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## Assembly, Processing, and Infectivity of Human Immunodeficiency Virus Type 1 Gag Mutants

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**We studied the effects of *gag* mutations on human immunodeficiency virus type 1 (HIV-1) assembly, processing, and infectivity by using a replication-defective HIV expression system. HIV mutants were screened for infectivity by transduction of a selectable marker and were examined for assembly by monitoring particle release from transfected cells. Gag protein processing and reverse transcriptase activities of mutant particles were also assayed. Surprisingly, most Gag protein mutants were assembled and processed. The two exceptions to this rule were a myristylation-minus mutant, and one *gag* matrix domain mutant which expressed proteins that were trapped intracellularly. Interestingly, a mutant with a 56-amino-acid deletion within the HIV *gag* capsid domain still could assemble and process virus particles, exhibited a wild-type retrovirus particle density, and had wild-type reverse transcriptase activity. Indeed, although most HIV-1 *gag* mutants were noninfectious or poorly infectious, they produced apparently normal particles which possessed significant reverse transcriptase activities. These results strongly support the notion that the HIV-1 Gag proteins are functionally involved in postassembly, postprocessing stages of virus infectivity.**

Assembly of human immunodeficiency virus (HIV) particles occurs at the plasma membranes of infected cells (41) and results in the incorporation of several viral components. They are the viral RNA genome, core (Gag) structural proteins, envelope (Env) glycoproteins, and virion-associated enzymes encoded by the viral *pol* gene, including protease, reverse transcriptase (RT), RNase H, and integrase (6, 36). In addition to the characteristic retroviral *gag-pol-env* genome (47), HIV type 1 (HIV-1) encodes several novel proteins. One small accessory protein, Vpu, is involved in the assembly process by assisting virus particle budding, although Vpu itself is not packaged into virions (7, 19, 43, 44). However, in the absence of other viral products, Gag polypeptides still can assemble as a virionlike particle, suggesting that *gag* is the only viral gene required for virus assembly (10, 39). The HIV Gag polypeptide is synthesized as a precursor Pr55, which is modified cotranslationally by methionine cleavage and the attachment of a myristic acid to the N-terminal glycine (45, 48). Myristylation is necessary for membrane association and virion formation (5, 13, 34). During or after budding, Pr55 is cleaved by the viral protease into p17 (matrix; MA), p24 (capsid; CA), p7 (nucleocapsid; NC), and p6 (17, 25, 29, 32). p17 is myristylated and membrane associated; p24 is the major capsid structure; p7, containing two zinc finger motifs, is a nucleic acid-binding protein (1, 2, 11, 40); and the p6 domain, located at the carboxyl end of Pr55, may play a functional role in the process of virus budding (10, 12).

Despite intensive efforts, the mechanism of HIV virus assembly is still unclear. One model, on the basis of previous studies, is that myristylated Gag precursors are targeted to the plasma membrane where they self-assemble into particles. Env proteins might be incorporated by binding to Gag proteins at the plasma membrane; Gag-Pol fusion proteins would be incorporated into virions by virtue of their N-ter-

minal *gag* determinants; and viral RNA might be encapsidated by interaction with Gag proteins at the RNA packaging signal (Psi) located around the initiation sequence of Gag (16, 27). While Gag proteins play a central role in the process of retroviral assembly, they also have been implicated in other functions. Studies have shown that mutations of murine leukemia virus (MLV) *gag* can block early stages of infection (8), and HIV *gag* mutants can interfere with replication of wild-type (wt) virus (46). This evidence suggests that Gag proteins also may affect the process of reverse transcription, nuclear transport, or integration (4, 37, 38).

To investigate the mechanism of HIV assembly and the potential functions of Gag proteins in other phases of the virus replication cycle, we have adapted a genetic approach. HIV *gag* mutations were created by deletion or linker insertion and were subcloned into a replication-defective HIV proviral genome (HIVgpt), which carries the drug-resistant *gpt* gene (30) in the *env* region (33). Cotransfections of wt or mutant HIVgpt constructs with an envelope expression plasmid permitted us to analyze how each *gag* mutation affected virus assembly and infectivity. Twelve HIV mutants were screened for infectivity, assembly, processing, and RT activity. Surprisingly, results showed that most mutants were assembled and released as particles. However, most mutants either were noninfectious or poorly infectious, although they had significant RT activities. Interestingly, a mutant with a 56-amino-acid deletion in the capsid domain was still assembled and processed. Indeed, the only mutants incapable of particle assembly were a myristylation mutant and a linker insertion mutant in the central portion of the matrix domain. Immunofluorescence studies showed that Gag proteins of this insertion mutant localized to the perinuclear area, indicating that the matrix domain may be involved in transport or assembly of Pr55<sup>gag</sup>. Overall, our results strongly support the notion that HIV Gag proteins are functionally involved in postassembly and postprocessing stages of viral infectivity.

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## MATERIALS AND METHODS

**Cell culture, transfections, infections, and infectivity assays.** HeLa and COS7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO). Confluent COS7 cells were split 1:10 onto 10-cm dishes 24 h before transfections. Fifteen micrograms of plasmid DNAs of *gag* mutants or wt HIVgpt was transfected onto COS7 cells by calcium phosphate precipitation (14). At 48 to 72 h after transfection, supernatants of COS7 cells were collected and filtered through a 0.45- $\mu$ m-pore-size filter and frozen at  $-80^{\circ}\text{C}$ . For infections, 15  $\mu$ g of plasmid DNAs of each mutant was cotransfected with 10  $\mu$ g of plasmid DNA of the MLV amphotropic *env* expression plasmid SV-A-MLV-*env* into COS7 cells. Two to three days later, cell supernatants of COS7 cells were used to infect HeLa cells, which had been split and grown to 10% confluence at the time of infection. Adsorption of virus was allowed to proceed at  $37^{\circ}\text{C}$  in the presence of 4  $\mu$ g of Polybrene per ml. Three days after infection, cells were trypsinized and split 1:8 onto 10-cm dishes containing selection medium. The selection medium was made of Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, penicillin plus streptomycin (GIBCO), 50  $\mu$ g of xanthine per ml, 3  $\mu$ g of hypoxanthine per ml, 4  $\mu$ g of thymidine per ml, 10  $\mu$ g of glycine per ml, and 150  $\mu$ g of glutamine plus 25  $\mu$ g of mycophenolic acid (GIBCO) per ml. Cells were refed every 3 to 4 days with selection medium until colonies of drug-resistant cells formed. The number of colonies was converted into titer (infectious units per milliliter). The infectivity of each mutant was determined by the ratio of its titer versus the titer of HIVgpt in parallel experiments.

**Recombinant plasmids.** The parent DNA in this study is pHXB2 (36). Plasmids HIVgpt and SV-A-MLV-*env* were generously provided by D. Littman (33). Construction and sequencing followed the protocols described in Maniatis et al. (28). The methods for engineering linker insertion mutants were described previously (15). Sequences in mutated regions are shown in Fig. 1.

**Protein analysis.** Supernatants of transfected COS7 cells were collected and filtered through a 0.45- $\mu$ m-pore-size filter. The filtered supernatants were centrifuged through 2 ml of 20% sucrose in TSE (10 mM Tris hydrochloride, 100 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) at  $4^{\circ}\text{C}$  for 45 min at  $274,000 \times g$  (SW41 rotor at 40,000 rpm). Pellets were suspended in 100  $\mu$ l of IPB (20 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, 1% Triton X-100, 0.02% sodium azide) plus 0.1 mM phenylmethylsulfonyl fluoride. Cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (PBS) and were collected in 1 ml of PBS for 10-cm plates. Cells then were pelleted and resuspended in 1 ml of IPB plus 0.1 mM phenylmethylsulfonyl fluoride followed by microcentrifugation to remove debris. Lysate was homogenized and saved. Supernatant and cell samples were prepared for loading by adding an equal volume of  $2\times$  sample buffer (12.5 mM Tris hydrochloride [pH 6.8], 2% SDS, 20% glycerol, 0.25% bromophenol blue) and 5%  $\beta$ -mercaptoethanol and boiling for 4 to 5 min. Samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (21) and were electroblotted onto a nitrocellulose filter. Procedures for immunodetection of nitrocellulose-bound proteins were described previously (18). Mouse anti-p24 monoclonal antibody (Epitope Inc., Beaverton, Ore.) was diluted at 1:20,000 as primary anti-

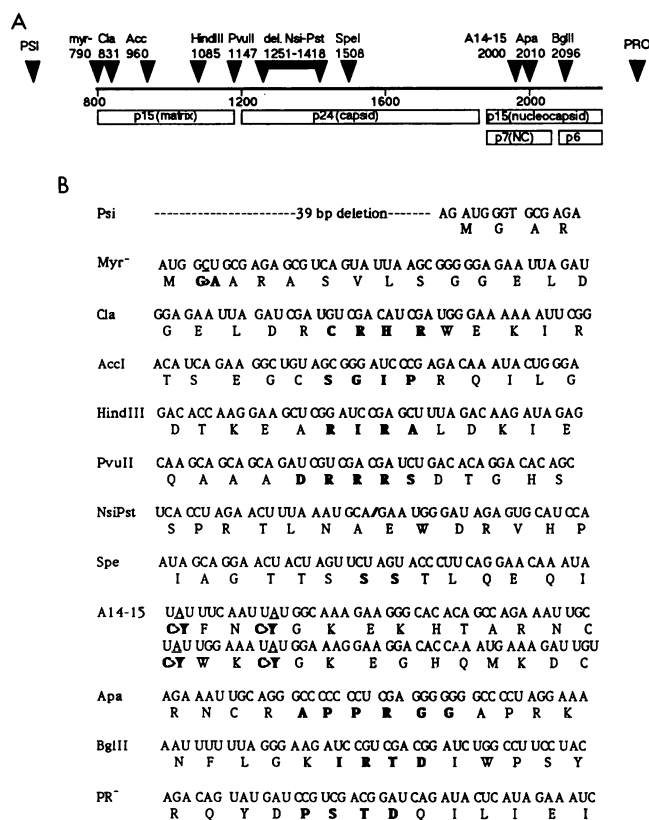


FIG. 1. HIV *gag* mutations. (A) Deletion, site-directed, and linker insertion mutations were generated at the designated nucleotide locations in the HIV-1 *gag* gene and introduced into the HIVgpt construct for analysis. HIVgpt, described by Page et al. (33), contains a simian virus 40 *ori* and the drug resistance *gpt* gene in place of *env* coding sequences. The *tat* and *rev* genes remain intact, and expression of HIVgpt in COS7 cells results in release of noninfectious virus particles. As shown, four mutations were created within the matrix domain by linker insertion at restriction sites of *Cla*I-831, *Acc*I-960, *Hind*III-1085, and *Pvu*II-1147. In addition, a *Myr*<sup>-</sup> version of HIVgpt was generated from a mutant kindly provided by L. Ratner (5). Two capsid mutations were generated, a mutation with a deletion from *Nsi*I-1251 to *Pst*I-1418 (dl.*Nsi*Pst) and a mutant with a linker insertion at *Spe*I-1508. Within the nucleocapsid domain, there were two linker-generated mutations, *Apa*I-2010 and *Bgl*II-2096. A third nucleocapsid mutation, A14-15, was a site-specific mutation at the zinc finger motifs and was a gift from R. Young (1). In addition, there were two mutations outside the *gag* coding region, including a protease mutation with a linker insertion at *Bcl*I-2429 (PRO) and a packaging signal (Psi [PSI]) deletion mutation, also from R. Young (1). (B) Viral RNA sequences and encoded protein sequences of mutated HIV *gag* regions. In the case of the Psi mutant, 39 bp between the first splice donor site and the *gag* initiation codon were deleted, while 56 amino acids were deleted in mutant dl.*Nsi*Pst. For all other mutants, inserted or changed amino acids are designated by boldface type.

body. The secondary antibody was a goat anti-mouse immunoglobulin G-alkaline phosphatase conjugate. HIV Gag proteins immunodetected on filters were quantitated by scanning densitometry with a Bio-Rad model 620 video densitometer on reflectance mode. For immunofluorescence, protocols were as described elsewhere (18), with anti-p24 antibody at a 1:1,000 dilution as primary antibody and rhodamine-conjugated rabbit anti-mouse antibody at a 1:100 dilution as secondary antibody.

**RT assay.** Supernatants of transfected COS7 cells were collected and pelleted as described above. Pellets were suspended in 1× PBS and were incubated for 2 h at 37°C with 2× cocktail mix [100 mM Tris buffer (pH 8.3), 40 mM dithiothreitol, 1.2 mM MnCl<sub>2</sub>, 120 mM NaCl, 0.1% Nonidet P-40, 20 mM dTTP, 10 mg of oligo(dT)<sub>12–18</sub> per ml, 20 mg of poly(A) per ml, and 20 µCi of Amersham [ $\alpha$ -<sup>32</sup>P]dTTP]. Samples were precipitated with 10% trichloroacetic acid after the 2-h incubation. The precipitates then were washed three times with 10% trichloroacetic acid, followed by liquid scintillation counting.

**Sucrose density gradient fractionation.** Linear sucrose gradients (20 to 50%) in TSE were poured into SW50.1 polyallomer tubes and allowed to sit at 4°C for 1 to 2 h. Cell supernatants from transfected COS7 cells were spun through a 20% sucrose cushion at 4°C for 45 min at 274,000 × *g*. Pellets were resuspended in 1× PBS and were carefully layered on top of the gradient. The gradients were centrifuged in an SW50.1 rotor overnight at 50,000 rpm (300,000 × *g*) at 4°C. Fractions of 0.4 ml were collected from top to bottom. Each fraction was measured for density and analyzed for Gag proteins by immunoblot.

## RESULTS

**Infectivity of HIV *gag* mutants.** In order to define the functional domains of the *gag* gene in the processes of HIV-1 virus assembly and infectivity, a series of *gag* mutations was generated and introduced into a replication-defective HIV vector, HIVgpt. As described previously (33), HIVgpt contains a deletion of the HIV-1 *env* coding region and an insertion of a fragment from pSV2gpt (30). This insertion fragment contains the simian virus 40 origin of replication and early promoter driving expression of the selectable bacterial *gpt* gene. The two regulatory protein coding regions for *tat* and *rev* that are essential for virus replication are intact, and transient expression of HIVgpt in COS7 cells results in assembly and release of noninfectious HIV virus particles. When HIVgpt is cotransfected into COS7 cells with a retrovirus envelope protein expression plasmid, virus particles are produced which can transduce the *gpt* gene in a single round of infection (33).

As described in Materials and Methods (Fig. 1), we constructed HIV mutations by deletion or linker insertion in the *gag* or *pol* gene of HXB2 (36). In addition, we were kindly provided with three previously created mutants (1, 5). All 12 mutations were introduced into the HIVgpt backbone, yielding the constructs illustrated in Fig. 1. As shown, the Psi<sup>−</sup> construct contains a packaging signal deletion (nucleotides 748 to 786 [1]) which inhibits viral RNA packaging. The myristylation-minus (Myr<sup>−</sup>) mutant construct possesses a second codon glycine-to-alanine mutation which reduces membrane association and blocks infectivity (5). The *Cla*I, *Acc*I, *Hind*III, and *Pvu*II mutations were linker insertions into the HIV-1 matrix coding region at nucleotides 831, 960, 1085, and 1147, respectively (Fig. 1B). Within the capsid domain, we generated a 56-amino-acid deletion (dl.NsiPst) and a two-codon linker insertion at nucleotide 1508 (*Spe*I). Further towards the Gag protein carboxy terminus are *Apa*I, a linker insertion in NC, and A14-15, a mutant which contains four individual mutations in the HIV-1 *gag* Cys-His motif, blocking RNA encapsidation (1). The final two mutations were *Bgl*II, located near the HIV-1 *gag-pol* frameshift region, and a linker insertion at the nucleotide 2429 *Bcl*II site, in the protease (PR) coding region.

For infectivity analysis, each mutant was cotransfected

into COS7 cells with the MLV amphotropic *env* expression plasmid SV-A-MLV-*env* (33). At 48 or 72 h after transfection, virus-containing supernatants were used to infect recipient HeLa cells; infections and selections for drug-resistant colonies were performed as described in Materials and Methods. In this regard, it should be noted that because cotransfections were not with an HIV *env* expression plasmid, potential *gag* mutations affecting HIV *env-gag* interactions could not be detected. Nevertheless, this system permitted analysis of *gag* mutants which perturbed other phases of the virus life cycle. With our protocol, the infectivity of each mutant was determined by the ratio of its titer versus the titer of wt HIVgpt in parallel experiments. Depending on the date of transfection, the wt HIVgpt construct yielded titers which varied from 263 to 2,080 CFU for virus collected at 48 h and varied from 3,428 to 4,916 for virus collected at 72 h. However, most of our *gag* mutant viruses were 20-fold less infectious than comparable wt stocks (Table 1). As shown in Table 1, several mutants (Myr<sup>−</sup>, *Acc*I, NsiPst, *Spe*I, A14-15, PR<sup>−</sup>) appeared noninfectious. Some mutants, such as Psi<sup>−</sup>, *Hind*III, *Apa*I and *Bgl*II, retained low levels of infectivity. The *Pvu*II mutant, within the *gag* MA domain, had levels of infectivity approximately 10% that of wt. Even higher levels of infectivity, averaging over 50% of wt levels, were demonstrated by the *Cla*I mutant, also within the MA domain.

On the basis of previous work, some of the results of our infection assays were expected. For instance, the Myr<sup>−</sup> mutation, which has been reported to block virus assembly (5, 34), was noninfectious. Similarly, RNA encapsidation mutants mapping to the packaging signal region (Psi<sup>−</sup>) and the nucleocapsid RNA binding motif also were poorly infectious. The other NC domain mutant, *Apa*I, which contained a six-amino-acid insertion between the two HIV Cys-His motifs, also had a low titer, as did our insertion within the protease region (PR<sup>−</sup>). However, infectious virus particles were produced on cotransfection of PR<sup>−</sup> with the noninfectious dl.NsiPst mutant, suggesting that NsiPst Gag-Pol proteins could provide a functional protease when incorporated into protease-minus particles. Also infectious were viruses produced by constructs with mutations just after the matrix myristylation signal (*Cla*I) and just before the MA-CA cleavage site (*Pvu*II). These results suggested that, unlike other Gag protein regions, the *Cla*I mutation (and to lesser extent the *Pvu*II mutation) defines an area where variation is tolerated.

**Expression and assembly of HIV *gag* mutants.** To assay Gag protein expression and to examine the effects of mutations on virus assembly, immunoblotting was performed to detect virus-associated Gag antigen in the medium versus inside the cells. At 48 h after transfection of COS7 cells, cell supernatants and cell samples were prepared and subjected to SDS-PAGE followed by electroblotting onto a nitrocellulose membranes as described in Materials and Methods. HIV Gag proteins then were immunodetected with anti-p24<sup>gag</sup> monoclonal antibody. As illustrated for several mutants in Fig. 2, the mature Gag product p24<sup>gag</sup>, and incompletely processed proteins Pr55<sup>gag</sup> and p41<sup>gag</sup> were detected in medium supernatants (lanes A to G). In cell samples, precursor Pr55<sup>gag</sup> and p41<sup>gag</sup> bands were visible (lanes I to O), while p24<sup>gag</sup> was variably detected (lanes I and O). In the case of dl.NsiPst (Fig. 2, lanes F and N) a band at 49 kDa and bands at 34 to 36 and 16 to 20 kDa were observed, consistent with a deletion of 56 amino acids. As shown, some mutant proteins were released efficiently from cells, whereas others

TABLE 1. Infectivity of HIV *gag* mutants

Construct <sup>a</sup>	Titer	HIVgpt titer <sup>b</sup>	% Infectivity <sup>c</sup>
HIVgpt	1,681	1,681	100
Psi <sup>-</sup>	8	263	3.0
Myr <sup>-</sup>	0	263	0
<i>Cla</i> I	72	544	13.2
	3,234	4,916	65.8
	3,687	4,916	75.0
<i>Acc</i> I	0	880	0
	0	1,660	0
<i>Hind</i> III	2	1,392	0.1
	183	3,428	5.3
<i>Pvu</i> II	66	544	12.1
	174	4,916	3.5
	508	4,916	10.3
dl.NsiPst	0	544	0
	0	660	0
<i>Spe</i> I	0	2,080	0
	0	2,080	0
<i>Apa</i> I	2	984	0.2
	251	3,428	7.3
A14-15	0	660	0
	0	660	0
<i>Bgl</i> II	0	1,392	0
	33	3,428	1.0
PR <sup>-</sup>	0	1,392	0
	0	660	0
dl.NsiPst + PR <sup>-</sup>	3	660	0.5

<sup>a</sup> Each construct was cotransfected with SV-MLV-A-env into COS7 cells. In most cases, 2 days later, cell supernatants were used to infect HeLa cells. In two experiments (those with HIVgpt titers of 3,428 and 4,916), cell supernatants were collected after 3 days rather than 2 days. Infections and selections for mycophenolic acid-resistant colonies were performed as described in Materials and Methods.

<sup>b</sup> The value for the HIVgpt titer was an average of 10 independent experiments. The averages for HIVgpt titers from 3- and 2-day collections were 4,172 and 1,058  $\pm$  611, respectively. Duplicate experiments were performed with different DNAs, at different times, or both.

<sup>c</sup> Infectivities for each mutant were determined by the ratio of its titer versus the wt HIVgpt titer (middle column) in a parallel experiment.

were present in medium supernatants at noticeably reduced levels (see *Acc*I, Fig. 2, lanes D and L).

Because these experiments involved collection of medium supernatant virus proteins which were pelleted in a 45-min spin through a 20% sucrose cushion, we believe that they represent virus-associated proteins. Based on centrifugation clearing rate estimates, the minimum size of a pelletable particle would be 165S, and >90% of our medium HIVgpt Gag protein was recoverable by this method (data not shown). Nevertheless, we were surprised to find that even dl.NsiPst, the 56-amino-acid deletion protein, was released as a high-molecular-weight complex, and thus we examined particles released from COS7 cells in more detail. To do so, medium supernatant proteins were fractionated by sucrose density gradient centrifugation, assayed by immunoblotting,

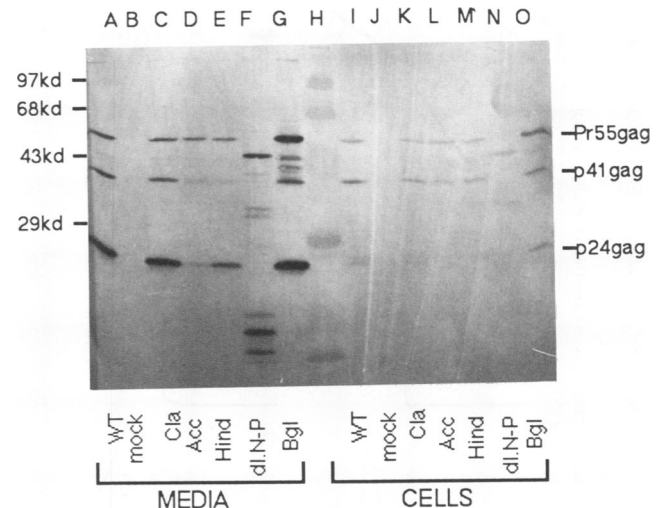


FIG. 2. Expression and release of HIV *Gag* proteins. COS7 cells were transfected with the designated plasmids. After 48 to 72 h, supernatants and cells were collected and prepared for protein analysis as described in Materials and Methods. Supernatant samples (lanes A to G, corresponding to 50% of the total sample) and cell samples (lanes I to O, corresponding to 5% of the total cell sample) were fractionated by SDS-PAGE and were electroblotted onto a nitrocellulose filter. HIV *Gag* proteins were detected with a mouse anti-p24 monoclonal antibody at a 1:2,000 dilution, followed by a secondary alkaline phosphatase-conjugated goat anti-mouse antibody at a 1:1,500 dilution and detection of alkaline phosphatase activity. Molecular size markers (lane H) are indicated on the left, and HIV *Gag* proteins Pr55, p41, and p24 are shown on the right. Lanes: A and I, wt; B and J, mock; C and K, *Cla*I; D and L, *Acc*I; E and M, *Hind*III; F and N, NsiPst; G and O, *Bgl*II.

and quantitated by densitometry. Because *Cla*I and *Pvu*II mutants were infectious (Table 1), Psi<sup>-</sup> and A14-15 mutants had been examined previously (1), and limited release of *Acc*I and *Hind*III mutant proteins prohibited their analysis, we focused our examination on wt, dl.NsiPst, *Spe*I, *Apa*I, *Bgl*II, and PR<sup>-</sup> medium proteins. As shown in Fig. 3, wt and all five mutant proteins banded in our fractions 7 and 8, at densities between 1.16 and 1.18 g/ml. This particle density reading is consistent with that for retrovirus particles (3, 18) and indicates that the levels of pelletable medium *Gag* proteins reflect the amount of virus particles released from cells.

To reliably evaluate the effects of *gag* mutations on HIV assembly and particle release, we quantitated *Gag* protein levels with a scanning densitometer and determined ratios of total *Gag* protein levels in the medium supernatants versus cells. To compare with wt HIVgpt, ratios obtained with each mutant were divided by wt ratios in parallel experiments. The results shown in Table 2 indicate that most mutants were capable of particle assembly and release because their ratios were comparable to that of the wt construct. Two mutants, *Cla*I and dl.NsiPst, possessed medium/cell *Gag* ratios that were even higher than the wt ratio; this result could be due either to increased levels of intracellular mutant *Gag* protein degradation or to increased assembly rates. In contrast, the ratio of the Myr<sup>-</sup> mutant was <1% of the wt ratio, a result consistent with the previous demonstration that Myr<sup>-</sup> HIV mutants are blocked in virus assembly (5, 34). With the exception of Myr<sup>-</sup>, the ratios of two matrix mutants, *Acc*I and *Hind*III, were lower than those of any

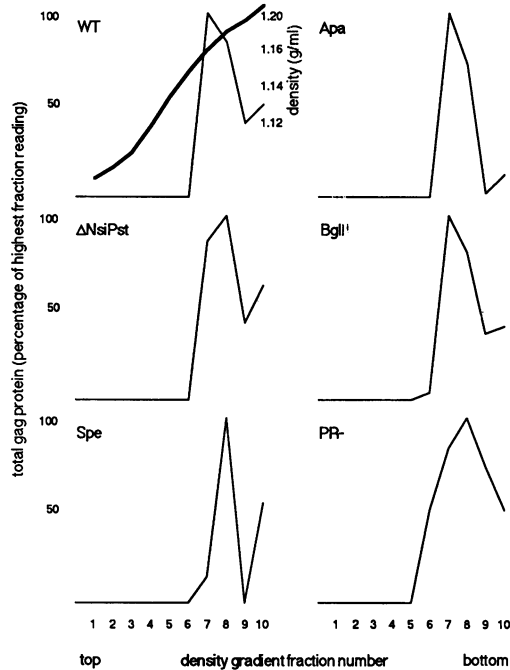


FIG. 3. Sucrose density gradient fractionation of HIV particles. Supernatants from transfected COS7 cells were collected and fractionated by sucrose density gradients (20 to 50%) as described in Materials and Methods. Fractions were collected from top to bottom, and virus-associated Gag proteins from each fraction were measured by immunoblot techniques and were quantitated by scanning densitometry. As shown, the peak Gag protein fractions had densities of 1.16 to 1.18 g/ml.

other mutants. Indeed, *AccI* Gag proteins were detected predominantly inside cells, with medium/cell ratios 8 to 30% of wt levels (Fig. 2 and Table 2). Theoretically, *AccI* Gag protein release values could be a consequence of protein instability in virus particles or inefficient particle budding. Because we have not observed major differences in particle-associated Gag protein degradation rates (data not shown), we favor the hypothesis that the *AccI* matrix mutation inhibits HIV-1 particle release. Support for this notion comes from indirect immunofluorescence studies. After transfection with wt HIVgpt or *Myr*<sup>-</sup>, *ClaI*, *AccI*, *HindIII*, *BglII*, or *PR*<sup>-</sup> constructs, COS7 cells on coverslips were processed for immunofluorescent localization of Gag antigen by using an anti-p24<sup>gag</sup> first antibody and a rhodamine-conjugated rabbit anti-mouse second antibody (see Materials and Methods). As illustrated in Fig. 4A, wt Gag proteins were present throughout the cytoplasm of transfected cells as a heterogeneously staining haze. Localization patterns of *ClaI*, *HindIII*, *BglII*, and *PR*<sup>-</sup> mutant Gag proteins could not be distinguished from wt patterns. In contrast, cells expressing the *AccI* mutant showed punctate staining asymmetrically located around cell nuclei (Fig. 4B). The pattern of the *AccI* mutant was different from that with the *Myr*<sup>-</sup> protein, which localized to the cytoplasm of transfected cells (Fig. 4C). These results suggest that *AccI* mutants demonstrated reduced particle assembly because the Gag proteins were trapped intracellularly.

**Characterization of virus particles.** The results of Table 1 indicate that many of our HIV *gag* mutants were poorly infectious, while data in Table 2 show that, for most mu-

TABLE 2. HIV Gag protein release from cells<sup>a</sup>

Construct	Expt	Total Gag protein in <sup>b</sup> :		Medium/cell ratio <sup>c</sup>	% wt
		Medium	Cells		
HIVgpt	1	4.96	3.35	1.46	100
	2	31.88	24.0	1.33	100
	3	9.89	15.2	0.65	100
	4	3.62	6.0	0.60	100
<i>Myr</i> <sup>-</sup>	1	<0.32	19.6	0.016	<1
<i>ClaI</i>	2	30.6	9.8	3.12	234
<i>AccI</i>	1	0.72	6.0	0.12	8
	2	3.74	9.2	0.41	30
	3	1.57	16.0	0.10	15
<i>HindIII</i>	2	10.8	10.0	1.08	81
	3	6.66	19.6	0.34	52
<i>PvuII</i>	4	2.91	7.2	0.40	67
dl.NsiPst	2	24.47	7.2	3.40	255
<i>SpeI</i>	3	9.20	4.6	2.0	130
<i>ApaI</i>	3	4.43	7.2	0.62	95
<i>BglII</i>	2	8.32	32.4	1.49	112
	3	34.4	18.6	1.85	286
<i>PR</i> <sup>-</sup>	2	18.23	9.6	1.90	143

<sup>a</sup> Supernatant and cell samples of wt and mutant constructs were analyzed by immunoblot techniques as described in Materials and Methods. Gag proteins from medium or cell samples were quantitated by scanning Pr55, p41, and p24 band densities from immunoblots. The results of four experiments were compiled.

<sup>b</sup> The sum of arbitrary density units (Pr55 plus p41 plus 24) indicated the total Gag protein in the medium or inside the cells. In the case of the *Myr*<sup>-</sup> mutant, no Gag proteins could be detected in the medium (the 0.32 value indicated the minimal detectable signal by densitometry).

<sup>c</sup> Ratios of total Gag protein in the medium versus total Gag inside the cells were indicators of virus particle assembly and release. For comparison with release of wt virus, the ratio of each mutant was divided by the ratio of wt in each parallel experiment and was multiplied by 100.

tants, virion release was unimpaired. An obvious explanation for this difference is that the virus particles assembled by our mutant Gag proteins were defective for one reason or another. Because reverse transcription is essential for HIV replication, *gag* mutants also were characterized by measuring RT activity levels. Assays were performed as described in Materials and Methods, and in each case, the counts per minute of incorporated nucleotide was at least threefold over background levels (Table 3). While nucleotide incorporation counts yielded some information on RT levels, to obtain specific activities for each mutant, the ratios of normalized counts per minute versus densitometer-determined virus-associated Gag protein levels were compared with wt levels in parallel experiments. The results in Table 3 show that most mutants showed at least 50% of the wt RT activity. The only exceptions to this rule were the *SpeI*, *BglII*, and *PR*<sup>-</sup> mutants. For the *BglII* and *SpeI* mutants, which retained 30 to 60% of wt activity (Table 3), reduced RT levels could be due to reduced stability, processing, or incorporation of Gag-Pol proteins into virions. With our protease-defective mutant, specific RT activities were 30 and 50% of wt levels, consistent with previous reports (35). Although unprocessed Gag-Pol fusion proteins may result in reduction of enzymatic

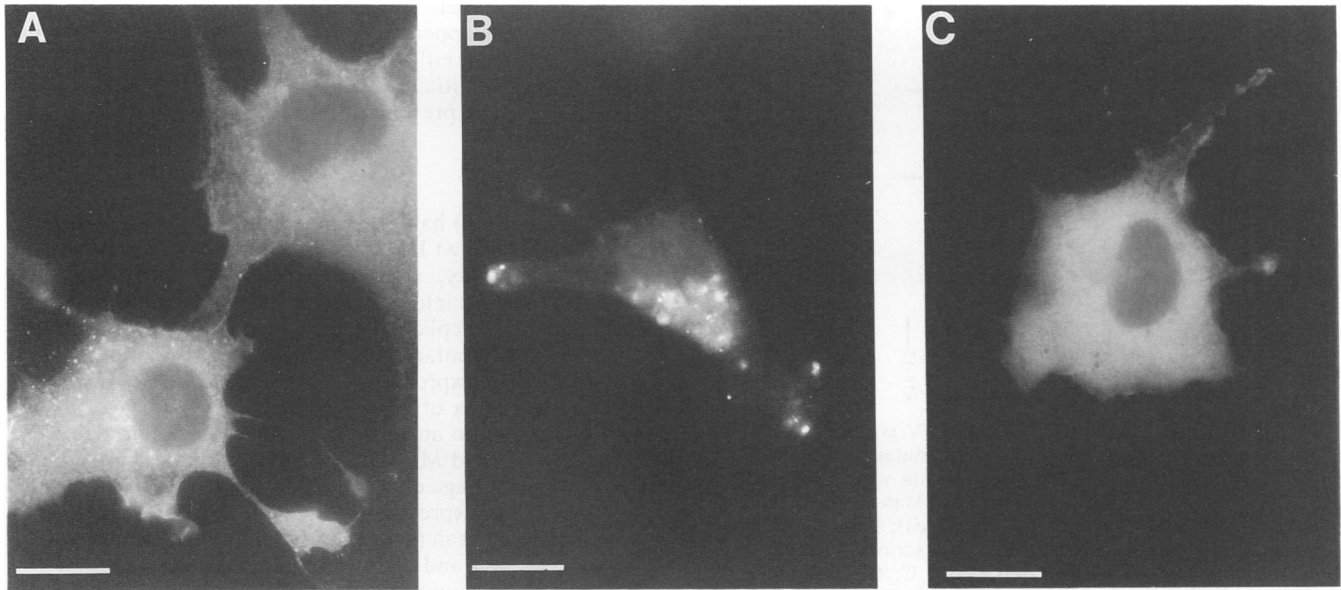


FIG. 4. Indirect immunofluorescence detection of HIV Gag proteins in COS7 cells. COS7 cells grown on coverslips were transfected with wt HIVgpt (A), Gag *AccI* (B), or Myr<sup>-</sup> (C) mutant constructs. Forty-eight hours after transfection, cells were fixed and permeabilized for immunofluorescence assays as described in Materials and Methods. The primary antibody was a mouse anti-p24 monoclonal antibody at a 1:1,000 dilution, and the secondary antibody was a 1:100 dilution of rhodamine-conjugated rabbit anti-mouse antibody. Mock-transfected COS7 cells and cells not exposed to the primary anti-Gag antibody yielded no signals (data not shown). Bars, 20  $\mu$ m.

activity (23, 24, 26), it is conceivable that the low enzymatic activity of the protease mutant may be due to the stability of immature virus cores (see below), and inefficient detergent release of Gag-Pol fusion proteins during RT assays.

Previous studies have shown that a functional protease is required for retrovirus infectivity (13, 20, 35), suggesting a requirement for HIV Gag protein processing. We analyzed processing of HIV *gag* mutants by immunoblotting of particle-associated proteins. Figure 5 shows that most mutants were processed as well as wt HIVgpt, in which p24<sup>gag</sup> is present, but precursor forms Pr55<sup>gag</sup> and p41<sup>gag</sup> were also observed. As expected, the clearest exception was the PR<sup>-</sup> mutant, in which the processing was blocked completely. In this experiment, medium *AccI* and *HindIII* Gag levels were below our levels of detection, consistent with their reduced release from cells, but results in Fig. 2 show that at least some processing of these proteins occurred. The dl.NsiPst construct with a 56-amino-acid deletion in the capsid domain still could be processed and detected as bands of 49, 35, and 18 kDa, corresponding to the wt Pr55<sup>gag</sup>, p41<sup>gag</sup>, and p24<sup>gag</sup> proteins, respectively. To assess the processing efficiency of each mutant, we quantitated the p24-associated Gag proteins in medium supernatant samples by densitometry and plotted the ratio of each Gag product versus total Gag proteins. As illustrated in Fig. 6, and as observed in Fig. 2 and 5, most mutants displayed a processing pattern similar to that of wt. However, there were several exceptions. In particular, mutants *AccI*, *SpeI*, and *BglIII* were processed incompletely, suggesting either that these mutant Gag proteins were resistant to cleavage, that mutant Gag-Pol proteins possessed defective protease moieties, or that mutant Gag-Pol proteins entered virions at reduced efficiencies (see Discussion).

Of all our mutants, only PR<sup>-</sup> was completely devoid of mature virus particle-associated mature proteins. This allowed us to investigate structural differences between immature and mature HIV particles. Previous studies have dem-

TABLE 3. RT activities of HIV *gag* mutants<sup>a</sup>

Construct	cpm incorporated	Relative activity (% wt) <sup>b</sup>
HIVgpt	3,090	100
	5,890	100
	1,781	100
	4,868	100
<i>ClaI</i>	5,700	101
	2,621	56
<i>AccI</i>	1,450	66
<i>HindIII</i>	5,420	83
	1,380	54
NsiPst	5,180	113
	3,453	90
<i>SpeI</i>	1,810	45
	1,526	43
A14-15	1,838	63
<i>BglIII</i>	3,010	30
	5,710	64
PR <sup>-</sup>	1,310	30
	1,352	50

<sup>a</sup> Preparation of supernatants and RT assays were performed as described in Materials and Methods. Note that activities (cpm incorporated) were at least threefold higher than that of background (396  $\pm$  6 cpm). For each sample, virus-associated Gag protein levels were determined as described in Table 2 footnotes. Results of four separate transfection experiments are given.

<sup>b</sup> Relative activities were determined as percentages of wt activities by the equation  $100 \times [(\text{mutant cpm} - \text{background}) / \text{mutant Gag protein} / (\text{wt cpm} - \text{background}) / \text{wt Gag protein}]$ .



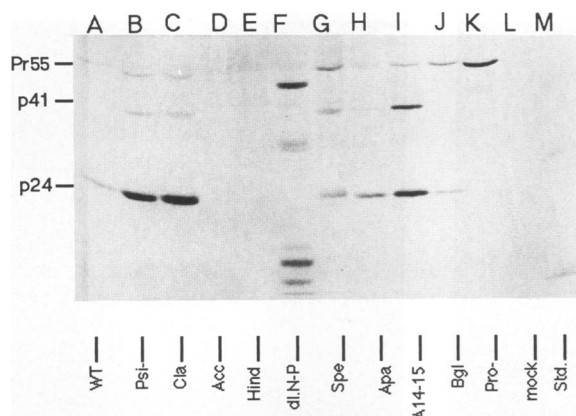


FIG. 5. Assembly and processing of HIV *gag* mutants. COS7 cells were transfected with wt HIVgpt and mutant plasmids. Forty-eight hours after transfections, supernatants were collected and prepared for protein analysis as described in Materials and Methods. Samples were fractionated by SDS-10% PAGE followed by immunoblot analysis with anti-p24 antibody as described in the legend to Fig. 2. Lanes: A, wt (HIVgpt); B,  $\Psi$ si<sup>-</sup>; C, *Cla*I; D, *Acc*I; E, *Hind*III; F, *Nsi*Pst; G, *Spe*I; H, *Apa*I; I, A14-15; J, *Bgl*II; K, *Pro*<sup>-</sup>; L, mock; M, standard. HIV Pr55, p41, and p24 Gag proteins are designated at the left. Note that particle release of *Acc*I and *Hind*III mutants was low in this experiment and that processing levels were determined from other transfections.

onstrated that immature virions of avian sarcoma-leukosis virus and MLV protease mutants were resistant to nonionic detergent (14a, 42). To investigate whether the HIV protease mutant had a similar property, culture supernatants of transfected cells were treated with 0.5% Triton X-100 followed by centrifugation through 20% sucrose cushions as described in Materials and Methods. Gag proteins recovered in the pellets were analyzed by immunoblotting. As shown in Fig. 7, most protease mutant virions were recovered in the pellet either with or without treatment of Triton X-100 (Fig. 7, lanes C and D). In contrast, p24<sup>gag</sup> in particles produced by wt HIVgpt was solubilized by Triton treatment (Fig. 7, lanes

A and B). However, it was noted that wt HIVgpt medium Pr55<sup>gag</sup> proteins appeared resistant to Triton X-100 solubilization, suggesting either that proteolytic processing is very rapid once it is initiated or that immature and mature Gag proteins were not present within the same particles.

## DISCUSSION

Trono et al. (46) have reported that coexpression of HIV *gag* mutants with wt HIV could result in a drastic reduction of virus infectivity, suggesting that mutant Gag proteins were incorporated into wt virus particles and interfered with subsequent viral replication. We have analyzed the infectivity of HIV *gag* mutants by cotransfection with an amphotropic envelope expression plasmid, SV-A-MLV-Env, into COS7 cells. Titers of generated virus particles were determined by infection and selection of drug-resistant colonies. Although HIV and MLV Env proteins do not share significant homology, Page et al. (33) reported that titers of virions generated by coexpression of HIVgpt and MLV Env were generally higher than those of virions generated by coexpression of HIVgpt and HIV Env in parallel transfections, indicating that pseudotyping of MLV Env with HIVgpt is an efficient process. However, a recent study demonstrated that the HIV matrix domain may be involved in the incorporation of HIV envelopes into virions (51), and we must note that because of our utilization of a murine envelope protein, we would be unable to detect specific perturbation of putative HIV Gag and Env interactions in our system.

We were surprised to discover the number of mutants that were able to assemble virus particles. To a certain extent, this may be attributable to use of a high-expression COS cell system. However, Western immunoblot analysis indicated a failure of mutants *Acc*I and *Myr*<sup>-</sup> to produce high levels of virus particles (Fig. 2 and Table 2). Our findings with the *Myr*<sup>-</sup> mutant corroborate previous reports that myristylation of HIV Gag is required for virus assembly and infectivity (5, 34). Also, previous work has shown that deletion mutations around the MA *Acc*I region could block virus assembly, because very little RT activity was released into the culture medium (51); however, these mutants were not

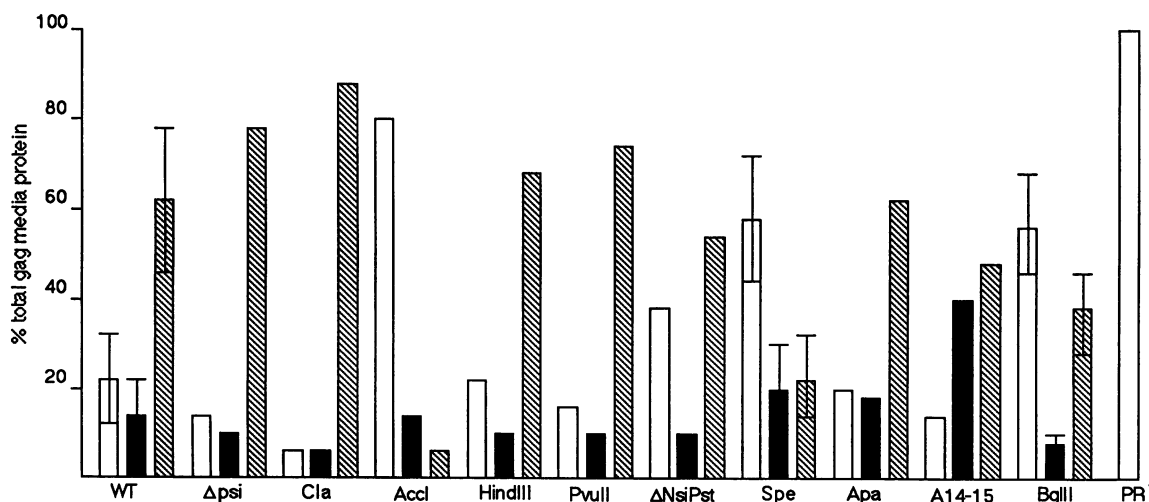


FIG. 6. Processing of HIV *gag* mutants. Gag proteins in medium supernatants were quantitated by scanning densitometry as described in the legend to Table 2. The percentages of Pr55<sup>gag</sup> (open bars), p41<sup>gag</sup> (solid bars), and p24<sup>gag</sup> (hatched bars) were obtained by dividing density units of individual bands by the total Gag protein density units and multiplying by 100. The wt values were derived from six independent trials, mutants *Spe*I and *Bgl*II were from three experiments each, and all others were derived from one experiment each.



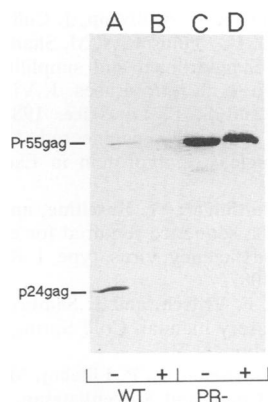


FIG. 7. Virus particle sensitivity to nonionic detergent treatment. Supernatants from wt or protease mutant transfected COS7 cells were filtered through a 0.45- $\mu$ m-pore-size filter. Cell supernatants then were mock treated (lanes A and C) or treated with 0.5% Triton-X100 (lanes B and D). After treatment, samples were centrifuged through 2 ml of 20% sucrose at 274,000  $\times$  g and 4°C for 45 min. Pellets were solubilized in sample buffer and separated on an SDS-10% polyacrylamide gel and were electroblotted onto a nitrocellulose filter. Immunodetection of Gag proteins was performed as described in the legend to Fig. 2. Core protein precursor Pr55<sup>gag</sup> and mature p24<sup>gag</sup> protein bands are indicated on the left.

further characterized. Indirect immunofluorescence revealed that mutant *AccI* Gag proteins appeared trapped around the perinuclear area (Fig. 3), indicating that mutation at this region may block a normal Gag transport route or cause the protein to mislocalize. Previous studies have suggested that the matrix domain of Moloney MLV Gag protein may interact with intracellular membranes prior to transport to the cell surface (15). Thus, it is conceivable that the HIV matrix domain also may interact with intracellular membranes under some circumstances.

Our linker-generated mutants *ClaI* (near the MA amino terminus, after the 15th codon of *gag*) and *PvuII* (after codon 120, 12 codons from the CA coding region) did not affect virus assembly or processing and had significant exogenous template RT activities, consistent with previous reports (50, 51). Our infectivity analysis showed that mutants *ClaI* and *PvuII* were somewhat infectious (*PvuII*) or up to half as infectious as wt (*ClaI*). However, we cannot exclude the possibility that the matrix domain may be involved in *in vivo* replication processes.

Several lines of evidence have suggested that retroviral capsid domains are key regions responsible for interactions among the Gag polyproteins (9, 37). Surprisingly, our HIV mutant containing a 56-amino-acid CA deletion did not affect virus assembly, budding, or processing (Fig. 2 and 6). The dl.NsiPst mutant RT activity was comparable to that of wt (Table 3), and this mutant even exhibited a characteristic wt retrovirus density of 1.16 to 1.18 g/ml (Fig. 3). Apparently, the amino-terminal HIV Gag CA of this region may be dispensable for HIV core assembly. The other capsid mutation (*SpeI*) containing only a two-amino-acid insertion, apparently eliminated viral infectivity (Table 1). Incomplete processing (Fig. 2 and 6) and low enzymatic activity (Table 3) may have rendered this mutant noninfectious. However, early postbinding steps in the infection process also may be affected by the *SpeI* mutation.

Both nucleocapsid mutants A14-15 and *ApaI* could assemble, release, and process Pr55<sup>gag</sup> (Fig. 2 and 6) but showed

reduced or no infectivity (Table 1). These observations are consistent with previous work, suggesting that NC (p7) does not play a role in core assembly (1). The inability of mutant A14-15 to replicate appears to be due to defective packaging of viral RNA into virions, because previous quantitation of viral RNA by slot blot hybridization (1) showed a nearly complete block in RNA encapsidation (1). However, preliminary results with our *ApaI* linker insertion between Cys-His finger motifs suggest that RNA incorporation is not inhibited (data not shown), although the mutation reduced infectivity.

As for the PR<sup>-</sup> mutant (*BcII*), our findings support previous studies (13, 35) which showed that protease-defective mutants are blocked for virus infectivity but not for assembly. The fact that maturation of immature virions by viral protease is required for viral infectivity is consistent with our findings with mutants *AccI*, *SpeI*, and *BglIII*, in which virions are both incompletely processed and noninfectious or poorly infectious (Table 1 and Fig. 6). It is possible that these mutations may lead to conformation changes in Gag precursors and subsequently may interfere with the exposure of cleavage sites to protease. Alternatively, conformation changes of Gag-Pol fusion proteins induced by these mutants may interfere with dimer formation of Gag-Pol molecules, which is required for activation of viral protease (22, 31, 49). The low RT activity of mutants *AccI*, *SpeI*, and *BglIII*, also may be a consequence of incomplete processing, defective Gag-Pol protein dimerization, or insufficient incorporation of Gag-Pol into virions (22, 31, 42).

Because the majority of our HIV *gag* mutants assembled noninfectious or poorly infectious particles, these studies strongly support the notion that retroviral Gag proteins function not only in driving assembly for the construction of virus particles but also in a variety of postassembly, post-processing events. It will be of interest to dissect the effects of these and other mutations on the processes of endogenous template reverse transcription, nuclear localization, and integration events.

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