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Characterization of Deletion Mutations in the Capsid Region of Human Immunodeficiency Virus Type 1 That Affect Particle Formation and Gag-Pol Precursor Incorporation

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The core of human immunodeficiency virus type 1 is derived from two precursor polyproteins, Pr55^{gag} and Pr160$^{gag\text{-}pol}$. The Gag precursor can assemble into immature virus-like particles when expressed by itself, while the Gag-Pol precursor lacks particle-forming ability. We have shown previously that the Gag precursor is able to "rescue" the Gag-Pol precursor into virus-like particles when the two polyproteins are expressed in the same cell by using separate simian virus 40-based plasmid expression vectors. To understand this interaction in greater detail, we have made deletion mutations in the capsid-coding regions of Gag- and Gag-Pol-expressing plasmids and assayed for the abilities of these precursors to assemble into virus-like particles. When we tested the abilities of Gag-Pol precursors to be incorporated into particles of Gag by coexpressing the precursors, we found that mutant Gag-Pol precursors lacking a conserved region in retroviral capsid proteins, the major homology region (MHR), were excluded from wild-type Gag particles. Mutant precursors lacking MHR were also less efficient in processing the Gag precursor in *trans*. These results suggest that the MHR is critical for interactions between Gag and Gag-Pol molecules. In contrast to these results, expression of mutated Gag precursors alone showed that deletions in the capsid region, including those which removed the MHR, reduced the efficiency of particle formation by only 40 to 50%. The mutant particles, however, were clearly lighter than the wild type in sucrose density gradients. These results indicate that the requirements for Gag particle formation differ from the ones essential for efficient incorporation of the Gag-Pol precursor into these particles.

The internal proteins of the human immunodeficiency virus (HIV) are principally derived from two precursor polyproteins, Pr55^{gag} and Pr160$^{gag\text{-}pol}$. These polyproteins are synthesized on membrane-free polysomes and are transported to the plasma membrane, the site of virus assembly (4, 32). Both precursors are translated from the same mRNA by a ribosomal frameshifting mechanism that allows the Pr160$^{gag\text{-}pol}$ precursor to be synthesized as a carboxy-terminal extension of Pr55^{gag}. Ribosomal frameshifting occurs once every 10 or 20 translation rounds and thus regulates the amount of the Gag-Pol precursor made relative to that of the Gag precursor (12). Recent studies suggest that the polyprotein precursors are cleaved by the viral protease encoded in Pr160$^{gag\text{-}pol}$ into their final products before the particles have budded from the cell surface (13). Pr55^{gag} is cleaved into p17 (matrix [MA]), p24 (capsid [CA]), p2, p9 (nucleocapsid [NC]), p1, and p6. Cleavage of Pr160$^{gag\text{-}pol}$ yields MA, CA, and NC, as well as p6*, protease, reverse transcriptase (RT), and integrase (IN) proteins (4, 32).

The precursor polyproteins have been expressed individually by several groups, and these studies have shown that Pr55^{gag} is capable of particle assembly in the absence of other viral proteins (6, 10, 18, 24, 26), while Pr160$^{gag\text{-}pol}$ is deficient in this regard (14, 19, 26). Since all of the essential viral enzymes are encoded in the Pr160$^{gag\text{-}pol}$ precursor, this precursor needs to be incorporated into the virus particle to obtain an infectious virus. We and others have previously shown that when the two proteins are coexpressed in the same cell from separate plasmid expression vectors, Pr55^{gag} enables Pr160$^{gag\text{-}pol}$ to be incorporated into particles (20, 26). These studies also showed that unmyristylated Pr160$^{gag\text{-}pol}$ can be incorporated into particles of Pr55^{gag}, despite the fact that particle formation by Pr55^{gag} was found to require myristylation. This suggests that Pr160$^{gag\text{-}pol}$ associates with Pr55^{gag} perhaps via a protein-protein-mediated interaction between the two precursors.

Little is known about these interactions or about how these precursors assemble to form the viral core. In this study, we have further analyzed the precursor interactions by taking advantage of our ability to express the polyproteins individually. To do this, we made several different deletions in the capsid regions of the two precursor polyproteins and assayed the mutated proteins for their abilities to assemble into virus-like particles when expressed individually or together in cells. Our results indicate that the "rescue" of Pr160$^{gag\text{-}pol}$ molecules into particles of wild-type Pr55^{gag} requires the presence of the major homology region (MHR) in Pr160$^{gag\text{-}pol}$. The MHR is a 20-amino-acid region that is conserved among all known retrovirus capsid proteins except those of spumaviruses. Mutant Gag-Pol precursors lacking the MHR were also less efficient in processing the Gag precursor in *trans*. In contrast to these results, we found that the MHR (and most of the capsid protein of Pr55^{gag}) could be removed without dramatically affecting the budding or release of the Gag precursor from cells, although the particles produced were slightly less dense than wild-type Pr55^{gag} particles on sucrose density gradients. Interestingly, when we tested the incorporation of Gag-Pol precursors into the mutant Gag particles, we found that the MHR was no longer required for this interaction.

MATERIALS AND METHODS

Plasmids. All of the plasmid expression vectors used in this study have been described previously (23, 25, 26). pBABY is the parental simian virus 40-based late replacement vector. It contains an *Xho*I cloning site positioned between the simian virus 40 late promoter and rabbit beta-globin intron and termination

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sequences. pSVGAGPOL-RRE-R contains the *gag-pol* region of HIV type 1 (HIV-1) and expresses both $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ in a ratio of about 20:1. pSVGAG contains the *gag* region of HIV-1 and expresses only $Pr55^{gag}$. pSVFS5T is derived from pSVGAGPOL-RRE-R but expresses only $Pr160^{gag\text{-}pol}$ as a result of being permanently frameshifted at the DNA level. pSVFS5TprotD25G is identical to pSVFS5T except that it contains an inactive viral protease gene. pCMVrev expresses the Rev protein of HIV-1 under control of the simian cytomegalovirus immediate-early promoter.

Mutants with deletion in the capsid regions of pSVGAG and pSVFS5T. Deletions were made in the p24-coding region of pSVFS5T by cleaving the plasmid at the unique *Spe*I site and digesting for various times with *Bal* 31 nuclease according to standard procedures (1). In-frame deletions were identified by double-stranded dideoxynucleotide DNA sequencing. The deletions were engineered into either the pSVFS5TprotD25G or pSVGAG background as described below.

To engineer the deletion mutations which were created in pSVFS5T into the pSVFS5TprotD25G background, an *Apa*I fragment from pSVFS5T, originating in the HIV-1 NC region and terminating in the rabbit beta-globin sequences, was replaced by the corresponding fragment from pSVFS5TprotD25G. It contained the mutated protease gene. To express the mutations in the context of $Pr55^{gag}$, the *Apa*I fragment was replaced by the corresponding fragment of pSVGAG.

Deletion of the MHR. Amino acids 152 through 172 (MHR) were deleted from the capsid protein of pSVFS5T by splicing overlap extension-PCR (9, 11). Oligonucleotides 5′-ACACCATGCTAAACACAGTGGG-3′ (top strand, outer) and 5′-GTCCAGATTGCTGGTAGGG-3′ (bottom strand, inner) were used in the first PCR; 5′-ACCAGCATTCTGAGAGCCGAGCAAGCTTCACAG-3′ (top strand, inner) and 5′-GGCCAGATCTTCCCTATTAGCCTGTCTCTCAG-3′ (bottom strand, outer) were used in the second PCR. pSVGAGPOL-RRE-r was used as the template for both PCRs. The PCR products from these reactions were purified on agarose gels and used together with the outer primers. The final PCR product was purified, cleaved with *Spe*I and *Bgl*II, and cloned into pSVFS5T($BglII^-$) digested with the same enzymes. pSVFS5T($BglII^-$) is identical to pSVFS5T except for the ablation of a *Bgl*II site in the beta-globin region of the plasmid. DNA sequencing indicated that during construction of this plasmid, an additional G-to-A mutation was created. This mutation changed the Met-Met cleavage site at the p2-p9 junction to Met-Ile. It should be noted that the cleavage site in the proviral clone, BH10, is TATIM-MQRGN, while that of pNL-4-3 is PATIM-IQKGN.

Cells and transfections. CMT3-COS cells are a derivative of CV1 cells that express simian virus 40 large T antigen under control of the metallothionein promoter (5). CMT3-COS cells were transfected by the DEAE-dextran method (8). Transfections were carried out in 100-mm-diameter tissue culture plates with 5 μg of the plasmid vectors or the amounts indicated in the figure legends and in the Results. In all cases the HIV-1 Rev protein was coexpressed by using pCMVrev.

Immunoprecipitation of labeled cells and media. Labeling with radioisotope was performed as previously described (26). In some instances, particles from the medium were precipitated by adding polyethylene glycol (PEG) to a final concentration of 7.5% in the presence of 1 M NaCl. The cell lysate and PEG-precipitated particles were immunoprecipitated by using 5 μl of serum from an HIV-infected individual and protein A-Sepharose beads (Pharmacia) as previously described (26). The immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorography. For some experiments the particles were pelleted through 20% (wt/vol) sucrose or recovered by PEG precipitation and analyzed by PAGE and fluorography without being subjected to an immunoprecipitation step.

Immunoblot analysis. The preparation of cell lysates for Western blots (immunoblots) was as previously described (26). For analysis of particles, the medium was cleared of floating cells by centrifugation at 2,500 rpm for 15 min at 4°C in an IEC Centra-8R centrifuge (International Equipment Co.). Particles from 8 ml of cleared medium were pelleted through 4 ml of 20% (wt/vol) sucrose by ultracentrifugation at 100,000 × *g* for 2 h at 4°C in a Beckman SW41 Ti rotor and resuspended in 80 μl of 1× SDS sample buffer. SDS samples of cell lysates or pelleted virions were heated in a boiling water bath for 10 min before being loaded onto SDS-PAGE gels.

The Western immunoblot analysis was done as described previously with minor modifications (26). Briefly, following transfer to Immobilon polyvinylidene difluoride membranes (Millipore), the membranes were blocked with 5% nonfat milk in phosphate-buffered saline (PBS) containing 0.3% Tween 20 (PBST). The membranes were washed with PBST and then probed with pooled human sera reactive against HIV-1 (1:200 dilution) in 2% nonfat milk–PBST. After overnight incubation at 4°C on a rocking platform, the membranes were washed with PBST containing 2% nonfat milk. The membranes were then reacted with a sheep anti-human immunoglobulin G–horseradish peroxidase conjugate (Amersham) at a 1:10,000 dilution in 2% nonfat milk–PBST. After a washing with PBS containing 0.5% Tween 20, the bound antibodies were detected with a chemiluminescence substrate (ECL; Amersham) and X-OMAT AR films (Kodak) or Reflection films (Dupont). Multiple exposures were taken at various time intervals after the addition of the substrate.

RT assays. RT assays were performed as previously described (26). Briefly, particles from 8 ml of medium were pelleted through 20% (wt/vol) sucrose at 100,000 × *g* in a Beckman SW41 Ti rotor for 2 h at 4°C. The particles were resuspended in 100 μl of 10 mM Tris–1 mM EDTA, pH 8.0 (TE), and a 10-μl aliquot was assayed for RT activity. For some experiments, the particles were pelleted from 1 ml of cleared medium in a Beckman TL100 microcentrifuge at 42,000 rpm for 2 h at 4°C and resuspended in 25 μl of TE prior to analysis.

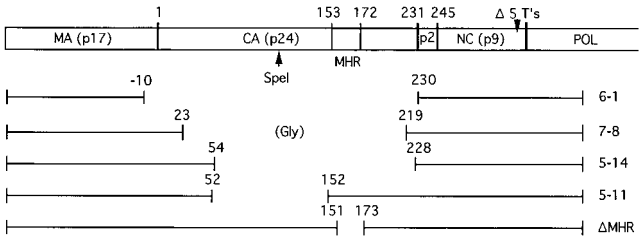

FIG. 1. Schematic representation of capsid deletion mutants of pSVFS5T. The names of the deletion mutations are indicated on the right. The numbers around the deletions refer to amino acid positions, with 1 being the first amino acid of the capsid protein in the BH10 clone of HIV-1. For example, the 6-1 mutant has a deletion that includes the last nine amino acids of p17 and extends to amino acid 229 of p24. The 7-8 mutation created a novel glycine residue at the junction site.

Density gradient analysis. CMT3-COS cells were transfected with pSVGAG and the various capsid deletion mutants of pSVGAG. At 72 h posttransfection, the medium was cleared of cell debris by centrifugation at 2,500 rpm for 15 min at 4°C in an IEC Centra-8R centrifuge (International Equipment Co.). The medium was then pelleted through 20% (wt/vol) sucrose as described above to collect particles, which were resuspended in PBS. The particles were then purified on a 20 to 60% (wt/vol) sucrose gradient that was centrifuged at 35,000 rpm for 16 h at 4°C in an SW50.1 rotor. Fractions (0.3 ml) were collected from the bottom of the tube. The densities of the fractions were determined by weighing 100 μl of each fraction. For Western immunoblot analysis, proteins in each fraction were then precipitated with 10% cold trichloroacetic acid after the fractions were spiked with 1 μg of glutathione-*S*-transferase protein, which was purified from *Escherichia coli* transformed with the plasmid pGEX-2T as recommended by the manufacturer (Pharmacia). The proteins were then analyzed by the Western immunoblot procedure as described above with the exceptions that a rabbit anti-glutathione-*S*-transferase serum at a dilution of 1:2,000 was added to the primary antibody and a donkey anti-rabbit–horseradish peroxidase conjugate at a dilution of 1:10,000 (Amersham) was added to the second antibody.

RESULTS

Incorporation of capsid region deletion mutants of $Pr160^{gag\text{-}pol}$ into particles. In a previous paper (26), we described a transient-expression system that allowed the individual expression of each core precursor polypeptide from a separate plasmid (26). We reasoned that this system could be used to map specific determinants in $Pr160^{gag\text{-}pol}$ that were required for its incorporation into virus particles. The strategy was to introduce deletion mutations into $Pr160^{gag\text{-}pol}$ and coexpress the mutant protein together with wild-type $Pr55^{gag}$. $Pr160^{gag\text{-}pol}$ incorporation into particles could then be monitored by collecting particles from the medium and analyzing them for the incorporation of the viral enzymes which would be present only if $Pr160^{gag\text{-}pol}$ was incorporated. Protease incorporation could be analyzed indirectly by monitoring the presence of p24 in the particles, while RT could be assayed directly.

A series of mutants with deletion mutations in the capsid region of $Pr160^{gag\text{-}pol}$ was made by *Bal* 31 exonuclease digestion from the unique *Spe*I site in the plasmid pSVFS5T. This plasmid expresses only the $Pr160^{gag\text{-}pol}$ precursor. After sequencing, several in-frame mutants were selected for further study. In addition to these mutants, we also constructed a mutant in which the 21 amino acids constituting the MHR of capsid were precisely removed by an in-frame deletion. These mutants are shown schematically in Fig. 1.

CMT3-COS cells were then transfected with the various capsid deletion mutants of pSVFS5T singly or together with pSVGAG. In all cases pCMVrev was also transfected to pro-

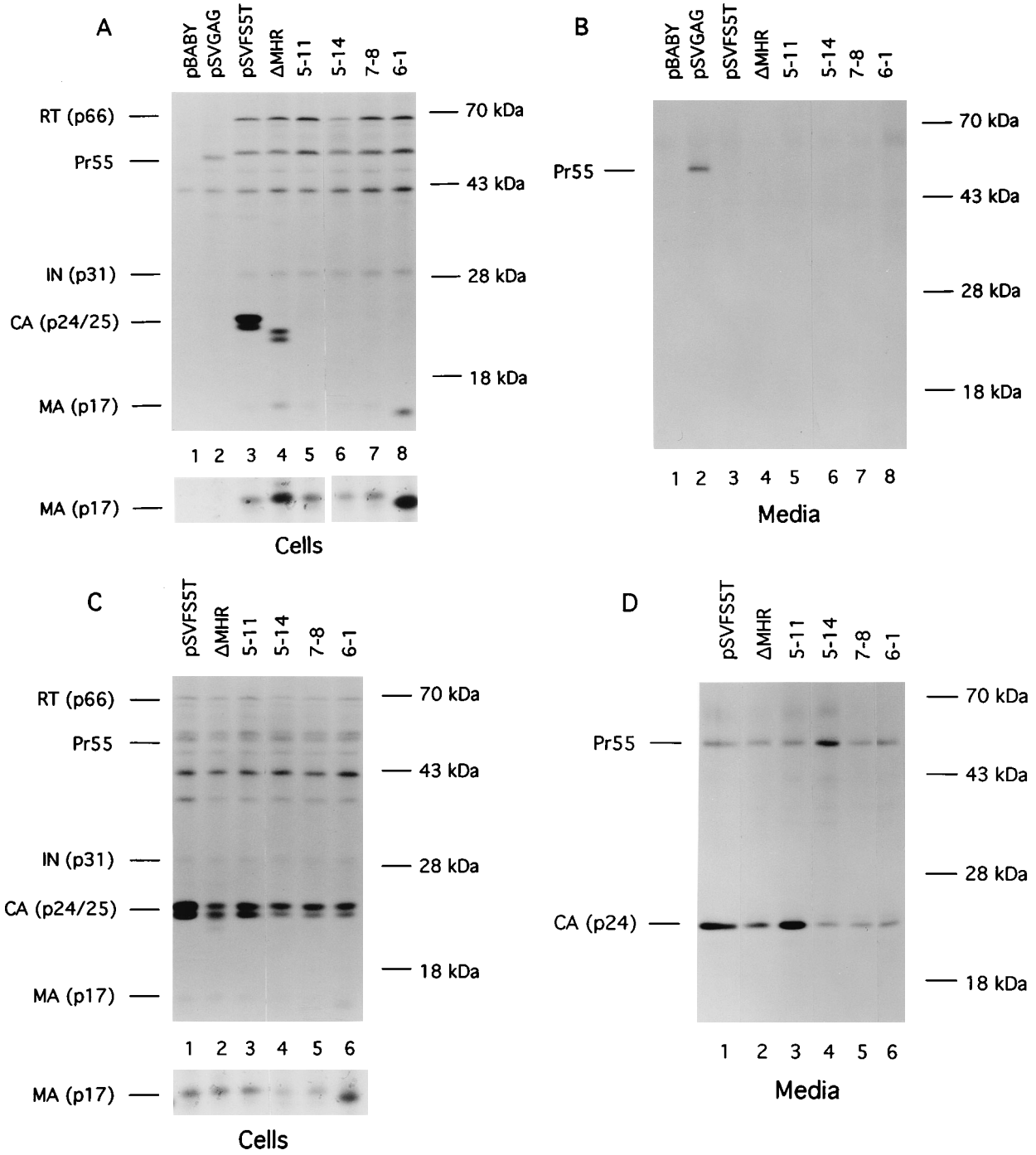

FIG. 2. Analysis of ^{35}S-labeled proteins produced by and released into the media of cells transfected with various plasmids. CMT3-COS cells were transfected with 5 μg of pSVGAG and 5 μg of various capsid deletion mutants of pSVFS5T either singly (A and B) or together (C and D). The cells were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine at 48 h posttransfection. After a 4-h pulse followed by a 4-hour chase, cells and media were harvested as described in Materials and Methods. Proteins were immunoprecipitated, using a human serum reactive with HIV-1, either from cell lysates (A and C) or from particles that were PEG precipitated from the medium (B and D) and analyzed by SDS-PAGE. Autoradiograms of the resulting gels are shown. In panels A and C an additional, longer exposure of the lower portion of the gel is shown to facilitate visualization of the MA band.

vide Rev protein, which is necessary for the expression of Gag and Gag-Pol from these plasmids. The transfected cells were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine at 48 h posttransfection. Cells and media were harvested after a 4-h pulse and 4-h chase as previously described (26). HIV-specific proteins were then immunoprecipitated from cell lysates or PEG-pelleted particles from the medium by using serum from an HIV-infected individual. The immunoprecipitated proteins were separated by SDS-PAGE and analyzed by fluorography.

Immunoprecipitation analysis of lysates from cells transfected with the various deletion mutants of Pr160$^{gag\text{-}pol}$ in the

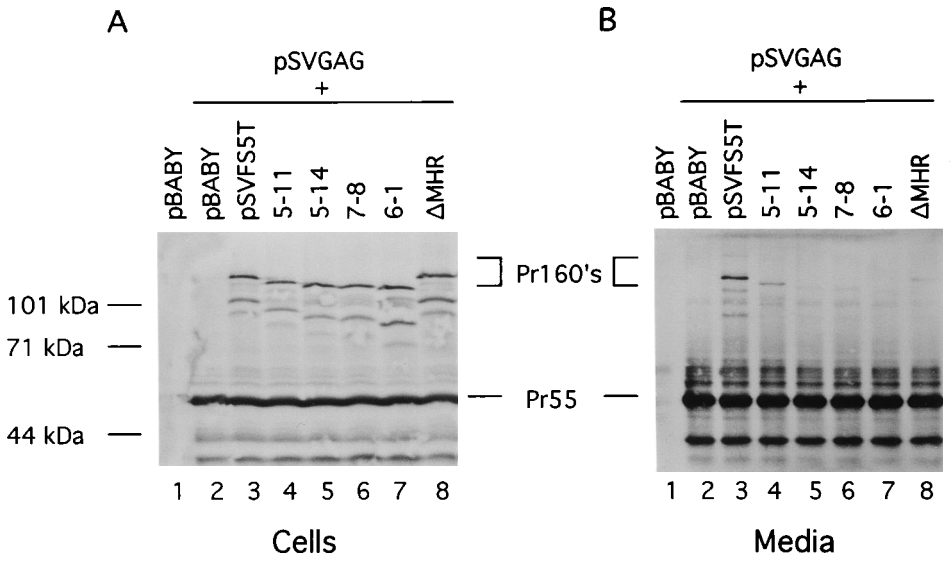

FIG. 3. Immunoblot analysis of proteins produced by and released into the media of cells transfected with various plasmids. CMT3-COS cells were transfected with 2 μg of pSVGAG and 5 μg of various capsid deletion mutants of pSVFS5TprotD25G. Cells and particles from the media were harvested at 72 h posttransfection as described in Materials and Methods. Proteins from cell lysates (A) and particles (B) were separated on 15% polyacrylamide gels and electrophoretically transferred onto Immobilon nylon membranes. The blot was probed with human pooled sera reactive with HIV-1 followed by anti-human immunoglobulin G–horseradish peroxidase conjugate. The bound conjugate was detected with a chemiluminescent substrate (ECL; Amersham) and Kodak XOMAT-AR X-ray films.

absence of $Pr55^{gag}$ showed that the precursors were efficiently processed in all cases, as indicated by the appearance of several cleavage products (Fig. 2A, lanes 3 to 8). In all lanes, bands corresponding to the mature matrix protein (p17), the RT (p66), and the integrase protein (p31), as well as an undefined intermediate, could be seen. These products were not seen in cells transfected with either the parental vector (Fig. 2A, lane 1) or a plasmid that expressed only $Pr55^{gag}$ (lane 2). The capsid proteins of the mutants with the larger deletions were not visualized under these conditions because of their small sizes (<15 kDa), but the shortened p24/p25 products from the ΔMHR mutant were clearly visible (lane 4). The p17 protein from cells transfected with the 6-1 deletion mutant migrated differently from the wild-type p17. This was expected, since the 6-1 deletion removed nine amino acids from p17, including the protease cleavage site, and positioned the p25 cleavage site directly at the end of p17. In accordance with previous observations (14, 20, 26) that the $Pr160^{gag\text{-}pol}$ precursor is not capable of particle formation on its own, viral proteins were not detected in the medium from cells transfected with any of the deletion mutants (Fig. 2B, lanes 4 to 8). However, control cells transfected with pSVGAG and pCMVrev released $Pr55^{gag}$-containing particles into the medium as expected (Fig. 2B, lane 2).

The results from cotransfection of pSVGAG with the various capsid deletion mutants of pSVFS5T and wild-type pSVFS5T are shown in Fig. 2C and D. Cotransfection, in all cases, resulted in cleavage of $Pr55^{gag}$ in *trans* by the protease encoded in $Pr160^{gag\text{-}pol}$, as judged by the appearance of p24/p25 in cells (Fig. 2C). The p24/p25 levels were higher when wild-type $Pr55^{gag}$ was coexpressed with wild-type $Pr160^{gag\text{-}pol}$. This was expected, since the p24/p25 doublet in this case contains the cleavage products from $Pr55^{gag}$ as well as those from $Pr160^{gag\text{-}pol}$. In all other cases, the p24/p25 doublet could arise only as a result of cleavage of $Pr55^{gag}$ in *trans* by the capsid deletion mutants of pSVFS5T, since all of them expressed a significantly smaller capsid protein. Relatively low levels of cleavage were observed with each mutant except the 5-11 mutant, which consistently gave higher levels of cleavage of $Pr55^{gag}$ into p24 in four independent experiments.

When the media from these transfections were analyzed, only the cotransfections with the 5-11 deletion mutant and wild-type pSVFS5T showed strong p24 bands (Fig. 2D, lanes 1 and 3), consistent with the results obtained with the cells. The other mutants all produced lower levels of p24 (Fig. 2D, lanes 2 and 4 to 6). Similar results were obtained in several independent transfections and also when the particles were analyzed by SDS-PAGE either after PEG precipitation or after recovery by ultracentrifugation of the medium (data not shown).

One interpretation of the above-described result is that all of the deletion mutants, with the exception of 5-11, were impaired in their interaction with $Pr55^{gag}$. This could lead to decreased incorporation of Gag-Pol into particles, resulting in decreased cleavage of the Gag precursor. Several other explanations are possible, however. For example, the decreased levels of p24 could be due to decreased efficiency of cleavage by the protease rather than to deficient incorporation of the deleted $Pr160^{gag\text{-}pol}$ precursors into the particles. Another possibility was that the deletion mutants could exert a transdominant effect on particle budding and release. This would then lead to fewer released particles, which would also lead to a reduced amount of p24 in the medium. A combination of all of the above-described consequences was also possible.

To distinguish between these possibilities, we decided to directly examine the incorporation of $Pr160^{gag\text{-}pol}$ into particles. To do this, we first created a plasmid that expressed a protease-inactive form of $Pr160^{gag\text{-}pol}$ by substituting a glycine for an asparate in the active site of the protease at residue 25. The various deletions were then moved into this background. This enabled us to determine the amounts of Gag and Gag-Pol precursors in the particles by Western blot analysis. CMT3-COS cells were transfected with 2 μg of pSVGAG and 5 μg of the various capsid deletion mutants of pSVFS5T carrying the protease mutation. Cells and particles from media were harvested at 72 h posttransfection and analyzed on Western blots

TABLE 1. RT levels in particles produced from cells transfected with pSVGAG and the $Pr160^{gag\text{-}pol}$ deletion mutants

Plasmid(s)[a] (no. of expts)	Normalized RT level (mean ± SD)
pSVGAG (5)	1.1 ± 2.4
pSVFS5T (5)	0
pSVFS5T 5-11 (4)	1.2 ± 2.3
pSVFS5T 7-8 (5)	0
pSVFS5T 6-1 (5)	0.2 ± 0.5
pSVFS5T 5-14 (2)	0
pSVFS5TΔMHR (2)	0
pSVGAG + pSVFS5T (6)	100
pSVGAG + pSVFS5T 5-11 (5)	49.1 ± 12.4
pSVGAG + pSVFS5T 7-8 (5)	6.2 ± 3.1
pSVGAG + pSVFS5T 6-1 (5)	6.3 ± 4.4
pSVGAG + pSVFS5T 5-14 (2)	9.9 ± 3.7
pSVGAG + pSVFS5TΔMHR (2)	6.9 ± 4.2

[a] Protease-negative versions of the pSVFS5T constructs were used in these experiments.

with pooled HIV-positive human sera. The results are shown in Fig. 3.

The analysis revealed that comparable amounts of $Pr55^{gag}$ were expressed in the cells in each of the different transfections and that comparable amounts of $Pr55^{gag}$ were released into the medium in each case. Thus, within the limits of sensitivity of the assay, there appears to be no significant difference in the efficiency of release of $Pr55^{gag}$ into the medium in the presence of the various deletion mutants of $Pr160^{gag\text{-}pol}$. Although long exposures of the blots are shown here to allow for visualization of the $Pr160^{gag\text{-}pol}$ bands (see below), shorter exposures of these blots also showed the same amounts of $Pr55^{gag}$ in each lane.

On the other hand, striking differences were observed for the release of $Pr160^{gag\text{-}pol}$. Although all of the mutant constructs appeared to be expressed in roughly similar amounts within the cells, only the wild-type pSVFS5T and 5-11 mutant were readily observed in the medium. However, the amount of 5-11 mutant precursor was significantly reduced compared with that of the wild type (Fig. 3B, lanes 3 and 4). Only trace amounts of the precursors of the other deletion mutants could be observed in the medium. This suggests that all of the mutants except 5-11 display severe incorporation defects.

In order to measure the incorporation of $Pr160^{gag\text{-}pol}$ precursors into particles in a more quantitative way, we decided to determine RT levels in particles produced from cells transfected with pSVGAG and the protease-negative variants of the $Pr160^{gag\text{-}pol}$ deletion mutants. In this way we could use RT activity as a measure of precursor incorporation. The experiment was performed in the context of an inactive protease to rule out any possible effect that variation in protease levels within each particle might have on RT activity. Previous studies have shown that the HIV RT is active, albeit at lower levels, when expressed in the context of an inactive protease (26).

To do this, CMT3-COS cells were transfected with pSVGAG or pSVFS5T constructs, individually or together. Particles were recovered from the medium at 72 h posttransfection, and aliquots were tested for RT activity. A summary of the results from several experiments is shown in Table 1. To enable a direct comparison of data from the different experiments, the results were in each case normalized relative to the level of RT incorporation into particles obtained with the wild-type $Pr160^{gag\text{-}pol}$-producing plasmid (pSVFS5T). The results demonstrated that no significant RT activity could be detected in the medium when the $Pr160^{gag\text{-}pol}$-expressing plasmids were transfected individually. Also, most of the deletion mutants produced less than 10% of the wild-type levels of RT when cotransfected with pSVGAG. However, when pSVFS5T 5-11 was coexpressed with pSVGAG, about 50% of the wild-type levels of RT was observed in particles.

These results thus correlated well with the p24 and Western blot data, and taken together, the results clearly demonstrated that the 5-11 deletion mutant is efficiently incorporated into particles, compared with the other constructs. Comparison of the sequences of the various mutants revealed that the 5-11 mutant retained the MHR, which all of the other mutants lacked (Fig. 1). Since this region is highly conserved among retroviral capsid proteins, it seemed likely that the MHR could be involved in the interaction of $Pr160^{gag\text{-}pol}$ with $Pr55^{gag}$. This hypothesis was strengthened by the fact that deletion of the MHR alone resulted in significantly reduced levels of p24 in the medium (Fig. 2D, lane 2) as well as reduced incorporation of Gag-Pol into particles (Fig. 3B, lane 8; Table 1). Thus, removal of the MHR resulted in particles with greatly reduced amounts of the Gag-Pol precursor. In contrast, a large deletion in p24 that did not affect the MHR (5-11), had relatively modest effects on Gag-Pol incorporation.

Effect of deletions in the capsid region on particle formation by $Pr55^{gag}$. The above-described experiments all examined the effect of deletion mutations within the context of $Pr160^{gag\text{-}pol}$. It was also of interest to test the effect of capsid deletions on the ability of $Pr55^{gag}$ to form released virus particles. To do this, the previously described deletions were moved into the context of $Pr55^{gag}$ by swapping appropriate restriction fragments between the $Pr160^{gag\text{-}pol}$- and $Pr55^{gag}$-producing plasmids. CMT3-COS cells were then transfected with these deletion mutants together with pCMVrev, and cells and media were harvested at 72 h posttransfection. Particles were prepared by pelleting through 20% sucrose. The pelleted particles and cells were analyzed on Western blots as described above (Fig. 4A and B). In these experiments, the appropriate bands were also quantitated by laser densitometry.

Figure 4A shows that all of the mutant plasmids expressed Gag precursors of the expected sizes. The wild-type and ΔMHR plasmids also expressed smaller products which could have resulted from proteolytic degradation or internal initiation. Similar species have been described by other investigators (6, 28). In several independent transfections, the 5-11 deletion mutant seemed to give slightly lower levels of protein than other mutants. This could be due to decreased stability of the protein or differential immune reactivity.

Analysis of the particles isolated from the medium (Fig. 4B) again showed the presence of the appropriate-size precursors. However, the 6-1 protein (Fig. 4B, lane 7) appeared to be present in greatly reduced amounts relative to those of the others. Again, several lower-molecular-mass species could be detected in both the wild-type and ΔMHR lanes. The efficiency of particle formation was determined by comparing the ratios of the amounts of precursor protein found in particles (as determined by laser densitometry) to those found in cells. This method of quantitation compensated for possible differences in efficiency of transfection, expression, and detection of the various mutant proteins. The results of such an analysis from two independent transfections are shown in Fig. 4C.

All of the deletion mutants, with the exception of 6-1, showed a decrease in the efficiency of particle formation and made particles at 40 to 50% of the wild-type level. Mutant 6-1 produced particles at only approximately 8% of the wild-type level. These results are quite different from those described for

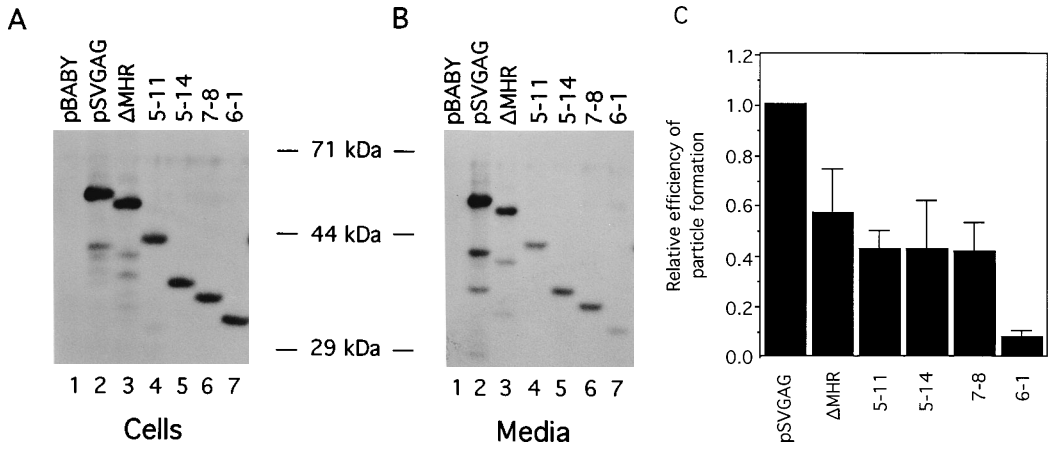

FIG. 4. Immunoblot analysis of proteins produced by and released into the media of cells transfected with various capsid deletion mutants of pSVGAG. CMT3-COS cells were transfected with 5 μg of plasmid of the indicated capsid deletion mutants of pSVGAG. Cells and particles from the media were harvested at 72 h posttransfection and analyzed by the immunoblot procedure as described in the legend to Fig. 3 and in Materials and Methods. (A) Cell lysates; (B) proteins released into the medium in particulate form. The bands were quantitated by using a densitometer and ImageQuant software (Molecular Dynamics). The efficiency of particle formation (C) was determined by calculating the ratio of the band present in the medium to the corresponding band in the cell lysate. Error bars indicate one standard deviation.

the incorporation of $Pr160^{gag\text{-}pol}$ into particles of wild-type $Pr55^{gag}$ in that the 5-11 mutant now behaved similarly to most of the others and the 6-1 mutant behaved differently.

The deletion in 6-1 encompasses the p17-p24 cleavage site at its N terminus, while this region is retained in the 7-8 mutant (Fig. 1). Thus, the poor particle release with 6-1 might have been due to the presence of an assembly domain (32) around the p17-p24 junction, as previously suggested by others (27, 28). To test this hypothesis directly, we made a construct that deleted only the last 9 amino acids of p17 and the first 22 amino acids of the capsid. Surprisingly, this mutant appeared to be fully competent in making particles (data not shown). This suggested that the defect in 6-1 was not due to the absence of amino acids around the p17-p24 junction.

Another possible reason for the poor particle formation obtained with 6-1 was that the deletion disrupted an assembly domain at the C-terminal end of the deletion. Interestingly, the endpoints of the 6-1 and 5-14 deletions differ by only two amino acids at their C termini, which lie just proximal to the p24-p2 cleavage site. The 6-1 mutation could thus have affected an assembly domain present around the p24-p2 junction. The p24-p2 cleavage site in the wild type is KARVL/AEAMS, whereas it is DTGVL/AEAMS in the 6-1 mutant and TARVL/AEAMS in the 5-14 mutant. To test the hypothesis that the sequences around this junction were important for particle assembly, we made another construct in which we mutated the region surrounding the p24-p2 cleavage site in $Pr55^{gag}$ from KARVL/AEAMS to KTGVL/AEAMS. Again, when we tested this construct for particle formation, the mutated Gag precursor was found to be released efficiently into the medium (data not shown). It thus appears that alteration of either region defined by the endpoints of the 6-1 deletion failed to affect particle formation.

To examine the densities of the particles produced by the various deletion mutants, a sucrose density gradient analysis was performed. To do this, pelleted particles from the individual transfections were pooled and layered onto the same sucrose density gradient. Fractions were collected, precipitated with 10% trichloroacetic acid, washed with cold acetone, and subjected to immunoblotting. As a marker for the gradient fractions and also as a control to monitor recovery, glutathione-*S*-transferase protein purified from *E. coli* transformed with the plasmid pGEX-2T was added to each fraction prior to the precipitation step. Since the different deletion mutants could be readily distinguished by size on the immunoblot, this method allowed a direct comparison of the various particle densities. A typical blot from such a gradient is shown in Fig. 5. Analysis of the blot clearly demonstrates that the particles formed with the wild-type $Pr55^{gag}$ produced a peak which banded at a density between 1.185 and 1.205 g/ml. On the other hand, the particles produced by each of the mutants displayed similar densities which appeared to be slightly less than that for the wild type, banding in the gradient between

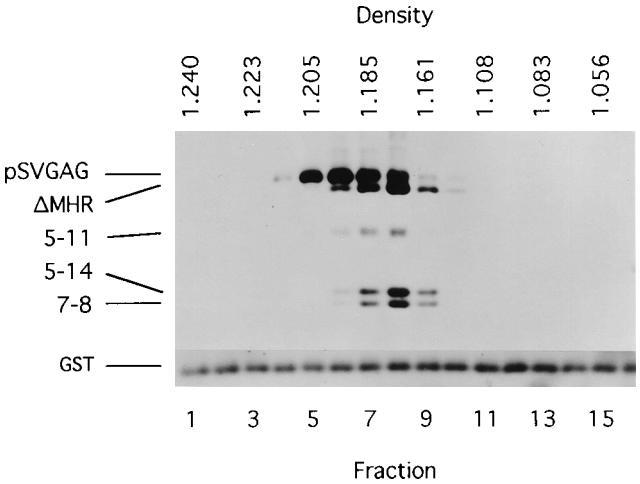

FIG. 5. Sucrose density gradient analysis of capsid deletion mutants of pSVGAG. CMT3-COS cells were individually transfected with the various capsid deletion mutants of pSVGAG. Particles were pelleted from 8 ml of medium through 4 ml of 20% sucrose. Pelleted particles corresponding to one-fourth plate of pSVGAG, one-half plate of ΔMHR, and two plates each of the other mutants were pooled in PBS and centrifuged on a 20 to 60% sucrose gradient at 26,000 rpm for 16 h at 4°C in an SW50.1 rotor in a Beckman ultracentrifuge. Fractions (300 μl) were collected from the bottom of the gradient and analyzed by the immunoblot procedure as described in Materials and Methods. GST, glutathione-*S*-transferase.

TABLE 2. RT levels resulting from cotransfections with pSVGAG or pSVGAG 6-1 and Gag-Pol-producing vectors

Test plasmid (5 μg)	Normalized RT level[a] with particle-producing vector:	
	pSVGAGPOL	pSVGAGPOLprotD25G
pBABY	100	100
pSVGAG	179	120
pSVGAG 6-1	38	53

[a] Derived from duplicate transfections.

1.161 and 1.185 g/ml. This was true even for ΔMHR, which had only a 21-amino-acid deletion.

The 6-1 deletion results in a Gag precursor that displays a dominant negative phenotype for particle production when cotransfected with a wild-type plasmid. As described above, the plasmid pSVGAG 6-1 was found to make particles with an efficiency of less than 10% of that of the wild type (Fig. 4C). It was therefore of interest to examine whether the Gag protein produced from this plasmid could interfere with particle production and/or release of wild-type particles.

To test this, CMT3-COS cells were cotransfected with pCMVrev and pSVGAG 6-1 together with either of the particle-producing vectors pSVGAGPOL-RRE-R and pSVGAG POLprotD25G. pSVGAGPOL-RRE-R and pSVGAGPOL protD25G are identical except that pSVGAGPOLprotD25G carries an inactive protease (26). These plasmids produce both $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$. As controls, these vectors were also cotransfected with a plasmid that produces wild-type $Pr55^{gag}$ (pSVGAG) or the parental vector without any insert (pBABY). The medium from the transfected cells was harvested at 72 h posttransfection, and RT activity was determined as described in Materials and Methods. The RT level obtained in control cotransfections with the pBABY plasmid was in each case set to 100. The results of this experiment are tabulated in Table 2. From the data, it is clear that in each instance transfection with the plasmid that produced wild-type $Pr55^{gag}$ increased the amount of RT present in the medium. In contrast, cotransfection with the plasmid that produced the 6-1 deletion mutant of $Pr55^{gag}$ resulted in RT levels which were 38 to 53% of that of the controls.

Rescue of capsid deletion mutants of $Pr160^{gag\text{-}pol}$ into particles of mutant $Pr55^{gag}$. We next examined the efficiency with which each mutant $Pr55^{gag}$ polypeptide incorporated each of the variously deleted $Pr160^{gag\text{-}pol}$ molecules into particles released into the medium of transfected cells. This was done by coexpressing each $Pr55^{gag}$ precursor polypeptide individually with each of the mutant $Pr160^{gag\text{-}pol}$ precursor polyproteins and assaying the medium for particle-associated RT activity. The results of this experiment are shown in Fig. 6. Once again, the wild-type $Pr55^{gag}$ preferentially incorporated wild-type $Pr160^{gag\text{-}pol}$ and the 5-11 deletion mutant protein compared with the other mutant proteins. In contrast, the $Pr55^{gag}$ mutant proteins showed no preference for the wild-type or the 5-11 deletion Gag-Pol precursors compared with the others. In fact, in some instances mutant forms of $Pr160^{gag\text{-}pol}$ appeared to be incorporated better than the wild type. This was most dramatic with the 7-8 Gag deletion mutant. In this case, the $Pr160^{gag\text{-}pol}$ molecule carrying the 7-8 deletion was incorporated into particles with an efficiency nearly twofold greater than that for the wild-type $Pr160^{gag\text{-}pol}$.

DISCUSSION

We have studied the effects of deletion mutations on the incorporation of the $Pr160^{gag\text{-}pol}$ precursor into virus-like particles by using our previously described simian virus 40-based expression system (26). The ability to express $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ individually allowed us to identify regions required for the interaction of the precursors in virion core morphogenesis. The results show that deletion of the MHR in the capsid region of $Pr160^{gag\text{-}pol}$ reduced the efficiency of incorporation of this precursor into wild-type particles. Deletion of the MHR within the $Pr160^{gag\text{-}pol}$ precursor also appeared to affect proteolytic processing of the wild-type $Pr55^{gag}$ precursor in *trans*. These results thus indicate that the deletion of MHR interferes with the ability of the $Pr160^{gag\text{-}pol}$ precursor to multimerize with the $Pr55^{gag}$ precursor. Our results are consistent with those of a study on the incorporation of Gag–β-galactosidase fusion proteins into particles that mapped the region of interaction to the C-terminal two-thirds of the capsid protein (30).

Several studies have shown that mutations in the capsid region can have an effect on proteolytic processing, probably by altering the ability of $Pr160^{gag\text{-}pol}$ to interact with itself and/or $Pr55^{gag}$. In one study, a series of linker insertions clustered towards the amino terminus of the capsid region appeared to affect the activity of the protease (16), and it was concluded that these mutations probably interfered with multimerization. That study utilized a bacterial expression system making a truncated $Pr160^{gag\text{-}pol}$ containing Gag and protease. Similar conclusions about the abilities of these mutants to multimerize were obtained from studies using a yeast two-hybrid system (15) and also when the same mutants were expressed in the context of a proviral clone (22). In our study, a deletion mutation (5-11) which deleted the region that contained these linker insertion mutations yielded a $Pr160^{gag\text{-}pol}$ that behaved nearly normally. Only when the deletion extended into the MHR was an effect on proteolysis and assembly of $Pr160^{gag\text{-}pol}$ into particles observed. It may be that deletion of this large region of the capsid is less deleterious to assembly than the alterations produced by the point mutations used in the previous study. Our results are consistent with

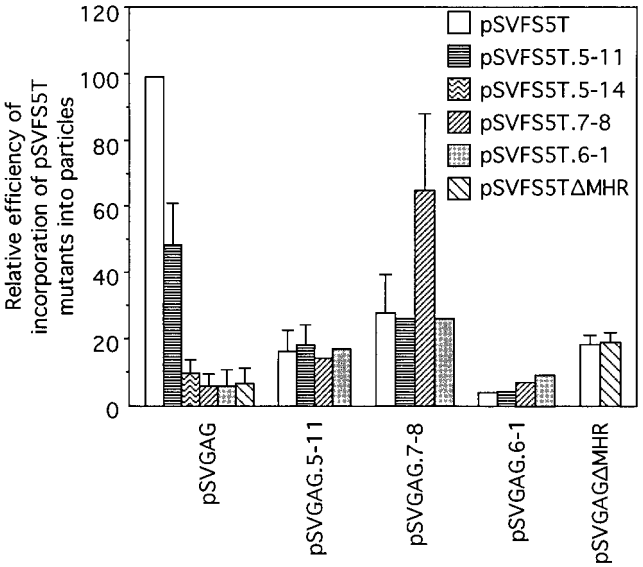

FIG. 6. Incorporation of deletion mutants of $Pr160^{gag\text{-}pol}$ into particles by deletion mutants of $Pr55^{gag}$. CMT3 cells were cotransfected with the different Gag (pSVGAG) and Gag-Pol (pSVFS5T) deletion mutants shown. Particles were collected and assayed for RT activity as described in Materials and Methods. In any given experiment, the results obtained were normalized to the value obtained for the cotransfection with wild-type Gag and Gag-Pol, which was set at 100. Error bars indicate one standard deviation.

those of other studies that have shown that mutations in the MHR appear to affect processing of the precursor polyproteins and precursor protein interactions in the context of the provirus (2, 17).

Our results with the capsid deletion mutations in the context of $Pr55^{gag}$ indicate that the majority of the capsid region, including the MHR, could be removed without affecting budding and release of the $Pr55^{gag}$ precursor, although the particles produced were less dense than wild-type particles. Surprisingly, an intact MHR in $Pr160^{gag\text{-}pol}$ was no longer needed for incorporation of mutated $Pr160^{gag\text{-}pol}$ molecules into these mutated particles (Fig. 6). Mutant precursors were incorporated into mutant particles as efficiently as or better than the wild-type Gal-Pol precursor.

Another study (3), which used a genetic assay to detect multimerization of Gag precursors, also showed that mutant Gag precursors were able to interact better with mutant precursors than with the wild type. The authors proposed that this was the result of a "spacing" problem between multimerization domains. Our results are consistent with their hypothesis, and we can now extend this idea as follows. A deletion mutation in $Pr160^{gag\text{-}pol}$ would be expected to create a precursor that is somewhat impaired in its ability to multimerize. In the presence of wild-type $Pr55^{gag}$ precursors, the mutant $Pr160^{gag\text{-}pol}$ molecules would be excluded from entering into particles because they compete less well in the assembly process than the $Pr55^{gag}$ molecules which form the particle. When mutant $Pr55^{gag}$ molecules form the budding particle, however, the mutant $Pr160^{gag\text{-}pol}$ molecules have a greater chance to become incorporated, since they can now compete better with the $Pr55^{gag}$-$Pr55^{gag}$ interactions.

Residues throughout the capsid protein have been implicated as being critical for particle formation (2, 3, 10, 11, 14, 20, 23, 24). In contrast, others (22, 29) have concluded from several linker insertion and deletion mutations that the capsid region was not essential for particle formation although it was essential for infectivity. Our results showing that deletions between residues 23 and 228 of the capsid protein do not affect particle budding and release are in agreement with those of these latter studies. Our results also agree well with those of studies carried out with avian retroviruses in which it was shown that large deletions in the Gag protein, including the capsid region, did not affect particle formation (31). It should be noted that our constructs have been tested only in the CMT3-COS cell system. The use of different expression systems by various investigators and different clones of HIV-1 may account for some of the discrepancies in the results.

One deletion mutant (6-1) that lacked the C-terminal nine residues of p17 (matrix protein) and almost the entire p24 capsid region was only about 8% as efficient as wild-type $Pr55^{gag}$ in particle production. This mutant was able to inhibit particle production in *trans*, consistent with the phenotypes of other previously described mutants that contained deletions extending across the p17-p24 junction (21, 27). Comparison of the sequence of the 6-1 mutant with those of our other deletion mutants that were efficient particle producers suggested two regions that could be important for assembly: one across the p17-p24 cleavage site and the other near the p24-p2 cleavage site. Previous studies had also implicated these regions as being important in assembly (7, 27, 28). Surprisingly, however, deletion of either of these putative assembly domains from an otherwise wild-type precursor did not affect particle production. Taken together, these results suggest the presence of multiple or redundant assembly domains throughout the $Pr55^{gag}$ precursor.

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