extents. In general, their data and ours agree: virus production is decreased and the particles formed are lighter, consistent with a defect in the I domain (20, 21, 77). However, we also found that this particle production defect could be suppressed by inactivating protease by protease deletion, mutation, or protease inhibitor treatment. This demonstrates that NC is not absolutely required for particle production.

One potential explanation for our result is that the DelNC mutation might slow the assembly and budding process. Since HIV-1 protease is activated during budding (44, 45), this putative decrease might cause the assembling and budding Gag to be prematurely cleaved, resulting in poor or aberrant budding. This potential mechanism has been previously proposed for other assembly defects (4, 8, 11). In support of this, the decreased production of bovine leukemia virus-like particles by an L-domain mutant is exacerbated by the presence of active protease (70). Furthermore, certain Rous sarcoma virus assembly and budding mutants with changes in p2b (the L-domain-containing region of its Gag) also can be complemented by inactivation of protease, especially those with decreased but not blocked assembly phenotypes (8, 73). Also, deletions in integrase can cause a decrease in HIV-1 budding that can be rescued by protease inactivation (11). Others have found that overexpression of protease, either by itself or within Gag-Pol, can drastically decrease retroviral particle formation and bud-

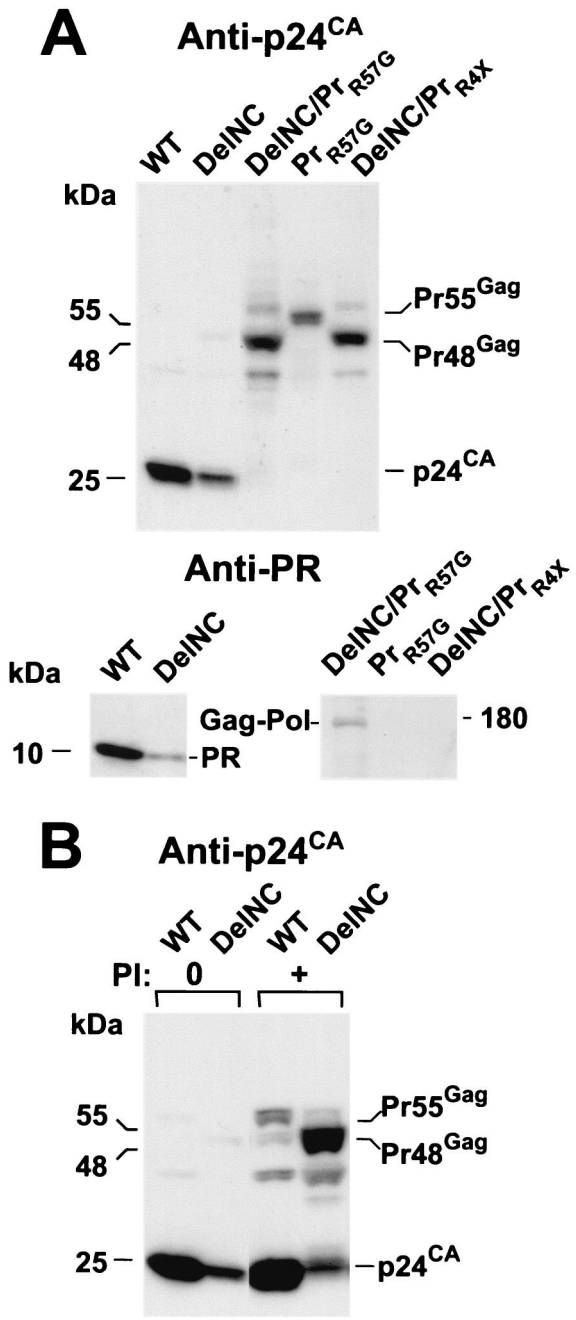

FIG. 7. Immunoblots of protease-deficient virions. Immunoblots of virion preparations produced from transfection of various constructs (A) and those of virion preparations produced by transfection in the presence or absence of 10 nM saquinavir (B) are presented. The antiserum or antibody used is indicated above the respective blot, and the samples analyzed are identified above the respective lanes. Molecular masses, as calculated from relative mobility, and identities of bands are indicated at the margins of the blots. The addition of saquinavir in the production of virus samples is indicated as PI + above the appropriate lanes. WT, wild type.

ding, suggesting an important regulation of protease activation during assembly (3, 4, 46, 48, 49, 52, 56). While we did observe a subtle processing defect, for the most part, processing of Gag did occur. Perhaps the presence of unprocessed Gag in both wild-type and mutant virion preparations is due to some virions budding without incorporating Gag-Pol or protease. The absence of partial cleavage products of Gag that are normally observed when processing is inhibited supports this explanation. If this is true, the increased amount of uncleaved Gag in DelNC preparations could be due to their ability to bud more efficiently in the absence of protease, similar to DelNC/PR_{R57G} particles. Given our data, the inefficiency of DelNC particle production could be due to a bottleneck at the assembly and budding stages that allows protease to prematurely process Gag rather than a simple failure to assemble.

An additional possibility is that NC itself plays a role in L-domain function. A minimal Gag construct required a portion of NC-SP2 in addition to the PTAP core L-domain sequence for efficient budding (66). Interestingly, this NC-SP2 region was not required when a PPPY sequence provided L-domain function for the minimal construct. Therefore, it is possible that sequences within NC might be part of the HIV-1 PTAP-based L domain.

While the budding of DelNC virions was reduced four- to sixfold, other groups have found that their NC deletion mutants make particles in approximately 10-fold-smaller amounts than the wild-type does (20, 21). Most experiments with virus-like-particle-expressing systems, which express Gag only, have found that Gag with NC deletions do not efficiently form particles (16, 27, 42, 61). The difference between our results and those with the virus-like particle systems cannot be explained by the presence of sequences within protease, RT, or IN, since the DelNC/PR_{R4X} mutation still produced particles in the absence of Pol. Also, many of the other viral studies on HIV-1 were based on HXB-2, which does not express full-length Vpr, Vpu, or Nef (59). Similarly, most of the virus-like particle studies do not express the full complement of viral proteins, while our NL4-3-based construct expresses the full repertoire of HIV-1 structural and accessory proteins (2). Perhaps the presence of Vpu, which assists viral budding (68), might explain this difference. Also, differences in the way NC was deleted from Gag could be another explanation.

Our results also show that NC and its corresponding sequences in the mRNA are not required for Gag-Pol expression, incorporation, or protease activation. This finding suggests that Gag-Pol can be brought into the assembling virion and that protease can dimerize by a means other than the NC-mediated assembly function. Experiments have shown that $Pr55^{Gag}$ can rescue the packaging of Gag-Pol myristylation mutants (65), a reflection of the critical nature of Gag and Gag-Pol interactions. Therefore, incorporation and activation seem to be carried out by interactions between regions of Gag and Gag-Pol other than NC.

As expected, our data reiterate the requirement for NC in genomic RNA packaging, since both the Zn^{2+} fingers and all but one of the basic residues are removed in this mutant. NC is the primary RNA binding portion of Gag, although the basic region of MA can also bind RNA in an apparently weaker interaction (12, 58). Since NC mutants did not package significant levels of genomic RNA, this basic region in MA is clearly insufficient for its incorporation. It remains to be determined whether this region or other unknown RNA binding sites might allow other cellular RNAs to be incorporated into DelNC mutants. These studies are complicated by potential

sources of RNA contamination from microvesicles, which also contain RNA (7).

The ability of DelNC/PR_{R57G} to efficiently form virions without NC probably reflects the sufficiency of the MA-CA-SP1 regions to interact in assembly (22, 36). In an absolute sense, this shows that NC-mediated scaffolding or protein-protein interactions are not required for particle production. Given our proposed mechanism for the budding defect, namely, that virions form slowly in the absence of NC, it still seems that NC has a very important function in assembly that can somehow be circumvented by another inefficient assembly pathway in the absence of protease.

NC has been identified as part of the I domain of retroviruses, although it seems that the portions of NC that are involved in I function vary among the viruses (68). This domain is commonly defined as being required for the formation of tightly packed, dense Gag structures within particles (26). Given that the DelNC/PR_{R57G} virions are as dense as their NL4-3 counterparts and efficiently produce particles, NC is not required for dense packaging of Gag in particles. Thus, NC is not necessarily part of the I domain in the context of full-length NL4-3.

Despite containing little genomic RNA and being profoundly replication deficient, DelNC particles contain Gag, Gag-Pol, and Env proteins. Therefore, these particles could be used as a starting point for a safe whole-particle vaccine.

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