JOURNAL OF VIROLOGY, Nov. 1995, p. 6810–6818 Vol. 69, No. 11
0022-538X/95/$04.00+0

$p6^{Gag}$ Is Required for Particle Production from Full-Length Human Immunodeficiency Virus Type 1 Molecular Clones Expressing Protease

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Received 21 April 1995/Accepted 20 July 1995

The human immunodeficiency virus type 1 (HIV-1) Gag protein precursor, $Pr55^{Gag}$, contains at its C-terminal end a proline-rich, 6-kDa domain designated p6. Two functions have been proposed for p6: incorporation of the HIV-1 accessory protein Vpr into virus particles and virus particle production. To characterize the role of p6 in the HIV-1 life cycle and to map functional domains within p6, we introduced a number of nonsense and single and multiple amino acid substitution mutations into p6. Following the introduction of the mutations into the full-length HIV-1 molecular clone pNL4-3, the effects on Gag protein expression and processing, virus particle production, and virus infectivity were analyzed. The production of mutant virus particles was also examined by transmission electron microscopy. The results indicate that (i) p6 is required for efficient virus particle production from a full-length HIV-1 molecular clone; (ii) a Pro-Thr-Ala-Pro sequence, located between residues 7 and 10 of p6, is critical for virus particle production; (iii) mutations outside the Pro-Thr-Ala-Pro motif have little or no effect on virus assembly and release; (iv) the p6 defect is manifested at a late stage in the budding process; and (v) mutations in p6 that severely reduce virion production in HeLa cells also block or significantly delay the establishment of a productive infection in the CEM(12D-7) T-cell line. We further demonstrate that mutational inactivation of the viral protease reverses the p6 defect, suggesting a functional linkage between p6 and the proteolytic processing of the Gag precursor protein during the budding of progeny virions.

Retroviral Gag proteins are initially synthesized as a polyprotein precursor which is proteolytically processed by the viral protease (PR) to produce the mature matrix (MA), capsid (CA), and nucleocapsid (NC) Gag proteins (33). In the case of human immunodeficiency virus type 1 (HIV-1), the Gag precursor and the MA, CA, and NC have been designated, on the basis of their apparent molecular weights, $Pr55^{Gag}$, p17, p24, and p7, respectively. A number of studies have demonstrated that the expression of retroviral Gag precursors, in the absence of other virally encoded proteins, is sufficient for the formation of immature, noninfectious virus-like particles (for a review, see reference 70).

The MA, CA, and NC domains of retroviral Gag protein precursors are each involved in various aspects of virus assembly. Mutations in the myristic acid acceptor sequence in the N-terminal MA domain of Gag block virus assembly by perturbing the association of the Gag precursor with cellular membranes (4, 7, 15, 19, 23, 42, 47, 56, 64). Single amino acid changes in the central portion of the HIV-1 MA domain either block virus assembly or redirect assembly to sites within the cytoplasm (15), and deletions in the MA of murine leukemia virus (24), Mason-Pfizer monkey virus (52, 53), Rous sarcoma virus (71), and HIV-1 (5, 63, 75) interfere with virus assembly. Single amino acid substitutions, multiple amino acid substitutions, and deletions in MA can also block the incorporation of HIV-1 envelope glycoproteins into virus particles (9, 14, 74). Mutations in the CA of murine leukemia virus (17, 35, 57), Mason-Pfizer monkey virus (59), and HIV-1 (5, 11, 40, 62) also disrupt virion formation. The NC is involved in RNA encapsidation into virus particles (10, 12, 18, 34, 43, 51) and in the formation of virions with normal structure and density (2, 16, 25, 42, 46, 65).

In addition to MA, CA, and NC, retroviruses encode a variety of other Gag proteins, the functions of which, in many cases, have not been fully elucidated (for a review, see reference 70). For example, the *gag* genes of the avian retrovirus Rous sarcoma virus and of murine leukemia virus contain sequences, mapping between the MA and CA domains, that encode proteins of 12 and 10 kDa, respectively. The type D Mason-Pfizer monkey virus encodes two proteins of 16 and 12 kDa, specified by sequences located between the MA and CA domains, and a protein of 4 kDa from a sequence situated at the C terminus of Gag. In the lentiviruses HIV-1, HIV-2, and the simian immunodeficiency viruses, a proline-rich, 6-kDa domain, designated p6, is located at the C-terminal end of Gag.

Two functions have been proposed for the lentiviral $p6^{Gag}$ protein. p6 appears to be required for the incorporation of the HIV-1 accessory protein Vpr and the HIV-2 accessory protein Vpx into virus particles (30, 37, 48, 73). It has also been reported that p6 plays a role in virus particle production at a late stage in the budding process (20). This latter function is still in question, however, as a number of groups, working with a variety of systems, have not observed a requirement for p6 in HIV-1 or HIV-2 particle production (21, 22, 25, 37, 38, 48, 55).

To assess the role of the HIV-1 $p6^{Gag}$ protein in virus particle production and to identify residues involved in p6 function, we introduced a number of truncation and amino acid substitution mutations into p6. The effects of the mutations on Gag expression and processing, virus particle production, and virus replication were analyzed in the context of a full-length

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HIV-1 molecular clone. Virus assembly and virion morphology were also examined by transmission electron microscopy. Our results confirm a role for p6 late in the virus assembly process and define a domain in p6 required for virion production. We also demonstrate that mutational inactivation of the viral protease (PR) reverses the effect of p6 mutations on virus particle production, suggesting a link between p6 and PR functions.

MATERIALS AND METHODS

Site-directed mutagenesis. The 1.2-kbp *Sph*I-*Bal*I fragment (nucleotide positions 1442 to 2619 [45]) from the full-length infectious molecular clone pNL4-3 (1) was subcloned into M13mp18, and oligonucleotide-directed mutagenesis was performed as described previously (32). A 473-bp *Apa*I-*Ppu*MI fragment (nucleotides 2006 to 2483) carrying the correct mutation was introduced back into pNL4-3. The entire *Apa*I-*Ppu*MI fragment was sequenced to confirm the presence of the desired changes and to determine that no additional changes had been introduced during mutagenesis.

Cell culture, transfections, infections, and RT assays. HeLa and CEM(12D-7) cells (54) were cultured as previously described (13). HeLa cells were transfected by calcium phosphate precipitation (13); CEM(12D-7) cells were transfected by the DEAE-dextran method (13). CEM(12D-7) cells were infected with equivalent amounts (normalized for reverse transcriptase [RT] activity) of input virus as previously described (15). RT assays were performed as reported previously (68), with the addition of 0.8 mM EDTA to the reaction cocktail. The method for measuring the release of particle-associated RT activity into the medium of transfected HeLa cells has also been described previously (15).

Metabolic labeling and radioimmunoprecipitation. The procedure used for metabolic labeling of transfected HeLa cells was described previously (13). Briefly, at approximately 48 h posttransfection, cells from transfected cultures were plated in 25-cm^2 tissue culture flasks in 2 ml of Met-free or Cys-free RPMI medium supplemented with 10% fetal bovine serum. [^{35}S]Met or [^{35}S]Cys (500 μCi) was added, and the cells were incubated overnight at 37°C. After the labeling period, the culture media were harvested, and unattached cells were pelleted with a brief spin in a microcentrifuge. The cell-free supernatants were filtered through a 0.45-μm-pore-size filter, and virion-associated material was harvested by pelleting for 30 min at 35,000 rpm in an SW50.1 rotor (Beckman).

Pulse-chase experiments were performed in suspension following the removal of transfected cells by scraping at approximately 40 h posttransfection. Cells from two transfected 60-mm-diameter plates for each clone were combined and transferred to 50-ml conical tubes. The remaining steps were performed as described previously (67). At the end of pulse and chase periods, the cells were pelleted and media were filtered. The virion-associated material was pelleted from the filtered cell supernatant in a microcentrifuge as previously described (15). The cell-free, virion-free supernatant fractions from the microcentrifugation were saved after each spin.

Immunoprecipitation was done as described previously (13, 66) with AIDS patient sera obtained from the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program (human HIV immune globulin; catalog no. 192). All precipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis in 10 or 12% acrylamide-AcrylAide (FMC) gels. The gels were fixed in methanol and acetic acid, treated with 1 M salicylic acid, dried at 100°C, and placed on X-Omat AR film (Eastman-Kodak).

Sucrose gradients. The analysis of labeled transfected-cell supernatants by sucrose gradient centrifugation was performed essentially as described previously (67). Media harvested from metabolically labeled cells were spun briefly in a microcentrifuge and filtered, as described above, to remove unattached cells and cell debris. Aliquots (500 μl) of the filtered medium were layered onto a 10 to 60% (wt/vol) discontinuous 4-ml sucrose gradient and were centrifuged in an SW50.1 rotor (Beckman) at 35,000 rpm for 90 min at 25°C. Twelve fractions of equal volume were collected from the top of the tube. Fraction 1 thus represents the material of lowest density; fraction 12 represents the most-dense material from the bottom of the gradient. The collected fractions were immunoprecipitated and analyzed by SDS-PAGE as described above.

Transmission electron microscopy. The procedures used to prepare and examine transfected cells by electron microscopy have been reported previously (15).

RESULTS

Mutagenesis of the HIV-1 p6^{Gag} protein. An amino acid sequence comparison of the p6 domains of Gag proteins from a number of HIV-1 isolates (45) revealed the presence of several highly conserved domains within p6, including a Pro-Thr-Ala-Pro (PTAP) motif between p6 residues 7 and 10 and a Leu-Phe-Gly-Ser-Asp sequence between residues 44 and 49 (45) (Fig. 1). To investigate potential functions of p6 and to determine the effects of mutations in highly conserved residues within p6, we introduced a number of mutations in the p6 open reading frame (Fig. 1A and B). Three truncation mutations were constructed by introducing stop codons at p6 residues 1 (L1Term), 25 (S25Term), or 36 (Y36Term). Single amino acid changes were introduced at highly conserved residues 2 (Q-2→A [Q2A]), 4 (R4S), 6 (E6G), 7 (P7L), 8 (T8I), 9 (A9R), 10 (P10L), 24 (P24L), 41 (L41R), 45 (F45C), 46 (G46D), and 48 (D48A). A 4-amino-acid substitution mutation (PTAP$^-$) in the highly conserved PTAP sequence was also made. Whenever possible, we avoided introducing changes into the overlapping *pol* open reading frame; however, for the L1Term and G46D mutants, such changes were unavoidable. To determine what effect, if any, the *pol* changes might elicit, the pol/LT and pol/R mutations were introduced into *pol* in the absence of p6 changes (Fig. 1B). All of the mutations were transferred to the full-length HIV-1 molecular clone pNL4-3 (1) as described in Materials and Methods. Mutations analogous to L1Term, pol/LT, and Y36Term have been reported previously (20).

Effects of p6 mutations on virus particle production. To determine whether p6 is required for virus particle production, we transfected HeLa cells in parallel with wild-type pNL4-3 or p6 mutant derivatives of pNL4-3. At 2 days posttransfection, the amount of virion-associated RT activity released into the medium of transfected cells was measured (Fig. 2). The L1Term mutation resulted in a greater than 20-fold reduction in the release of virion-associated RT activity. This reduction was due to the p6 truncation and not to the mutations in *pol*, since the pol/LT mutant, which contains the *pol* mutations alone, produced wild-type levels of RT activity. Truncation of p6 at amino acid 25 (S25Term) reduced RT production about twofold, whereas truncation of p6 at residue 36 caused only a slight reduction in the release of particle-associated RT activity, in agreement with a previous report (20). The domain responsible for the effect of p6 on virus particle production therefore appeared to reside near the N terminus of p6. Consistent with this conclusion, mutations in highly conserved residues near the C terminus of p6 (L41R, F45C, G46D, and D48A) had little effect on virus particle production, whereas single amino acid changes (P7L, T8I, A9R, and P10L) or a multiple amino acid change (PTAP$^-$) in the highly conserved PTAP sequence resulted in a dramatic reduction in virus particle release. Other N-terminal point mutations (Q2A, R4S, E6G, and P24L) did not significantly alter virus particle production. These data suggest that p6 is required for virion production and that the PTAP motif between p6 residues 7 and 10 is critical for this function. These results are in agreement with those of a previous report (20), which demonstrated that simultaneous mutation of the two Pro residues at p6 positions 10 and 11 significantly reduced particle production from transfected COS-7 cells.

Expression of cell- and virion-associated mutant p6^{Gag} proteins. To exclude the possibility that the reduction in virion-associated RT observed for the p6 mutants (Fig. 2) reflected a defect in RT activity in virions rather than a failure to produce virus particles, we examined by radioimmunoprecipitation the cell-associated and virion-associated proteins expressed by wild-type- and mutant-transfected HeLa cells (Fig. 3). The results of this analysis demonstrated comparable levels of cell-associated Pr55^{Gag} expression in HeLa cells transfected with wild-type or p6 mutant (P7L, T8I, A9R, P10L, PTAP$^-$, and L1Term) molecular clones. An examination of the original autoradiogram shown in Fig. 3A revealed an increase in the ratio of p25 to p24 with these p6 mutants, consistent with a previous report (20). Variations in the mobility of the Gag precursor Pr55^{Gag} also resulted from the p6 truncation muta-

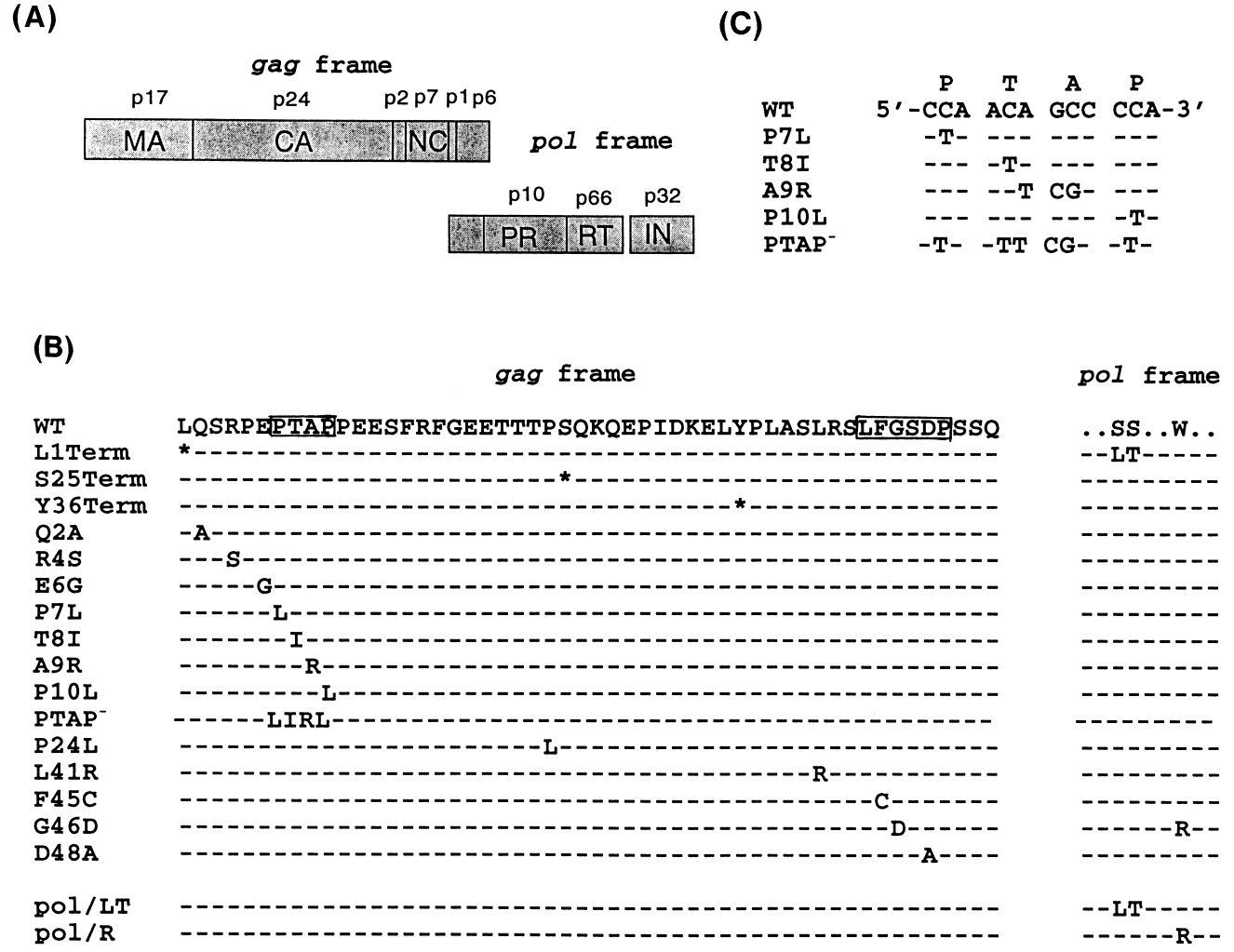

FIG. 1. Mutagenesis of HIV-1 p6. (A) Organization of the matrix (MA), capsid (CA), p2, nucleocapsid (NC), p1, p6, protease (PR), RT, and integrase (IN) domains of the HIV-1 *gag* and *pol* open reading frames. (B) Predicted amino acid sequences of wild-type (WT) and mutant p6 and the overlapping *pol* open reading frames. The p6 sequences highly conserved among HIV-1 isolates are boxed. Dashes indicate amino acids identical to those of the wild type; asterisks indicate the positions of stop codons. Residues are numbered with respect to the first amino acid after the cleavage site between p1 and p6, or the first amino acid in the *pol* frame (45). (C) Nucleotide sequences encoding the wild-type PTAP domain and the PTAP domain mutants.

tion (L1Term) and from several of the PTAP domain mutations.

In contrast to the comparable levels of cell-associated protein expression (Fig. 3A), the amounts of virion-associated protein released into the medium of cells transfected with the p6 mutants were markedly reduced (Fig. 3B). These results, in agreement with those obtained by analyzing particle-associated RT activity (Fig. 2), confirm that truncation of p6 or mutations within the PTAP motif of p6 significantly reduced virus particle production from transfected HeLa cells.

The proteins released into the medium of transfected, metabolically labeled HeLa cells were also examined by ultracentrifugation through a 10 to 60% (wt/vol) sucrose gradient (see Materials and Methods). Twelve fractions of equal volume were collected from the top of the gradient and were immunoprecipitated with AIDS patient sera. Free proteins were present in the top gradient fractions, and virions were found primarily in fractions 6 to 8, as reported previously (67). In the wild-type gradient (Fig. 3C, top panel), significant amounts of both free and virion-associated proteins were observed. In the $PTAP^-$ gradient (Fig. 3C, bottom panel), the amounts of free HIV-1 proteins were comparable to those observed with the wild type (compare fractions 1 to 3 in the top and bottom panels of Fig. 3C). However, consistent with the data presented in Fig. 3B, the amount of virion-associated protein present in the $PTAP^-$ gradient (compare fractions 6 to 8 in the top and bottom panels of Fig. 3C) was markedly reduced.

To analyze further the effect of p6 mutations on the kinetics of Gag protein synthesis, processing, and virus particle production, we performed a pulse-chase analysis of HeLa cells transfected with either wild-type pNL4-3 or the $PTAP^-$ mutant (Fig. 4). HeLa cells transfected with wild-type pNL4-3 or the $PTAP^-$ mutant were metabolically labeled with [^{35}S]Cys for 30 min and then chased in unlabeled medium for 2, 4, 8, 20, or 30 h. Three fractions were immunoprecipitated with AIDS patient sera (Fig. 4): the cell-associated fraction, the virion-associated fraction obtained by pelleting virions from the labeled cell medium in a microcentrifuge, and the cell-free, virion-free fraction obtained from the supernatant of the microcentrifuge

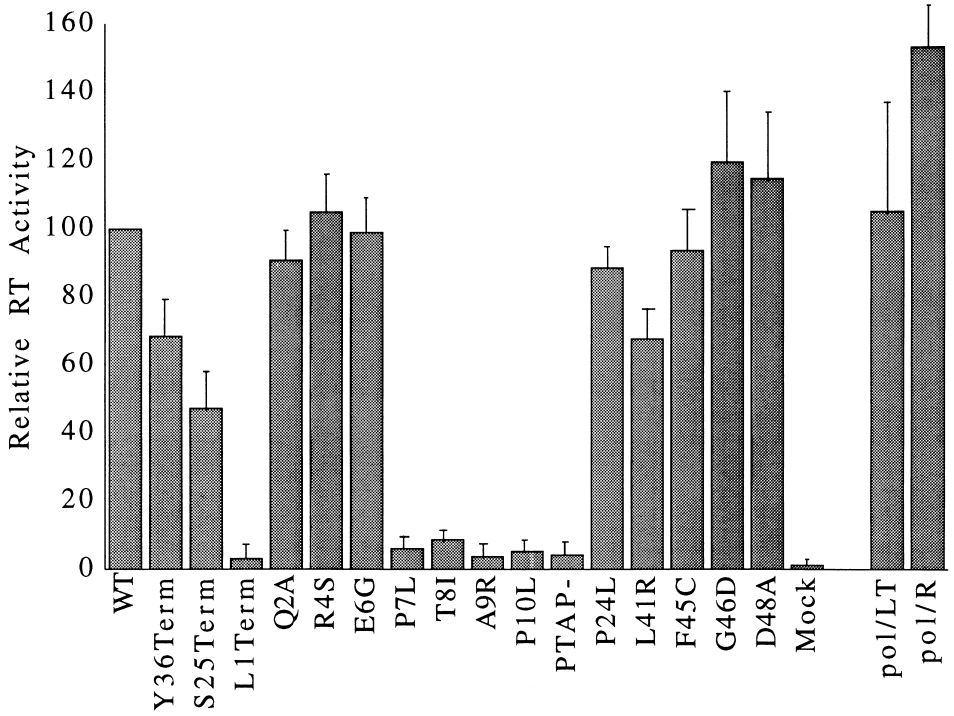

FIG. 2. Production of virion-associated RT activity from HeLa cells transfected with the p6 and *pol* mutants. HeLa cells were transfected with wild-type (WT) or mutant pNL4-3 molecular clones as indicated. Virions were pelleted from the transfected-culture supernatant and were analyzed for RT activity as described in reference 15 and in Materials and Methods. The data represent the means from at least three experiments; error bars represent standard deviations.

spin. In cells transfected with wild-type pNL4-3, expression of Pr55^{Gag} was evident during the pulse period, and p24 and p17 were detected during the chase (Fig. 4, top left panel). Release of p24 and p17, much of which was particle associated, occurred during the chase period (Fig. 4, left middle and bottom panels). The intracellular expression and processing of the PTAP$^-$ mutant Pr55^{Gag} were normal, except that an increased p25/p24 ratio was observed. As anticipated from the data presented in Fig. 2 and 3, there was no accumulation of virion-associated proteins in the medium of mutant-transfected cells (Fig. 4, right middle panel). The majority of p24 and p17 released into the medium of mutant-transfected cells was not virion associated but rather was present in the cell-free, virion-free fraction (Fig. 4, bottom panel).

Ultrastructural analysis of virus assembly by electron microscopy. Compared with wild-type transfectants, in which all of the characteristic stages of HIV-1 morphogenesis were seen, transmission electron microscopic examination of HeLa cells transfected with the p6 mutant molecular clones revealed an accumulation of late budding forms tethered to the plasma membrane (data not shown). This phenotype has been previously reported for HIV-1 p6 mutants (20). The few cell-free particles observed had an immature morphology characterized by an uncondensed, spherical nucleoid. These results are consistent with the defect in virus particle release noted above (Fig. 2 to 4) and suggest that the p6 mutant phenotype is manifested at a very late stage in the budding process, immediately prior to release from the plasma membrane.

Effects of p6 mutations on virus infectivity. To examine the effect of p6 mutations on the establishment of a productive infection, we transfected the mutants, in parallel with wild-type pNL4-3, into the CEM(12D-7) T-cell line (Fig. 5). The S25Term and Y36Term mutations caused a delay to peak RT production of several days relative to that of the wild type. Single amino acid changes outside the PTAP domain (e.g., Q2A, R4S, E6G, P24L, L41R, F45C, G46D, and D48A) had no significant effect on the kinetics of virus replication. The L1Term and PTAP$^-$ mutations resulted in a complete block in the establishment of a productive infection, as no progeny virus was detected in either transfected culture for up to 3 months posttransfection (Fig. 5; data not shown). Single amino acid changes in the PTAP domain resulted in significant delays to peak RT production relative to that of the wild type. These results suggest that p6 mutations cause defects in virus particle production not only in HeLa cells (Fig. 2 to 4) but also in the CEM(12D-7) T-cell line.

To determine whether the appearance of RT activity in the cultures transfected with the P7L, T8I, A9R, or P10L mutant was due to the emergence of virus revertants, virus from wild-type- and mutant-transfected cultures was harvested around the peak of RT production (Fig. 5D) and was used to infect fresh CEM(12D-7) T cells. The repassaged virus derived from cultures transfected with the P7L, T8I, and P10L mutants replicated with kinetics comparable to those of the wild type; virus derived from cultures transfected with the A9R mutant replicated with a 1-week lag relative to the wild type but considerably faster than the 6-week delay observed with the original mutant (Fig. 5E). The marked increase in the kinetics of virus spread observed upon repassage suggested that the virus production observed in the cultures transfected with the P7L, T8I, A9R, and P10L mutants was due to the emergence of viral revertants. We currently do not know whether these revertants arose as a result of changes back to the original, wild-type sequence or as a result of second-site changes. The fact that the A9R repassaged virus grew with kinetics which were much more rapid than those of the original mutant but slower than those of the wild type suggests that second-site change(s) may have occurred in the cultures transfected with the A9R mutant.

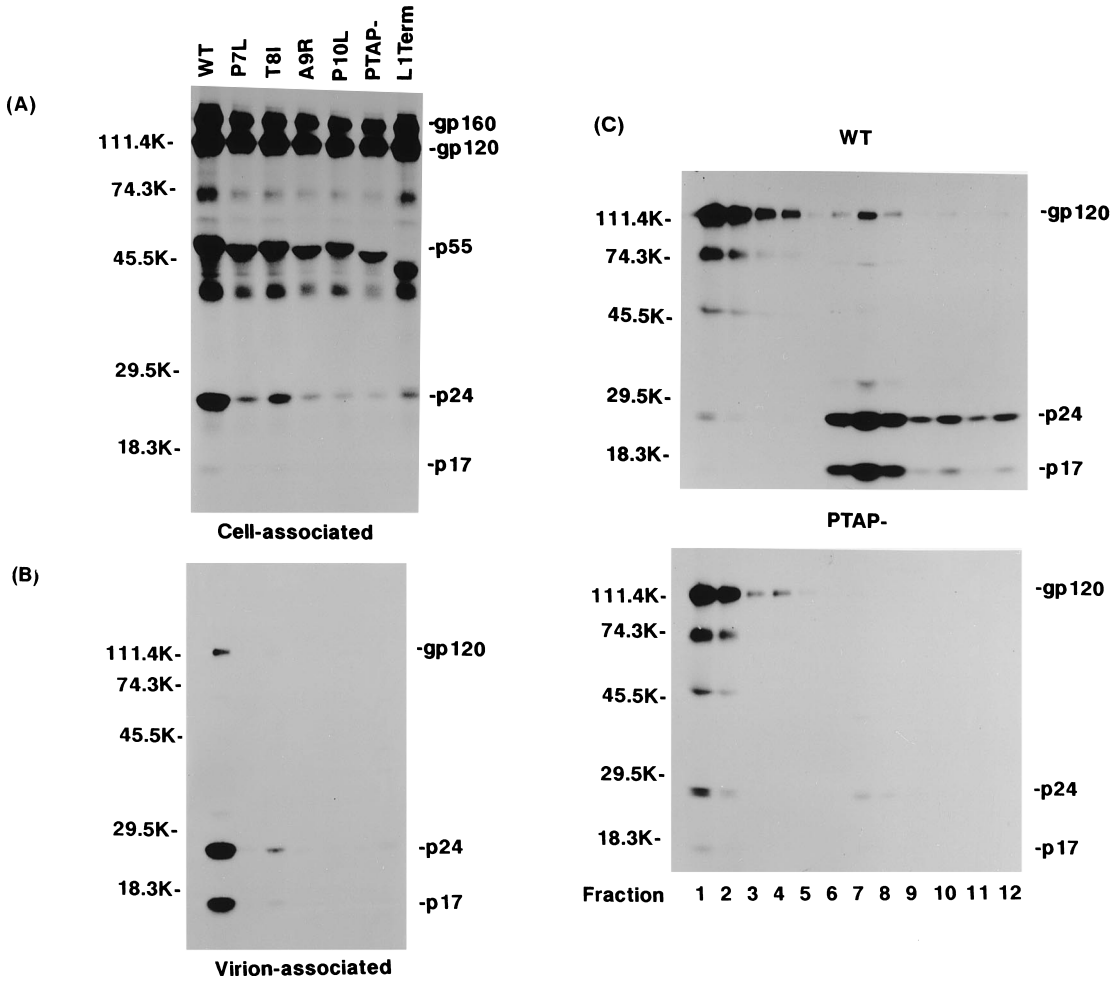

FIG. 3. Radioimmunoprecipitation analysis of p6 mutants. HeLa cells transfected with wild-type (WT) or mutant pNL4-3 molecular clones were metabolically labeled with [^{35}S]Cys. Cell-associated (A), virion-associated (B), and sucrose gradient-fractionated supernatant (C) proteins were immunoprecipitated with AIDS patient sera and resolved on SDS–12% polyacrylamide-AcrylAide gels (see Materials and Methods). The mobilities of the envelope glycoprotein precursor (gp160), surface envelope glycoprotein (gp120), Pr55^{Gag} precursor (p55), CA (p24), and MA (p17) are indicated on the right; positions of molecular weight standards are shown on the left.

Mutation of PR reverses the effect of p6 mutations on particle production. As mentioned in the introduction, even in the absence of the viral PR, the expression of retroviral Gag proteins results in the production of virus particles (70). However, several studies have indicated that perturbation of Gag precursor processing can severely impair the assembly of infectious virions (27, 28, 39, 42, 50). It is noteworthy that many studies which concluded that p6 played no role in virus particle production used expression systems in which PR was absent (21, 22, 25, 37, 38, 55). Furthermore, from our experiments, several observations suggested a link between PR activity and the p6 defect: (i) the ratio of p25 to p24 was higher in p6 mutant-transfected cells than in wild-type-transfected cells, implying an alteration in the pattern of Pr55^{Gag} processing; (ii) the p6-defective particles had an immature morphology as determined by transmission electron microscopy (data not shown), reminiscent of particles formed in the absence of PR (29, 49, 72); and (iii) the p6 defect appeared to manifest itself at a late stage in the assembly and budding process when PR is most active (26).

To understand the mechanism by which p6 mutations block virus particle production in our assays and to determine whether PR function was required for the effect of p6 mutations to be revealed, we examined PTAP$^-$ virus particle production in the absence of PR activity. An Asp→Ala substitution at PR residue 25, which was previously shown to block PR activity (36), was introduced in the context of both the wild-type pNL4-3 (PR$^-$) and the PTAP$^-$ mutant (PR$^-$/PTAP$^-$). Following transfection of PR$^-$ or PR$^-$/PTAP$^-$ clones into HeLa cells, viral proteins were labeled, immunoprecipitated, and analyzed by SDS-PAGE. As anticipated for a PR mutant, no p24 or p17 could be detected (data not shown). Interestingly, the ratio of cell-associated to particle-associated Pr55^{Gag} produced by the PR$^-$/PTAP$^-$ mutant was comparable to that produced by the PR$^-$ mutant (Fig. 6, right panel), an observation that was confirmed by Phosphorimager analysis of the bands shown in Fig. 6 (data not shown). This finding demonstrated that the PR mutation reversed the effect of the p6 mutation on virus particle production; p6 was not required for virus particle assembly and release in the absence of PR activity. We also noted that the level of virion gp120 was reduced in the PR$^-$/PTAP$^-$ mutant relative to the PR$^-$ mutant (Fig. 6, right panel). This effect was less pronounced, however, than

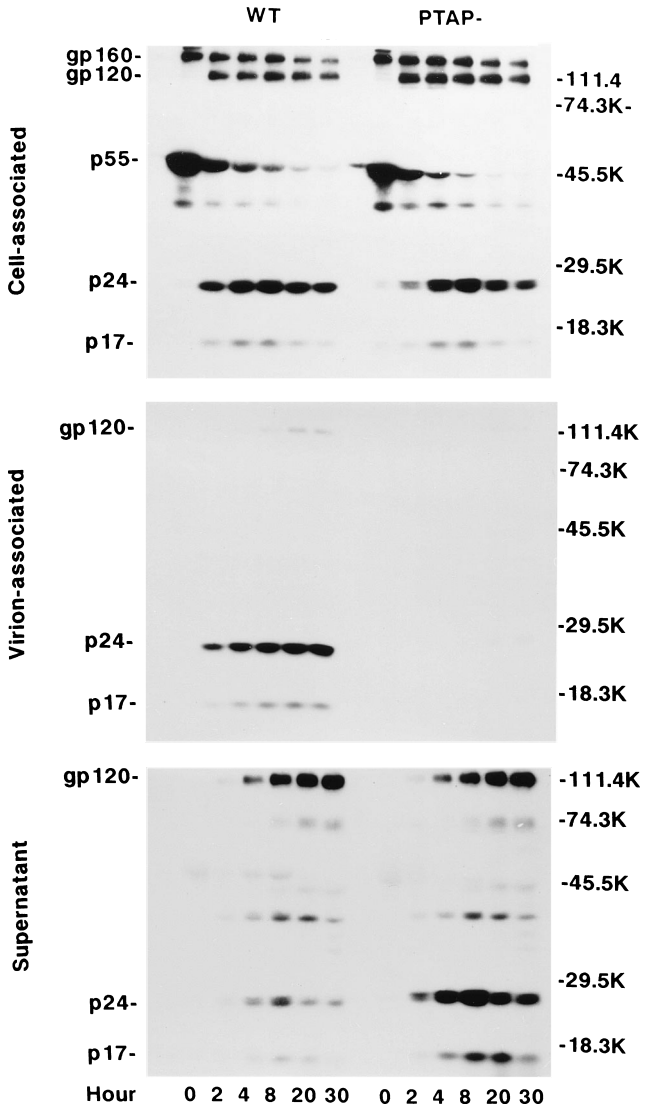

FIG. 4. Pulse-chase analysis of viral protein expression and release from transfected HeLa cells. HeLa cells transfected with wild-type (WT) or PTAP$^-$ mutant pNL4-3 molecular clones were pulse-labeled with [^{35}S]Cys for 30 min and chased in unlabeled medium for the times indicated. At each time point, equal fractions were removed and separated into cell-associated, virion-associated, and supernatant fractions as described in Materials and Methods. Lysates from each fraction were immunoprecipitated with AIDS patient sera. The mobilities of the envelope glycoprotein precursor (gp160), surface envelope glycoprotein (gp120), Pr55^{Gag} precursor (p55), CA (p24), and MA (p17) are indicated on the left; positions of molecular weight standards are shown on the right.

that we observed previously with specific single amino acid changes in MA (14).

DISCUSSION

Conflicting reports concerning the role of p6 in virus particle production have been published. Gottlinger et al. (20) reported that mutations in p6, in the context of a full-length HIV-1 molecular clone, blocked or reduced virus particle production from transfected cells. A number of other studies, however, found that expression of p6-deleted HIV-1 or HIV-2 Gag proteins resulted in efficient particle formation (21, 22, 25, 37, 38, 48, 55). In most of these latter studies, particle production was driven by the expression of Gag in the absence of other virally encoded proteins (21, 22, 25, 37, 38, 55).

In this study, we introduced a number of single and multiple amino acid substitution and nonsense mutations into HIV-1 p6 in the context of a full-length, infectious HIV-1 molecular clone and analyzed the effects on the virus life cycle. Our results indicate that p6 is required for efficient virus particle production from HeLa cells transfected with a full-length HIV-1 molecular clone and that a PTAP sequence, located between p6 residues 7 and 10, is critical for efficient virus particle production. The p6 defect occurs at a late stage in the budding process, and mutations in p6 that severely reduce virion production in HeLa cells also block or significantly delay virus replication in the CEM(12D-7) T-cell line. Finally, mutational inactivation of the viral PR reverses the effect of a p6 mutation on virion production.

As indicated in Fig. 1, proteolytic processing of HIV-1 Gag occurs at five sites in Pr55^{Gag}: between MA and CA, CA and the spacer peptide p2, p2 and NC, NC and the spacer peptide p1, and p1 and p6. Because of amino acid sequence differences at the cleavage sites and differences in the accessibility of the cleavage sites to PR, cleavage at each site occurs at a distinct rate (44, 50, 61). As a result, Pr55^{Gag} processing occurs as a sequential, stepwise process. Several studies have demonstrated that even subtle alterations in Gag precursor processing can lead to severe defects in the assembly of infectious virus particles. Partial inhibition of HIV-1 PR function with PR inhibitors or overexpression of PR in *trans* blocked virus assembly or resulted in the formation of aberrant, noninfectious virions (27, 28, 39, 41, 42, 58). Furthermore, mutations in the HIV-1 p2 spacer peptide, which altered ordered Gag precursor cleavage, resulted in defective virus particle production (31, 50), and premature Gag processing resulting from mutations in the p2 spacer peptide between MA and p10 of Rous sarcoma virus reduced particle release (3). In the latter study, the defect caused by the p2 mutations could be reversed by abolishing PR function (3). Thus, it appears that excessive, inadequate, or mistimed PR activity can block the assembly and release of infectious virions.

Several observations reported here suggest a link between the role of p6 in particle production and PR function. First, mutagenesis of p6 resulted in an increased ratio of p25 to p24, presumably as a result of a reduced rate of cleavage between CA and p2. It has been suggested that the cleavage of p25 to p24 occurs very late in the assembly process, during or shortly after budding (22). Second, transmission electron microscopy indicated that p6-deficient virions possess an immature morphology, reminiscent of PR-defective particles (29, 49, 72). Third, and most important, mutational inactivation of PR reversed the virus production defect caused by p6 mutations. The link between p6 and PR could result from a direct enhancement or suppression of PR activity by p6, although a previous study reported no effect of p6 on PR activity in vitro (60). Alternatively, removal or mutagenesis of p6 might affect the conformation of Pr55^{Gag}, as has been suggested previously (55), thereby altering PR-mediated Gag processing. In any case, these results indicate that p6 is not required for efficient particle production from constructs expressing only the Gag precursor protein but rather is required in the context of the complete viral genome when PR is present. This observation reconciles the finding that p6 deletion does not affect the production of HIV-1 and HIV-2 Gag-only particles (21, 22, 25, 37, 38, 55) with results demonstrating that p6 is critical for virion production from full-length HIV-1 molecular clones (reference 20 and this report). These results also indicate that data obtained with subgenomic Gag expression systems may

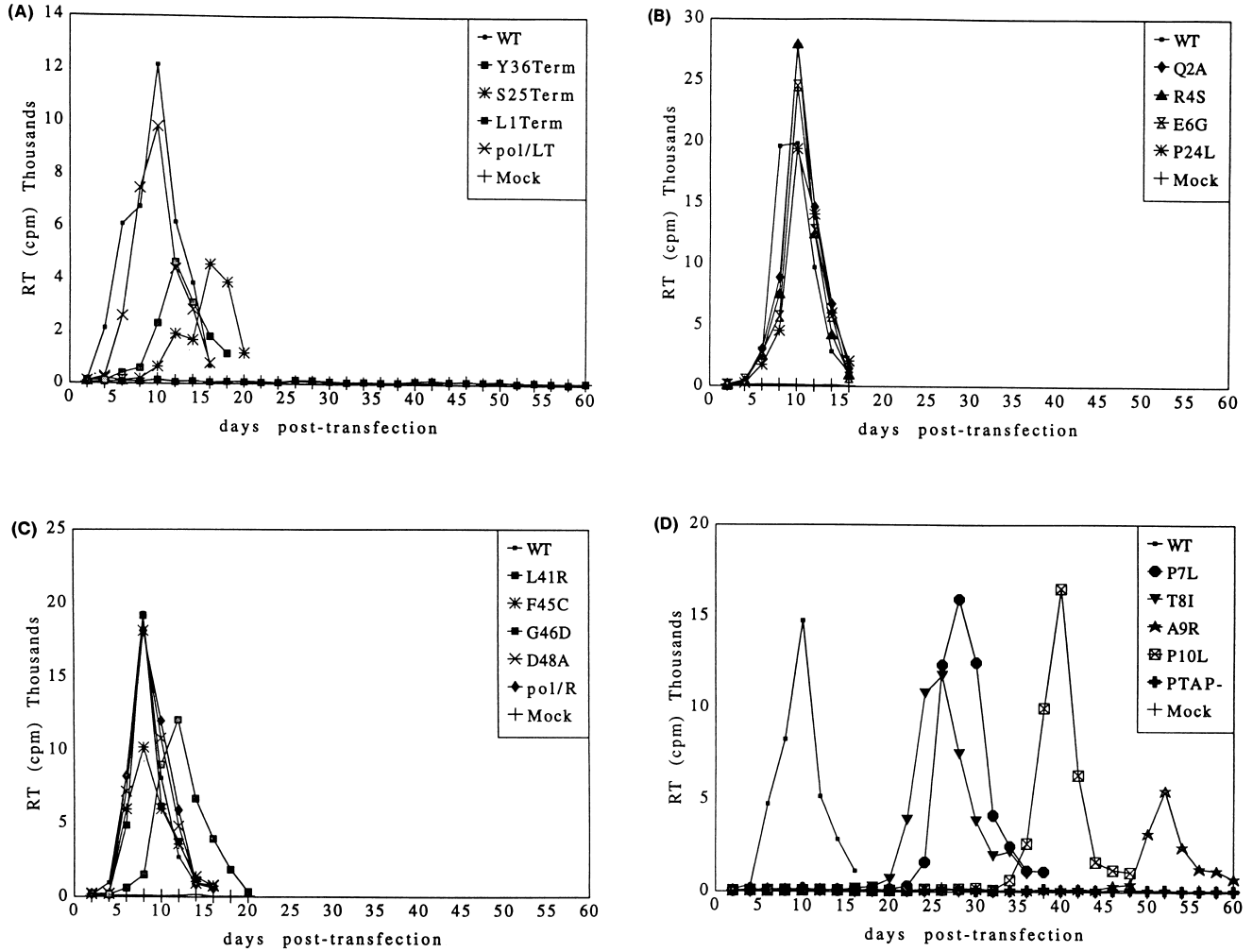

FIG. 5. Replication kinetics of p6 and *pol* mutants in the CEM(12D-7) T-cell line. CEM cells were transfected (A, B, C, and D) or infected (E) with the indicated proviral DNAs or viruses as described in Materials and Methods. Cells were split 1:3 every 2 days, and RT activity in the supernatant was monitored at each time point. WT, wild type.

not apply to systems using full-length, infectious molecular clones.

The transmission electron microscopy observations (data not shown) suggest that the p6 mutants proceeded through the typical stages of morphogenesis but failed to complete the budding process and remained tethered to the plasma membrane. A similar $p6^-$ phenotype was observed previously (20). The electron microscopy data are consistent with our biochemical data, which demonstrate that a severe reduction in virus particle production results from p6 mutations (Fig. 2 to 4). The presence of a significant amount of soluble, virally encoded protein in the medium of cells transfected with the $PTAP^-$ mutant (Fig. 4) raises the possibility that some p6 mutant particles are released from the cell and then rapidly degraded or are degraded while still tethered to the plasma membrane.

Recent studies have highlighted the importance of P-X-X-P motifs (where P is Pro and X is a variable amino acid) in protein-protein interactions involved in signal transduction pathways (for a review, see reference 6). Since a domain that is critical for p6 function (PTAP) is a P-X-X-P motif, it is possible that this region might interact with other viral or cellular proteins during the assembly process. Alternatively, the Pro-rich nature of the PTAP region (which is located within a 7-amino-acid sequence containing four Pro residues [Fig. 1]) suggests that it may function as a molecular hinge (8) during assembly and budding. Such a hinge model has been proposed

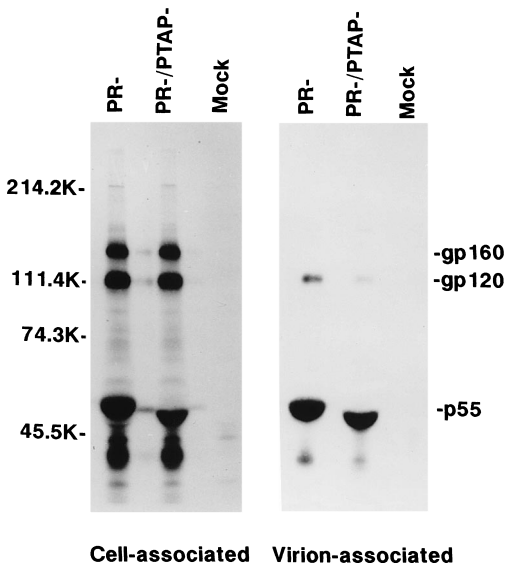

FIG. 6. Radioimmunoprecipitation analysis of viral protein expression and release from HeLa cells transfected with the PR-deficient proviruses. HeLa cells were transfected with the PR$^-$ or PR$^-$/PTAP$^-$ mutant molecular clones and were metabolically labeled with [^{35}S]Met. Cell-associated and virion-associated proteins were immunoprecipitated with AIDS patient sera. The mobilities of the envelope glycoprotein precursor (gp160), surface envelope glycoprotein (gp120), and Pr55^{Gag} precursor (p55) are indicated on the right; positions of molecular weight standards are shown on the left.

for a Pro-rich assembly domain in the Rous sarcoma virus p2 Gag peptide (69). Future studies will further characterize the role of p6 in virus assembly and in other aspects of the HIV-1 life cycle.

ACKNOWLEDGMENTS

We thank R. Willey, G. Englund, and L. Derr for critical review of the manuscript, R. Willey for helpful discussions, and Alicia Buckler-White for DNA sequencing. The HIV immunoglobulin (from A. Prince) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Bennett, R. P., T. D. Nelle, and J. W. Wills.** 1993. Functional chimeras of the Rous sarcoma virus and human immunodeficiency virus Gag proteins. J. Virol. **67:**6487–6498.
3. **Bowles, N., D. Bonnet, F. Mulhauser, and P.-F. Spahr.** 1994. Site-directed mutagenesis of the p2 region of the Rous sarcoma virus *gag* gene: effects on Gag polyprotein processing. Virology **203:**20–28.
4. **Bryant, M., and L. Ratner.** 1990. Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. Proc. Natl. Acad. Sci. USA **87:**523–527.
5. **Chazal, N., C. Carriere, B. Gay, and P. Boulanger.** 1994. Phenotypic characterization of insertion mutants of the human immunodeficiency virus type 1 Gag precursor expressed in recombinant baculovirus-infected cells. J. Virol. **68:**111–122.
6. **Cohen, G. B., R. Ren, and D. Baltimore.** 1995. Modular binding domains in signal transduction proteins. Cell **80:**237–248.
7. **Copeland, N. G., N. A. Jenkins, B. Nexo, A. M. Schultz, A. Rein, T. Mikkelsen, and P. Jorgensen.** 1988. Poorly expressed endogenous ecotropic provirus of DBA/2 mice encodes a mutant Pr65^{gag} protein that is not myristylated. J. Virol. **62:**479–487.
8. **DeLuca-Flaherty, C., D. B. McKay, P. Parham, and B. L. Hill.** 1990. Uncoating protein (hsc70) binds a conformationally labile domain of clathrin light chain LC$_a$ to stimulate ATP hydrolysis. Cell **62:**875–887.
9. **Dorfman, T., A. Bukovsky, A. Ohagen, S. Hoglund, and H. G. Gottlinger.** 1994. Functional domains of the capsid protein of human immunodeficiency virus type 1. J. Virol. **68:**8180–8187.
10. **Dorfman, T., J. Luban, S. P. Goff, W. A. Haseltine, and H. G. Gottlinger.** 1993. Mapping of functionally important residues of a cysteine-histidine box in the human immunodeficiency virus type 1 nucleocapsid protein. J. Virol. **67:**6159–6169.
11. **Dorfman, T., F. Mammano, W. A. Haseltine, and H. G. Gottlinger.** 1994. Role of the matrix protein in the virion association of the human immunodeficiency virus type 1 envelope glycoprotein. J. Virol. **68:**1689–1696.
12. **Dupraz, P., S. Oertle, C. Meric, P. Damay, and P.-F. Spahr.** 1990. Point mutations in the proximal Cys-His box of Rous sarcoma virus nucleocapsid protein. J. Virol. **64:**4978–4987.
13. **Freed, E. O., and M. A. Martin.** 1994. Evidence for a functional interaction between the V1/V2 and C4 domains of human immunodeficiency virus type 1 envelope glycoprotein gp120. J. Virol. **68:**2503–2512.
14. **Freed, E. O., and M. A. Martin.** 1995. Virion incorporation of envelope glycoproteins with long but not short cytoplasmic tails is blocked by specific, single amino acid substitutions in the human immunodeficiency virus type 1 matrix. J. Virol. **69:**1984–1989.
15. **Freed, E. O., J. M. Orenstein, A. J. Buckler-White, and M. A. Martin.** 1994. Single amino acid changes in the human immunodeficiency virus type 1 matrix protein block virus particle production. J. Virol. **68:**5311–5320.
16. **Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. De Wilde.** 1989. Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. Cell **59:**103–112.
17. **Goff, S. P., and L. I. Lobel.** 1987. Mutants of murine leukemia viruses and retroviral replication. Biochim. Biophys. Acta **907:**93–123.
18. **Gorelick, R. J., L. E. Henderson, J. P. Hanser, and A. Rein.** 1988. Point mutants of Moloney murine leukemia virus that fail to package viral RNA: evidence for specific RNA recognition by a zinc finger-like protein sequence. Proc. Natl. Acad. Sci. USA **85:**8420–8424.
19. **Gottlinger, H. G., T. Dorfman, J. G. Sodroski, and W. A. Haseltine.** 1989. Role of capsid precursor processing and myristylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86:**5781–5785.
20. **Gottlinger, H. G., T. Dorfman, J. G. Sodroski, and W. A. Haseltine.** 1991. Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. Proc. Natl. Acad. Sci. USA **88:**3195–3199.
21. **Hockley, D. J., M. V. Nermut, C. Grief, J. B. M. Jowett, and I. M. Jones.** 1994. Comparative morphology of Gag protein structures produced by mutants of the *gag* gene of human immunodeficiency virus type 1. J. Gen. Virol. **75:**2985–2997.
22. **Hoshikawa, N., A. Kojima, A. Yasuda, E. Takayashiki, S. Masuko, J. Chiba, T. Sata, and T. Kurayta.** 1991. Role of the *gag* and *pol* genes of human immunodeficiency virus in morphogenesis and maturation of retrovirus-like particles expressed by recombinant vaccinia virus: an ultrastructural study. J. Gen. Virol. **72:**2509–2517.
23. **Jorgensen, E. C., N. O. Kjeldgaard, F. S. Pedersen, and P. Jorgensen.** 1988. A nucleotide substitution in the *gag* N terminus of the endogenous ecotropic DBA/2 virus prevents Pr65^{gag} myristylation and virus replication. J. Virol. **62:**3217–3223.
24. **Jorgensen, E. C. B., F. S. Pedersen, and P. Jorgensen.** 1992. Matrix protein of AKV murine leukemia virus: genetic mapping of regions essential for particle formation. J. Virol. **66:**4479–4487.
25. **Jowett, J. B. M., D. J. Hockley, M. V. Nermut, and I. M. Jones.** 1992. Distinct signals in human immunodeficiency virus type 1 Pr55 necessary for RNA binding and particle formation. J. Gen. Virol. **73:**3079–3086.
26. **Kaplan, A. H., M. Manchester, and R. Swanstrom.** 1994. The activity of the protease of human immunodeficiency virus type 1 is initiated at the membrane of infected cells before the release of viral proteins and is required for release to occur with maximum efficiency. J. Virol. **68:**6782–6786.
27. **Kaplan, A. H., J. A. Zack, M. Knigge, D. A. Paul, D. J. Kempf, D. W. Norbeck, and R. Swanstrom.** 1993. Partial inhibition of the human immunodeficiency virus type 1 protease results in aberrant virus assembly and the formation of noninfectious particles. J. Virol. **67:**4050–4055.
28. **Karacostas, V., E. J. Wolffe, K. Nagashima, M. A. Gonda, and B. Moss.** 1993. Overexpression of the HIV-1 Gag-Pol polyprotein results in intracellular activation of HIV-1 protease and inhibition of assembly and budding of virus-like particles. Virology **193:**661–671.
29. **Katoh, I., Y. Yoshinaka, A. Rein, M. Shibuya, T. Odaka, and S. Oroszlan.** 1985. Murine leukemia virus maturation: protease region is required for conversion from "immature" to "mature" core form and for virus infectivity. Virology **145:**280–292.
30. **Kondo, E., F. Mammano, E. A. Cohen, and H. G. Gottlinger.** 1995. The p6^{gag} domain of human immunodeficiency virus type 1 is sufficient for the incorporation of Vpr into heterologous viral particles. J. Virol. **69:**2759–2764.
31. **Krausslich, H.-G., M. Facke, A.-M. Heuser, J. Konvalinka, and H. Zentgraf.** 1995. The spacer peptide between human immunodeficiency virus capsid and nucleocapsid proteins is essential for ordered assembly and viral infectivity. J. Virol. **69:**3407–3419.
32. **Kunkle, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient

site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
33. **Leis, J., D. Baltimore, J. M. Bishop, J. Coffin, E. Fleissner, S. P. Goff, S. Oroszlan, H. Robinson, A. M. Skalka, H. M. Temin, and V. Vogt.** 1988. Standardized and simplified nomenclature for proteins common to all retroviruses. J. Virol. **62:**1808–1809.
34. **Leis, J., and J. Jentoft.** 1983. Characteristics and regulation of interaction of avian retrovirus pp12 protein with viral RNA. J. Virol. **48:**361–369.
35. **Lobel, L. I., and S. P. Goff.** 1984. Construction of mutants of Moloney murine leukemia virus by suppressor-linker insertional mutagenesis: positions of viable insertion mutations. Proc. Natl. Acad. Sci. USA **81:**4149–4153.
36. **Loeb, D. D., R. Swanstrom, L. Everitt, M. Manchester, S. E. Stamper, and C. A. Hutchinson III.** 1989. Complete mutagenesis of the HIV-1 protease. Nature (London) **340:**397–400.
37. **Lu, Y.-L., P. Spearman, and L. Ratner.** 1993. Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. J. Virol. **67:**6542–6550.
38. **Luo, L., Y. Li, S. Dales, and C. Y. Kang.** 1994. Mapping of functional domains for HIV-1 *gag* assembly into virus-like particles. Virology **205:**496–502.
39. **Luukkonen, B. G. M., E. M. Fenyo, and S. Schwartz.** 1995. Overexpression of human immunodeficiency virus type 1 protease increases intracellular cleavage of Gag and reduces virus infectivity. Virology **206:**854–865.
40. **Mammano, F., A. Ohagen, S. Hoglund, and H. G. Gottlinger.** 1994. Role of the major homology region of human immunodeficiency virus type 1 in virion morphogenesis. J. Virol. **68:**4927–4936.
41. **McQuade, T. J., A. G. Tomasselli, L. Liu, V. Karacostas, B. Moss, T. K. Sawyer, R. L. Heinrikson, and W. G. Tarpley.** 1990. A synthetic HIV-1 protease inhibitor with antiviral activity arrests HIV-like particle maturation. Science **247:**454–456.
42. **Mergener, K., M. Facke, R. Welker, V. Brinkmann, H. R. Gelderblom, and H.-G. Krausslich.** 1992. Analysis of HIV particle formation using transient expression of subviral constructs in mammalian cells. Virology **186:**25–39.
43. **Meric, C., and P.-F. Spahr.** 1986. Rous sarcoma virus nucleic acid-binding protein pp12 is necessary for viral 70S RNA dimer formation and packaging. J. Virol. **60:**450–459.
44. **Mervis, R. J., N. Ahmad, E. P. Lillehoj, M. G. Raum, F. H. R. Salazar, H. W. Chan, and S. Venkatesan.** 1988. The *gag* gene products of human immunodeficiency virus type 1: alignment within the *gag* open reading frame, identification of posttranslational modifications, and evidence for alternative *gag* precursors. J. Virol. **62:**3993–4002.
45. **Myers, G., S. Wain-Hobson, B. Korber, R. F. Smith, and G. N. Pavlakis (ed.).** 1993. Human retroviruses and AIDS. A compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, N.Mex.
46. **Ottmann, M., C. Gabus, and J.-L. Darlix.** 1995. The central globular domain of the nucleocapsid protein of human immunodeficiency virus type 1 is critical for virion structure and infectivity. J. Virol. **69:**1778–1784.
47. **Pal, R., M. S. Reitz, Jr., E. Tschachler, R. C. Gallo, M. G. Sarngadharan, and F. D. M. Veronese.** 1990. Myristylation of gag proteins of HIV-1 plays an important role in virus assembly. AIDS Res. Hum. Retroviruses **6:**721–730.
48. **Paxton, W., R. I. Connor, and N. R. Landau.** 1993. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of *gag* and mutational analysis. J. Virol. **67:**7229–7237.
49. **Peng, C., B. Ho, T. Chang, and N. Chang.** 1989. Role of human immunodeficiency virus type 1 specific protease in core maturation and viral infectivity. J. Virol. **63:**2550–2556.
50. **Pettit, S. C., M. D. Moody, R. S. Wehbie, A. H. Kaplan, P. V. Nantermet, C. A. Klein, and R. Swanstrom.** 1994. The p2 domain of human immunodeficiency virus type 1 Gag regulates sequential proteolytic processing and is required to produce fully infectious virions. J. Virol. **68:**8017–8027.
51. **Prats, A., L. Sarith, C. Gabus, S. Litvak, G. Keith, and J.-L. Darlix.** 1988. Small finger protein of avian and murine retroviruses has nucleic acid annealing activity and positions the replication primer tRNA onto genomic RNA. EMBO J. **7:**1777–1783.
52. **Rhee, S. R., and E. Hunter.** 1990. Structural role of the matrix protein of type D retroviruses in Gag polyprotein stability and capsid assembly. J. Virol. **64:**909–917.
53. **Rhee, S. R., and E. Hunter.** 1991. Amino acid substitutions within the matrix protein of type D retroviruses affect assembly, transport, and membrane association of a capsid. EMBO J. **10:**535–546.
54. **Ross, E. K., A. J. Buckler-White, A. B. Rabson, G. Englund, and M. A. Martin.** 1991. Contribution of NF-κB and Sp1 binding motifs to the replicative capacity of human immunodeficiency virus type 1: distinct patterns of viral growth are determined by T-cell types. J. Virol. **65:**4350–4358.
55. **Royer, M., M. Cerutti, B. Gay, S.-S. Hong, G. Devauchelle, and P. Boulanger.** 1991. Functional domains of HIV-1 *gag*-polyprotein expressed in baculovirus-infected cells. Virology **184:**417–422.
56. **Schultz, A. M., and A. Rein.** 1989. Unmyristylated Moloney murine leukemia virus $Pr65^{gag}$ is excluded from virus assembly and maturation events. J. Virol. **63:**2370–2373.
57. **Schwartzberg, P., J. Colicelli, M. L. Gordon, and S. P. Goff.** 1984. Mutations in the *gag* gene of Moloney murine leukemia virus: effects on production of virions and reverse transcriptase. J. Virol. **49:**918–924.
58. **Sommerfelt, M. A., S. R. Petteway, Jr., G. B. Dreyer, and E. Hunter.** 1992. Effect of retroviral proteinase inhibitors on Mason-Pfizer monkey virus maturation and transmembrane glycoprotein cleavage. J. Virol. **66:**4220–4227.
59. **Strambio-de-Castilla, C., and E. Hunter.** 1992. Mutational analysis of the major homology region of Mason-Pfizer monkey virus by use of saturation mutagenesis. J. Virol. **66:**7021–7032.
60. **Stys, D., I. Blaha, and P. Strop.** 1993. Structural and functional studies in vitro on the p6 protein from the HIV-1 *gag* open reading frame. Biochim. Biophys. Acta **1182:**157–161.
61. **Tritch, R. J., Y.-S. E. Cheng, F. H. Yin, and S. Erickson-Viitanen.** 1991. Mutagenesis of protease cleavage sites in the human immunodeficiency virus type 1 *gag* polyprotein. J. Virol. **65:**922–930.
62. **Von Poblotzki, A., R. Wagner, M. Niedrig, G. Wanner, H. Wolf, and S. Modrow.** 1993. Identification of a region in the $Pr55^{gag}$-polyprotein essential for HIV-1 particle formation. Virology **193:**981–985.
63. **Wang, C.-T., and E. Barklis.** 1993. Assembly, processing, and infectivity of human immunodeficiency virus type 1 Gag mutants. J. Virol. **67:**4264–4273.
64. **Weaver, T. A., and A. T. Panganiban.** 1990. N myristoylation of the spleen necrosis virus matrix protein is required for correct association of the Gag polyprotein with intracellular membranes and for particle formation. J. Virol. **64:**3995–4001.
65. **Weldon, R. A., and J. W. Wills.** 1993. Characterization of a small (25-kilodalton) derivative of the Rous sarcoma virus Gag protein competent for particle release. J. Virol. **67:**5550–5561.
66. **Willey, R. L., J. S. Bonifacino, B. J. Potts, M. A. Martin, and R. D. Klausner.** 1988. Biosynthesis, cleavage and degradation of the human immunodeficiency virus type 1 envelope glycoprotein gp160. Proc. Natl. Acad. Sci. USA **85:**9580–9584.
67. **Willey, R. L., T. Klimkait, D. M. Frucht, J. S. Bonifacino, and M. A. Martin.** 1991. Mutations within the human immunodeficiency virus type 1 gp160 envelope glycoprotein alter its intracellular transport and processing. Virology **184:**319–329.
68. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62:**139–147.
69. **Wills, J. W., C. E. Cameron, C. B. Wilson, Y. Xiang, R. P. Bennett, and J. Leis.** 1994. An assembly domain of the Rous sarcoma virus Gag protein required late in budding. J. Virol. **68:**6605–6618.
70. **Wills, J. W., and R. C. Craven.** 1991. Form, function, and use of retroviral Gag proteins. AIDS **5:**639–654.
71. **Wills, J. W., R. C. Craven, R. A. Weldon, Jr., T. D. Nelle, and C. R. Erdie.** 1991. Suppression of retroviral MA deletions by the amino-terminal membrane-binding domain of $p60^{src}$. J. Virol. **65:**3804–3812.
72. **Witte, O. W., and D. Baltimore.** 1978. Relationship of retrovirus polyprotein cleavages to virion maturation studied with temperature-sensitive murine leukemia virus mutants. J. Virol. **26:**750–761.
73. **Wu, X., J. A. Conway, J. Kim, and J. C. Kappes.** 1994. Localization of the Vpx packaging signal within the C terminus of the human immunodeficiency virus type 2 Gag precursor protein. J. Virol. **68:**6161–6169.
74. **Yu, X., X. Yuan, Z. Matsuda, T.-H. Lee, and M. Essex.** 1992. The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. J. Virol. **66:**4966–4971.
75. **Yuan, Y., X. Yu, T.-H. Lee, and M. Essex.** 1993. Mutations in the N-terminal region of human immunodeficiency virus type 1 matrix protein block intracellular transport of the Gag precursor. J. Virol. **67:**6387–6394.