

## Effect of Mutations in Gag on Assembly of Immature Human Immunodeficiency Virus Type 1 Capsids in a Cell-Free System

Aalok R. Singh,<sup>\*†</sup> Rebecca L. Hill,<sup>\*</sup> and Jaisri R. Lingappa<sup>‡1</sup>

<sup>\*</sup>Department of Physiology and <sup>†</sup>Department of Pediatrics, University of California at San Francisco, San Francisco, California 94117; and <sup>‡</sup>Department of Pathobiology, University of Washington, Box 357238, Seattle, Washington 98195

Received July 29, 1999; returned to author for revision September 15, 1999; accepted October 11, 2000

Studies of HIV-1 capsid formation in a cell-free system revealed that capsid assembly occurs via an ordered series of assembly intermediates and requires host machinery. Here we use this system to examine 12 mutations in HIV-1 Gag that others studied previously in intact cells. With respect to capsid formation, these mutations generally produced the same phenotype in the cell-free system as in cells, indicating the cell-free system's high degree of fidelity. Analysis of assembly intermediates reveals that a mutation in the distal region of CA (322 LΔS) and truncations proximal to the second cys-his box in NC block multimerization of Gag at early stages in the cell-free capsid assembly pathway. In contrast, mutations in the region of amino acids 56–68 (located in the proximal portion of MA) inhibit assembly at a later point in the pathway. Other mutations, including truncations distal to the first cys-his box in NC and mutations in the distal half of MA (88HΔG, 85YΔG, Δ104–115, and Δ115–129), do not affect formation of immature capsids in the cell-free system. These data provide new information on the role of different domains in Gag during the early events of capsid assembly. © 2001 Academic Press

### INTRODUCTION

The assembly of the HIV-1 core, or capsid, involves a complex series of events (reviewed in Wills and Craven, 1991; Gelderblom, 1991; Krausslich and Welker, 1996; Boulanger and Jones, 1996; Freed, 1998; Garnier *et al.*, 1998). Pr55 Gag precursor polypeptides are synthesized in the cytoplasm of the host cell, myristoylated cotranslationally, and then targeted to the cytoplasmic side of the plasma membrane. There they multimerize to form spherical immature capsids that subsequently become enveloped by the plasma membrane. As the enveloped immature particles are released, the HIV-1 protease cleaves Pr55 Gag into Pr17 (matrix or MA), Pr24 (capsid or CA), Pr7 (nucleocapsid or NC), and Pr6. These late cleavage events transform the immature spherical capsid into the mature, bullet-shaped core.

When HIV-1 Gag is the only viral protein expressed in cells, particles closely resembling enveloped immature capsids are produced, indicating that Gag is the only viral gene product required for immature capsid assembly. Numerous studies expressing mutations in Gag within mammalian cells revealed that specific regions of Gag are important for assembly (reviewed in Wills and Craven, 1991; Gelderblom, 1991; Krausslich and Welker, 1996; Boulanger and Jones, 1996; Freed, 1998; Garnier *et al.*, 1998). Exactly how these regions of Gag act to promote the proper multimerization of approximately 1500

Gag polypeptides into a single immature capsid has not yet been elucidated. This significant gap in our understanding of the assembly process exists because early events in capsid formation are difficult to detect and study in cellular systems.

Recently, insights into these events were gained by taking a biochemical approach to capsid assembly, using cell-free systems. In these experiments, cell-lysates, which can be readily manipulated, are used to support synthesis of Gag and its assembly into particles that closely resemble immature capsids produced in mammalian cells (Spearman and Ratner, 1996; Lingappa *et al.*, 1997). Dissection of this system revealed that ATP and a host protein or protein complex are required for posttranslational events in cell-free capsid formation, demonstrating for the first time the importance of host machinery in capsid assembly (Lingappa *et al.*, 1997). Furthermore, in the same study pulse-chase analysis showed that Gag multimerization in the cell-free system occurs through stepwise formation of discrete assembly intermediates that progress from 10S to 80S to 150S and possibly 500S in size, culminating in the formation of the completely assembled 750S immature capsid. These posttranslational intermediates, composed of Gag polypeptides as well as one or more host proteins (J. R. Lingappa, unpublished data), can be distinguished by their relatively discrete sizes upon velocity sedimentation (Lingappa *et al.*, 1997). Together, they define an ordered pathway of assembly intermediates, as previously described (Lingappa *et al.*, 1997). Complexes resembling these cell-free assembly intermediates were

<sup>1</sup> To whom correspondence and reprint requests should be addressed. Fax: (206) 543-3873. E-mail: jais@u.washington.edu.



also identified in mammalian cells expressing HIV-1 Gag (Lingappa *et al.*, 1997; Lee and Yu, 1998; Lee *et al.*, 1999). In comparison to cellular systems, the cell-free system accumulates much larger quantities of assembly intermediates, making it advantageous for studying the capsid assembly process.

To gain a better understanding of the role of domains in Gag during the early events in capsid formation, we analyzed cell-free reactions programmed with mutants in Gag whose effects on particle formation in cellular systems were previously described by others. An initial examination of four well-known mutants in Gag revealed that their phenotypes, with respect to capsid assembly, were faithfully reproduced in the cell-free system (Lingappa *et al.*, 1997). Interestingly, that study revealed that different mutants result in accumulation of specific subsets of assembly intermediates, suggesting that different domains of Gag are required for progression from one assembly intermediate to the next. In the present study, we examined the phenotypes with respect to capsid assembly and the formation of assembly intermediates for 12 additional mutations in Gag (diagrammed in Fig. 1), seven of which involve point mutations (87V $\Delta$ E, 85Y $\Delta$ G, 88H $\Delta$ G, and 59I $\Delta$ E) or deletions ( $\Delta$ 56–68,  $\Delta$ 104–115, and  $\Delta$ 115–129) in MA. One is located in the distal third of CA (322 L $\Delta$ S) and four involve progressive truncations in NC between residues 390 and 430 (Pr41.9, Pr44.2, Pr44.3, and Pr45). One striking finding of this study is that the assembly phenotypes found in the cell-free system correspond closely to the phenotypes that other investigators found in cells. In addition, analysis of intermediates produced in the cell-free system suggests that residues in the distal region of CA and the adjacent proximal region of NC are involved in the early stages of Gag multimerization. In contrast, other residues (for example, in certain regions of the MA domain and in the proximal portion of CA) appear to have effects on late events in immature capsid formation.

## RESULTS

In the cell-free system, Gag transcripts are translated in the presence of a cellular extract that contains soluble factors necessary for translation and assembly, as well as membranes (plasma membranes and other cellular membranes) required for proper capsid formation, as described in Lingappa *et al.* (1997). The formation of *bona fide* capsids in this system was previously confirmed by a variety of different techniques (Lingappa *et al.*, 1997). In the current study, cell-free reactions were programmed with wild-type vs mutant forms of Gag, and allowed to translate and assemble for 150 min. The total amount of wild-type or mutant Gag synthesized in each reaction was the same (see bar graphs in Figs. 2–6). Upon completion of the assembly reaction, Nonidet P-40 (NP-40), a

nonionic detergent, was added to 1% to remove membranes associated with capsids, allowing protein complexes containing Gag to be studied in isolation. The reaction products were then subjected to velocity sedimentation on 13-ml gradients. We previously showed that this technique allows posttranslational Gag-containing complexes of different sizes (approximately 10S, 80S, 150S, 500S, and completed 750S capsids) to be resolved, that these complexes appear in an ordered fashion over time, and that those less than 750S in size most likely correspond to assembly intermediates. In addition, we previously demonstrated by both biochemical and ultrastructural analyses that the 750S complex closely resembles completely assembled immature capsids produced in transfected mammalian cells (Lingappa *et al.*, 1997). To further confirm that the assembly intermediates represent protein complexes of different S values, we examined the migration of these complexes when subjected to a much longer centrifugation that should place complexes of 10S and greater toward the bottom of the gradient. As predicted, all the assembly intermediates migrated together in the pellet under these conditions (Fig. 1B, open circles). This indicates that in our standard gradients, assembly intermediates are being separated on the basis of their S values, not their densities.

### Effect of mutations in NC and distal CA on capsid formation in the cell-free system

One group of mutations we examined is located in the NC region of Gag, which contains two cysteine-histidine (cys-his)-rich regions as well as highly basic residues flanking either side of the first cys-his box. The NC region of Gag is thought to play an important role in both particle formation and RNA packaging (reviewed in Berkowitz *et al.*, 1996; Freed, 1998). Studies using expression in insect cells demonstrated that, when Gag is truncated just proximal to the second cys-his box in NC, particle formation closely resembles that of wild-type Gag by biochemical and morphological criteria (Jowett *et al.*, 1992; Hockley *et al.*, 1994). These studies also showed that particles of the correct density and morphology fail to form when both cys-his boxes and all their adjacent basic residues are removed. Similar results were obtained using transfected mammalian cells (Zhang and Barklis, 1997; Dawson and Yu, 1998). Further investigation revealed that multiple regions of NC are important for Gag–Gag interactions, including the N-terminal of NC, the first cys-his box, and residues distal to the first cys-his box (Sandefur *et al.*, 2000). Since the cell-free assembly pathway allows for a detailed analysis of such Gag–Gag interactions, we examined the capsid assembly phenotypes that result from truncations in NC.

Truncation mutants were constructed by inserting a stop codon after residues located in and around the two

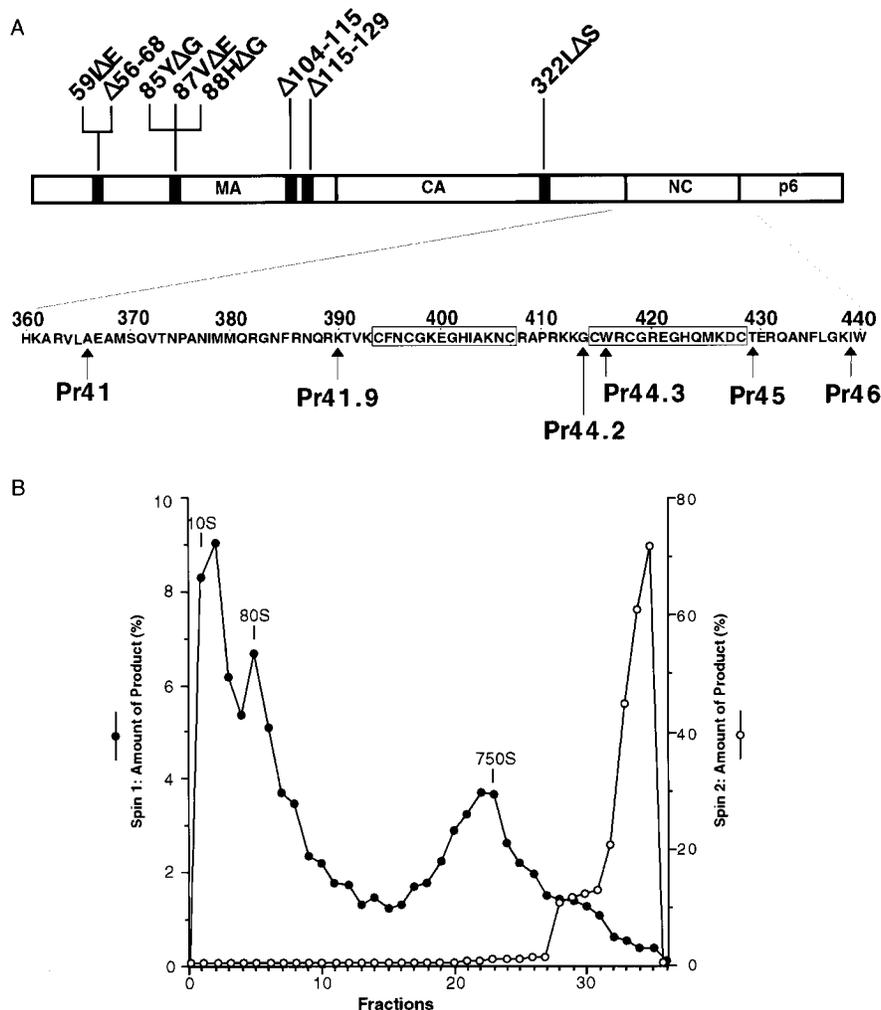


FIG. 1. (A) Diagram showing location of mutations expressed in the cell-free HIV capsid assembly system. HIV-1 Gag (Pr55) consists of four domains: MA, CA, NC, and Pr6, whose junctions are indicated with narrow lines. The NC domain starts at residue 380. Mutations that were constructed and expressed for this study include seven mutations in MA (59  $\Delta$ E,  $\Delta$  56–68, 85 Y $\Delta$ G, 87 V $\Delta$ E, 88 H $\Delta$ G,  $\Delta$ 104–115, and  $\Delta$ 115–129), and one mutation in CA (322 L $\Delta$ S), as indicated by filled boxes in the diagram on top. The inset below shows the exact position of four truncation mutants in NC that were also examined in this study (Pr41.9, Pr44.2, Pr44.3, and Pr45), and two truncation mutants in NC (Pr41 and Pr46) that were studied previously in the cell-free system (Lingappa *et al.*, 1997). The arrows indicate the last amino acid before the stop codon for each mutation. Open boxes indicate the residues that comprise the first and second cys-his boxes of Gag, respectively. (B) Analysis of assembly intermediates by velocity sedimentation. Cell-free translation and assembly reactions were programmed with transcript encoding wild-type Gag and incubated for 150 min. At the end of the incubation, NP-40 was added to 1%. One aliquot of products (spin 1, filled circles) was analyzed by velocity sedimentation on 13-ml linear sucrose gradients containing 1% NP-40 using parameters (35,000 rpm using a Beckman SW40 Ti rotor for 95 min at 4°C) that we previously showed to optimize separation of HIV-1 capsid assembly intermediates (Lingappa *et al.*, 1997). An equivalent aliquot (spin 2, open circles) was centrifuged using the same gradients and rotor at 40,000 rpm for 140 h. The latter centrifugation would be expected to pellet particles of 10S or greater. The amount of radiolabeled translation product in each fraction was determined and expressed as a percentage of total synthesis for each reaction.

cys-his boxes, as described under Materials and Methods and diagrammed in Fig. 1. The nomenclature we used for these mutations was similar to that used by Jowett *et al.* (1992). We previously demonstrated that the mutant Pr41, which is truncated proximal to the first residue of NC, is assembly-defective in the cell-free system, and results in accumulation of large amounts of only the first (10S) assembly intermediate (Lingappa *et al.*, 1997). Similar results were obtained when cell-free reactions were programmed with the longer truncation mutant Pr41.9 (Fig. 2), which removes both cys-his

and most but not all of the adjacent basic residues of NC, as shown in Fig. 1. In contrast, accumulation of the 750S completed capsid was observed when cell-free reactions were programmed with Pr44.2 (Fig. 2), in which the entire first cys-his box and its highly basic flanking residues are intact but the second cys-his box is completely removed. Successive truncations that express part or all of the second cys-his box (Pr44.3 and Pr45, shown in Fig. 2) also resulted in accumulation of the completed 750S capsid. Together, these data demonstrate that, in the cell-free system, completed immature capsids of the

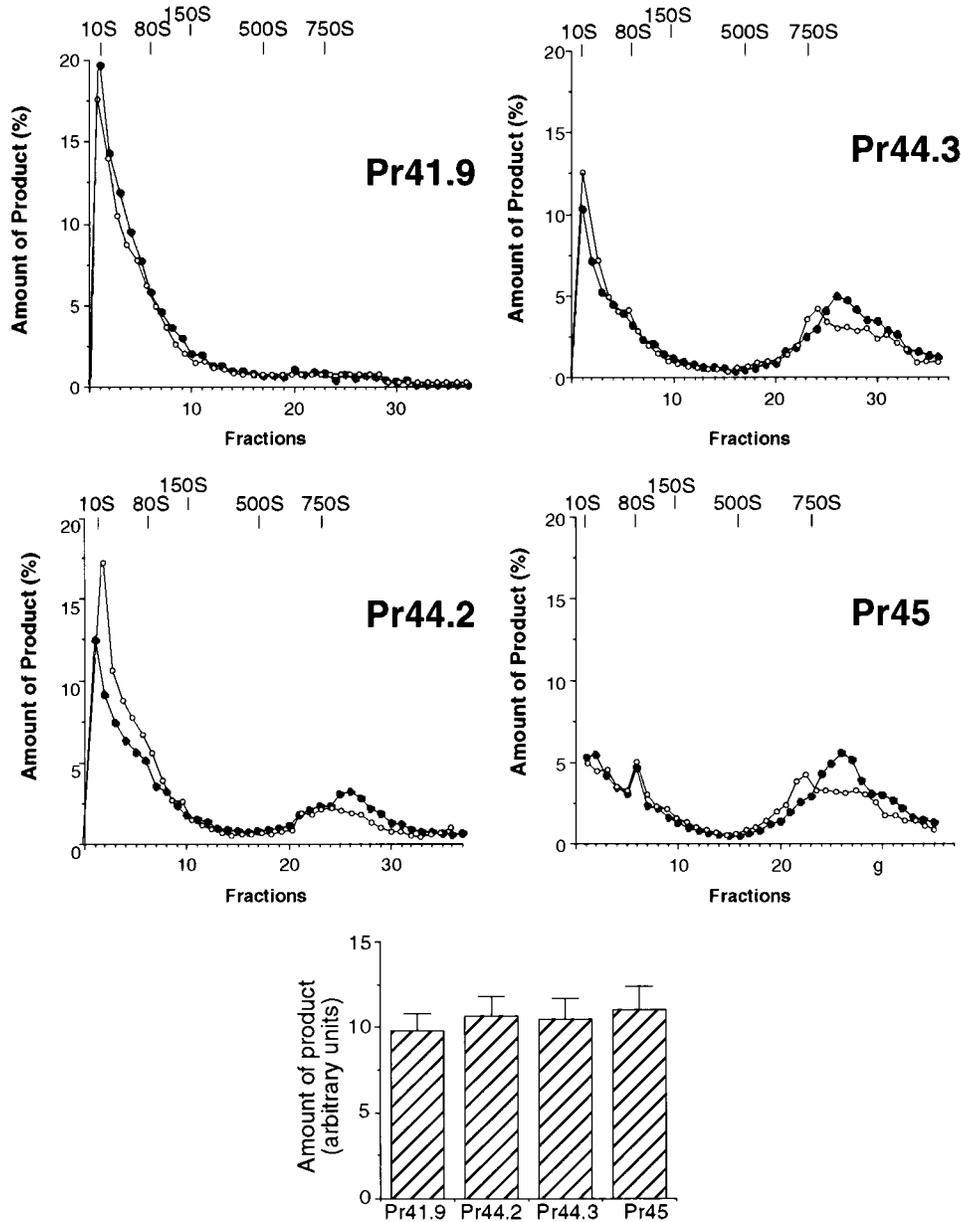
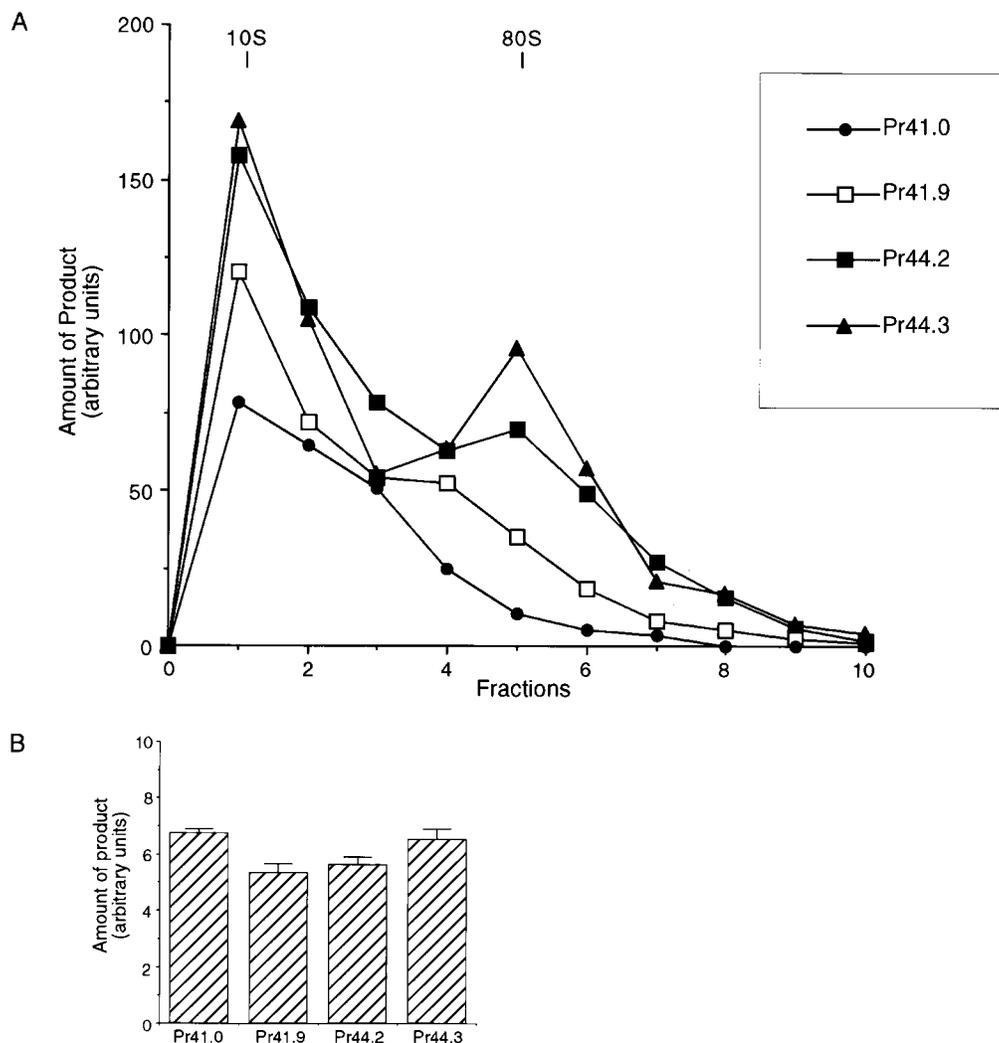


FIG. 2. Velocity sedimentation analysis of truncation mutants in the NC region of Gag. Cell-free translation and assembly reactions ( $12 \mu\text{l}$ ) were programmed with transcript encoding the Pr41.9, Pr44.2, Pr44.3, and Pr45 mutations in HIV-1 Gag (see Fig. 1A). At the end of the 150-min reaction NP-40 was added to 1%, and reaction products were analyzed by velocity sedimentation on 13-ml sucrose gradients containing 1% NP-40, as described under Materials and Methods. The amount of radiolabeled translation product in each fraction was determined and expressed as a percentage of total synthesis for each reaction. Reactions and gradient analyses were performed in parallel with a wild-type control three independent times; two representative experiments are shown (open circles vs filled circles). Total cell-free reaction products were quantitated as shown in the bar graph and described under Materials and Methods.

correct size are produced in the absence of the second cys-his box, but not when both cys-his boxes and their adjacent basic residues are removed.

In addition, we observed that cell-free expression of the assembly-competent truncation mutants Pr44.2, Pr44.3, and Pr45 resulted in accumulation of variable amounts of residual early assembly intermediates (10S and 80S), as is seen with wild-type Gag. Inclusion of progressively more of the second cys-his box appears to

shift the reaction toward production of larger amounts of the 750S capsid and 80S intermediate, and smaller amounts of the 10S assembly intermediate (Fig. 2). Since resolution of the 10S and 80S peaks is limited using the gradients shown in Fig. 2, we optimized the analysis of these two peaks by examining their appearance at different time points using velocity sedimentation gradients that optimize separation of the 10S and 80S intermediates (described under Materials and Methods). These



**FIG. 3.** Analysis of NC truncations and the production of the 80S assembly intermediate. Cell-free translation and assembly reactions were programmed with transcripts encoding the Pr41.0, Pr41.9, Pr44.2, and Pr44.3 mutations in HIV-1 Gag. The amount of the 80S intermediate produced by each of these reactions was examined at 30, 45, 60, and 90 min using velocity sedimentation gradients, and found to be maximal at 45 min (data not shown). Reactions were then programmed for all four mutations in parallel. Reactions were incubated for 45 min, EDTA was added to 10 mM to eliminate polysomes, NP-40 was added to 1%, and reaction products were subjected to velocity sedimentation analysis on 2-ml linear sucrose gradients containing 1% NP-40 as described under Materials and Methods. The position of an 80S complex was determined using S-value standards as previously described (Lingappa *et al.*, 1994). (A) The experiment was repeated three times, and one representative experiment is shown. (B) Total cell-free reaction products were quantitated as shown in the bar graph and described under Materials and Methods.

experiments revealed that the amount of the 80S intermediate is greatest at 30–45 min into the cell-free reaction (data not shown), as would be expected from our previous pulse-chase data (Lingappa *et al.*, 1997). Comparison of the different truncation mutants in parallel at 45 min (using gradients that resolve in the 10S to 100S range) demonstrates that including additional amino acids in the proximal region of NC leads to increasing formation of the 80S intermediate. Thus, Pr41 forms no 80S intermediate (Figs. 2 and 3, and Lingappa *et al.*, 1997), and Pr41.9 resembles Pr41 (Fig. 3). In contrast, Pr44.2 and Pr44.3 have progressively larger peaks in the 80S region. The 80S peak was even more prominent for Pr45 and Pr46 than for Pr44.3 (Fig. 2 and data not

shown). Together, these data suggest that residues in NC between 390 and 414 play a role in allowing Gag chains to progress beyond the first assembly intermediate in the cell-free capsid assembly pathway.

We examined whether mutations in other regions of Gag, besides NC, might also result in blockade at early steps in the assembly pathway. We constructed a point mutation, 322  $\Delta$ S (in the distal region of CA) that was previously found to be assembly-defective when expressed in insect cells (Hong and Boulanger, 1993). When the cell-free system was programmed with transcript for 322  $\Delta$ S, no completed 750S capsids were produced (Fig. 4). Analysis of assembly intermediates in Fig. 3 revealed that the 322  $\Delta$ S mutation results pre-

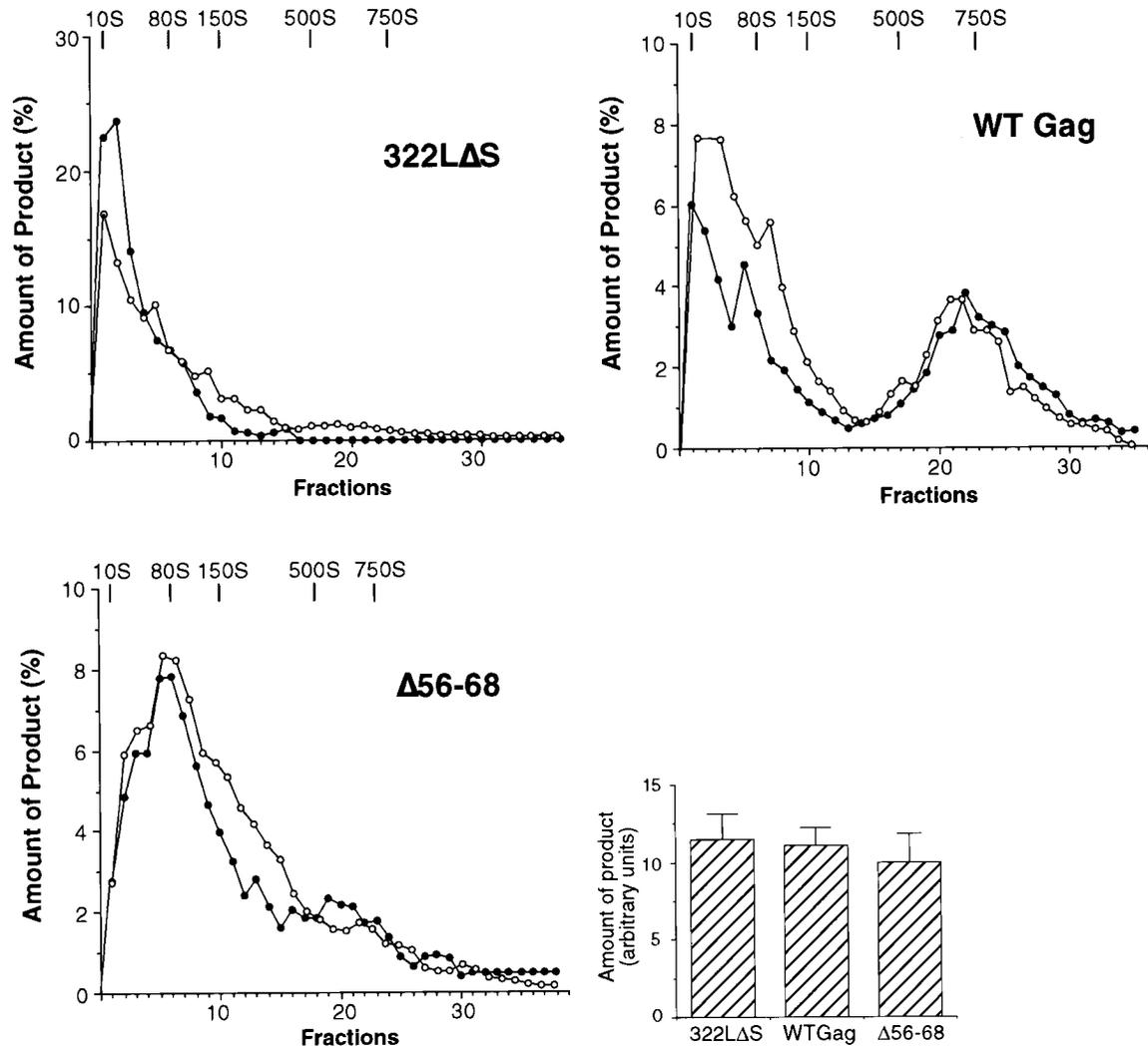


FIG. 4. Velocity sedimentation analysis of the 322 LΔS and Δ56-68 mutations in HIV-1 Gag. Cell-free translation and assembly reactions were programmed with transcript encoding wild-type Gag or the 322 LΔS and Δ56-68 mutations, located in the CA and MA regions of Gag, respectively. At the end of the reaction, NP-40 was added to 1% and reaction products were analyzed by velocity sedimentation on 13-ml sucrose gradients containing 1% NP-40, as described under Materials and Methods. The amount of radiolabeled translation product in each fraction was determined and expressed as a percentage of total synthesis for each reaction. Reactions and gradient analyses were performed in parallel with a wild-type control three independent times; two representative experiments are shown (open circles vs filled circles). Total cell-free reaction products were quantitated as shown in the bar graph and described under Materials and Methods.

dominantly in the accumulation of the 10S assembly intermediate, with a small amount of Gag migrating in the region of the second (80S) intermediate. These findings were confirmed on gradients that produce optimal separation between the 10S and 80S peaks (data not shown). Thus, it appears that residues in the distal region of CA around amino acid 322 also play a role in promoting Gag-Gag interactions during the early steps in the assembly pathway.

#### Effect of mutations in MA on capsid formation in the cell-free system

Having found that regions of CA and NC are important for progression of assembly during the early stages of

Gag multimerization, we examined whether deletions and mutations in the amino-terminal MA region of Gag have similar consequences. We had previously examined the effect of mutating the myristoylation signal at the extreme N-terminus of MA on cell-free capsid assembly, and found that it plays a role in progression beyond the second (80S) assembly intermediate (Lingappa *et al.*, 1997). In the current study, we examined four additional mutations in MA that were previously studied by others in intact cells, which included point mutations in the region of the first two cysteines in MA (residues 56 and 86, respectively) and deletions of two regions located in the distal end of MA (residues 104-115 and residues 115-129, respectively).

Deletions and point mutations adjacent to either the first or the second cysteine residue of MA were shown to block viral particle release in mammalian and insect cells (Freed *et al.*, 1994; Chazal *et al.*, 1995). Interestingly, ultrastructural examination revealed that these blockades occur for different reasons. When the region around cys 56 is mutated, no completely formed capsids are seen within cells by EM, while mutations surrounding cys 86 result in high levels of capsid formation occurring almost exclusively at cytoplasmic vacuoles rather than at the plasma membrane (Freed *et al.*, 1994). Deletion of amino acids 104–115 in MA was also shown to redirect the location of capsid assembly (Chazal, 1995), as was deletion of even larger sections of MA (Facke *et al.*, 1993). In contrast, deletion of amino acids 115–129 within MA has no effect on the amount or location of particle assembly or on particle release (Chazal *et al.*, 1995). These findings indicate that mutations surrounding cys 56 produce defects in capsid assembly per se, while mutations surrounding cys 86 as well as deletion of residues 104–115 primarily cause defective targeting of Gag polypeptides, with a resulting failure in particle release. Particles formed when assembly is redirected to cytoplasmic vacuoles appear to be morphologically aberrant by EM (Freed *et al.*, 1994; Facke *et al.*, 1993). It is unclear whether this abnormal morphology is a consequence of altered budding and envelopment, or whether it results from an additional, independent defect in capsid formation that is also caused by these mutations. Examination in the cell-free system could be useful for identifying whether mutations that redirect targeting of Gag chains also produce defects in Gag–Gag interactions.

Cell-free assembly reactions were programmed with transcript encoding wild-type Gag or transcript encoding mutations  $\Delta 56-68$ , 85Y $\Delta$ G, and 88H $\Delta$ G,  $\Delta 104-115$ , or  $\Delta 115-129$ . Each cell-free reaction was subjected to analysis by sucrose gradient sedimentation. As shown in Fig. 5, no discrete peak is seen in the region of 750S completed capsids when the reaction is programmed with the  $\Delta 56-68$  mutation. Instead there are large accumulations of Gag-containing complexes in the previously defined 10S, 80S, and 500S positions, and a trail extending into higher S-value ranges. Similar results were obtained when reactions were programmed with the point mutant 59 I $\Delta$ E (data not shown). The absence of completed capsids is consistent with findings other investigators obtained upon ultrastructural examination of intact cells expressing these mutations. The accumulation of Gag-containing complexes having values of  $S \geq 80$  indicates that mutations in the region of cys 56 block the formation of immature HIV-1 capsids by preventing proper multimerization of Gag chains at a relatively late point in the cell-free assembly pathway. This differs from findings obtained with the assembly-defective mutations in NC and the distal portion of CA described above, in which all

or the majority of Gag chains were found in 10S complexes.

Cell-free reactions expressing mutations surrounding cys 86 (Fig. 5) and deletions in more distal regions of MA (Fig. 6) had a very different outcome than reactions programmed with transcript encoding mutations surrounding cys 56. Point mutations 85Y $\Delta$ G and 88H $\Delta$ G resulted in accumulation of 750S completed capsids to an extent similar to that with wild-type Gag (Fig. 5). Programming cell-free reactions with the point mutant 87V $\Delta$ E (data not shown) gave results identical to those shown for 85Y $\Delta$ G and 88H $\Delta$ G. In each case, residual amounts of assembly intermediates were seen, as with wild-type Gag (Fig. 5). Deletion of residues 104–115 also gave results in the cell-free system that were indistinguishable from that of wild-type Gag (Fig. 6). These data suggest that the mutations surrounding cys 86 as well as deletion of residues 104–115, both of which are known to redirect targeting of Gag chains to internal membranes, do not alter Gag multimerization and assembly of the immature capsid per se. Finally, we examined a deletion in MA ( $\Delta 115-129$ ) that has no effect on Gag targeting or HIV-1 capsid formation in insect cells (Chazal *et al.*, 1995). We found that this mutation closely resembles wild-type Gag when expressed in the cell-free system (Fig. 6). Thus, the phenotype of this assembly-competent mutation in Gag is also faithfully reproduced in the cell-free system.

To determine whether the assembly-defective mutants were stably arrested at specific points in the capsid assembly pathway, or whether they slowly progress to form completed capsids, cell-free reactions were analyzed by velocity sedimentation at both 150 and 300 min after the start of the cell-free reactions. As shown in Fig. 7A, the total amount of 750S completed capsids produced in the cell-free reactions did not change significantly after 150 min of incubation. Furthermore, assembly-defective mutants were still blocked at the same points in the assembly pathway when profiles were compared at 150 vs 300 min into the reactions (Fig. 7B). These findings demonstrate that assembly-defective mutants do not progress further along the capsid assembly pathway after the standard 150-min incubation period, and confirm that assembly-defective mutants are indeed trapped at specific stages of capsid assembly.

## DISCUSSION

In this study, 12 well-characterized mutations in the MA, CA, and NC regions of Gag were examined by velocity sedimentation for their ability to form immature capsids in the cell-free system. Previously only five mutations in HIV-1 Gag were analyzed in cell-free systems and reported (Spearman and Ratner, 1996; Lingappa *et al.*, 1997). Thus, this study adds significantly to our understanding of how mutations in Gag affect the behavior

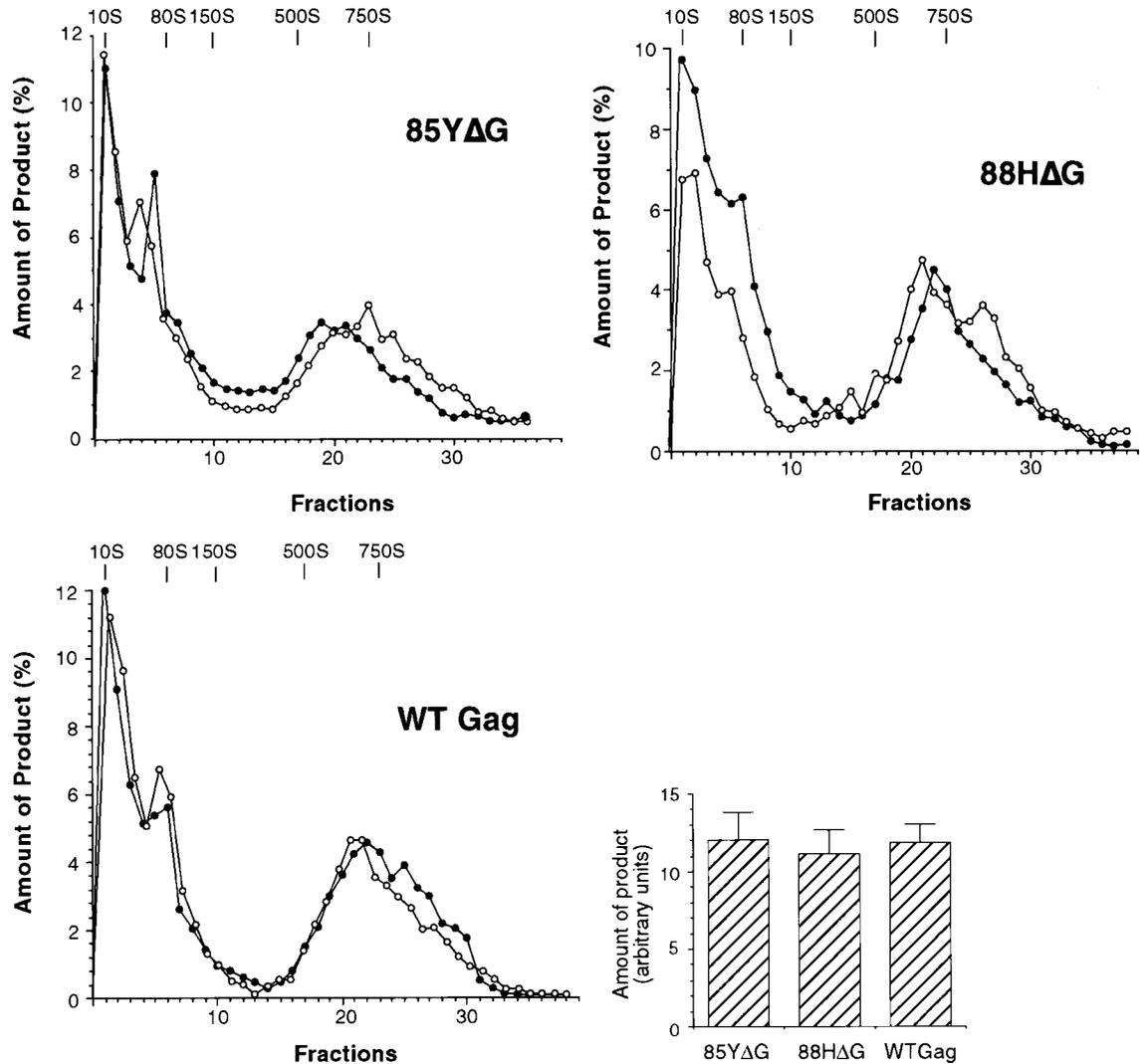


FIG. 5. Velocity sedimentation analysis of the 85Y $\Delta$ G and 88H $\Delta$ G mutations in HIV-1 Gag. Cell-free translation and assembly reactions were programmed with transcript encoding wild-type Gag or the 85Y $\Delta$ G and 88H $\Delta$ G mutations, located in the MA region of Gag. At the end of the reaction, NP-40 was added to 1%, and reaction products were analyzed by velocity sedimentation on 13-ml sucrose gradients containing 1% NP-40, as described under Materials and Methods. The amount of radiolabeled translation product in each fraction was determined and expressed as a percentage of total synthesis for each reaction. Reactions and gradient analyses were performed in parallel with a wild-type control three independent times; two representative experiments are shown (open circles vs filled circles). Total cell-free reaction products were quantitated as shown in the bar graph and described under Materials and Methods.

of newly synthesized Gag chains in eukaryotic cell lysates. A chart of mutations in Gag that were studied in both cellular and cell-free systems reveals that they generally produce the same assembly phenotypes in the cell-free system as they do in cells (Table 1). This finding confirms and extends previous observations (Lingappa *et al.*, 1997) that the cell-free system faithfully reproduces cellular events in immature capsid assembly and, therefore, constitutes an excellent system for understanding posttranslational biochemical events in capsid formation.

Analysis in cellular systems by numerous groups resulted in identification of mutations in Gag that block the proper formation of immature capsids. However, to date such studies have not defined the mechanisms underly-

ing such inhibition of capsid assembly. Our analysis of posttranslational assembly intermediates in the cell-free system indicates that assembly defects fall into relatively distinct biochemical categories. The first category consists of mutations that block progression beyond the first and smallest (10S) posttranslational assembly intermediate (which is likely a Gag dimer). Truncations that remove both cys-his boxes in NC as well as some or all of the adjacent highly basic residues (i.e., Pr41 and Pr41.9) block progression beyond the first (10S) assembly intermediate in the cell-free system. In contrast, truncations that leave intact the entire first cys-his box and its adjacent highly basic residues (Pr44.2 and Pr44.3) result in proper formation of the 80S intermediate as well as completed capsids (Fig. 2).

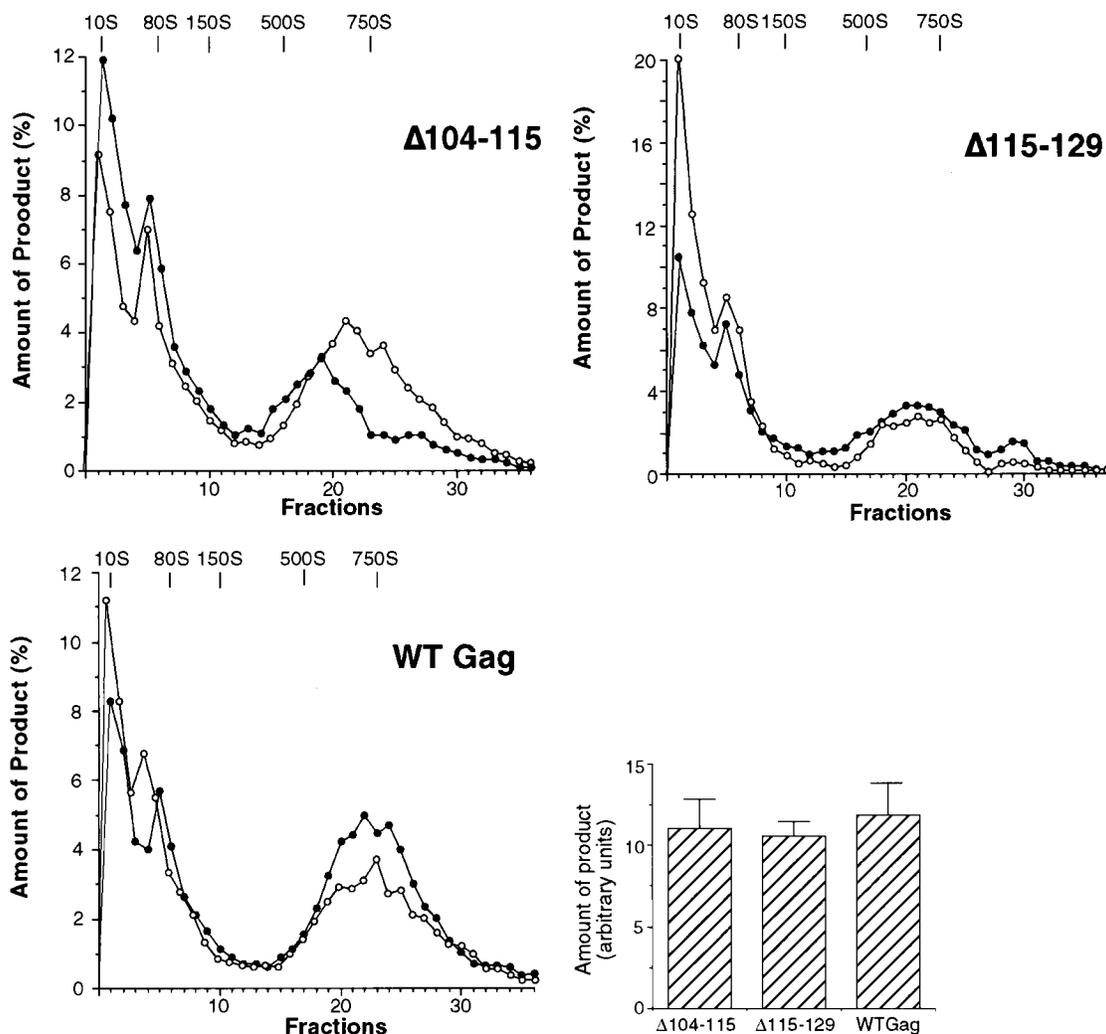
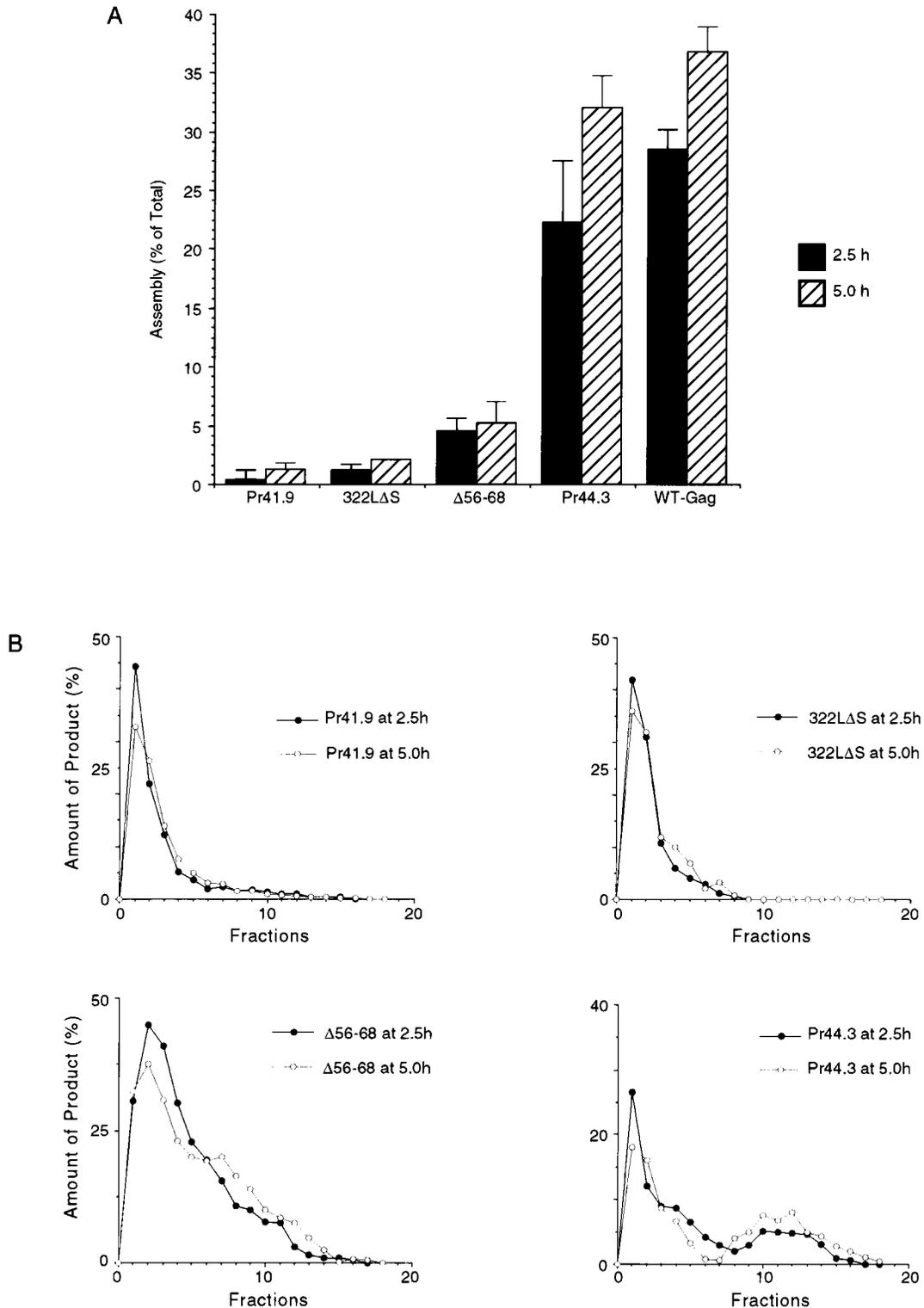


FIG. 6. Velocity sedimentation analysis of the  $\Delta 104-115$  and  $\Delta 115-129$  mutations in HIV-1 Gag. Cell-free translation and assembly reactions were programmed with transcript encoding wild-type Gag or the mutations  $\Delta 104-115$  and  $\Delta 115-129$ , located in the MA region of Gag. At the end of the reaction, NP-40 was added to 1%, and reaction products were analyzed by velocity sedimentation on 13-ml sucrose gradients containing 1% NP-40, as described under Materials and Methods. The amount of radiolabeled translation product in each fraction was determined and expressed as a percentage of total synthesis for each reaction. Reactions and gradient analyses were performed in parallel with a wild-type control three independent times; two representative experiments are shown. Total cell-free reaction products were quantitated as shown in the bar graph and described under Materials and Methods.

In general, our results and those of other investigators localize the domain important for Gag–Gag interactions (I domain) to the N-terminal residues of NC (Jowett *et al.*, 1992; Zhang and Barklis, 1997; Dawson and Yu, 1998; Sandefur *et al.*, 1998, 2000). However, there are some differences in findings obtained with truncation around residue 390. One group, studying expression in insect cells, found that truncation of Gag at residue 390 results in formation of particles that migrate quite differently from wild-type particles in velocity sedimentation gradients (migrating in 30 vs 45% sucrose) (Jowett *et al.*, 1992). Two other groups found that, when Gag is terminated at residue 391, the density of resulting immature particles is similar to that of comparable wild-type particles (Zhang and Barklis, 1997; Sandefur *et al.*, 1998, 2000). Finally, our results indicate that proper capsids fail to form in the cell-free system upon truncation at residue 390. Note

that our analysis examines the velocity sedimentation characteristics of *detergent-resistant* complexes, and therefore measures the integrity of the Gag-containing complexes and capsids in the absence of lipids. In contrast, the two other investigators measured velocity sedimentation (Jowett *et al.*, 1992) or density (Zhang and Barklis, 1997; Sandefur *et al.*, 1998, 2000) of lipid-enveloped capsids. Thus, the differences are likely to be due to different methods of analysis, with velocity sedimentation revealing abnormalities that are not appreciated using density measurements. Together the data suggest that truncation around residue 390 leads to formation of particles that contain some abnormalities, most likely in the structure of the capsid. In general, all of these studies agree that the residues at the extreme N-terminus of NC are important for Gag–Gag interactions.

Our demonstration that increasing the length of NC



**FIG. 7.** Time course of assembly. Cell-free reactions were programmed with each of the assembly-defective mutants described in Figs. 2 through 6, as well as with wild-type Gag and one assembly-competent mutant (Pr44.3). Equivalent aliquots were removed at 2.5 and 5 h into the reaction. NP-40 was added to 1%, and reaction products were subjected to velocity sedimentation on 5-ml linear sucrose gradients, as described under Materials and Methods. The amount of radiolabeled translation product in each fraction was determined. (A) Bar graph shows the amount of completely assembled capsid (750S peak, fractions 8–15) present in each reaction at 2.5 h (solid bars) and at 5 h (hatched bars), expressed as a percentage of the total product. Values shown are the means of three experiments, and error bars indicate standard error. (B) Line graphs show the amount of radiolabeled product (x-axis) present in each fraction (y-axis) as a percentage of total, at 2.5 h (filled circles) and at 5 h (open circles) for three assembly-defective mutants (Pr41.9,  $\Delta$ 56–68, and 322 L $\Delta$ S) and one assembly-competent mutant (Pr44.3). Reactions were performed three independent times; data are from two representative experiments.

TABLE 1

The Effect of Mutations in HIV Gag on Capsid Formation in the Cell-Free System and in Intact Cells

Plasmid	Capsid assembly	
	Cell-free	Cells
WT Gag	+	+
NC		
Pr46	+	+
Pr45	+	+
Pr44.3	+	+
Pr44.2	+	+
Pr41.9/Pr42	–	±
Pr41.0	–	–
CA		
322 $\Delta$ S	–	–
$\Delta$ 250–260	–	–
MA		
$\Delta$ 115–129	+	+
$\Delta$ 104–115	+	+
88H $\Delta$ G	+	+
87V $\Delta$ E	+	+
85Y $\Delta$ G	+	+
$\Delta$ 56–68	–	–
59I $\Delta$ E	–	–
2G $\Delta$ A	–	–

*Note.* Sixteen mutations in Gag were analyzed in the cell-free system as well as in intact cells. Analyses of cell-free reactions programmed with four of these mutants (Pr46, Pr41,  $\Delta$ 240–250, and 2G $\Delta$ A) were described previously (Lingappa *et al.*, 1997) and the remaining mutations are described in this report. Cellular studies were performed by others, as follows: *NC truncation mutants*: Gheysen *et al.*, 1989; Royer *et al.*, 1991; Jowett *et al.*, 1992; Hockley *et al.*, 1994; Spearman *et al.*, 1994; Zhang and Barklis, 1997; Dawson and Yu, 1998; Sandefur, *et al.*, 1998; Sandefur *et al.*, 2000; *CA mutations*: Hong and Boulanger, 1993; Hockley *et al.*, 1994; Zhao *et al.*, 1994; *MA mutations*: Gottlinger *et al.*, 1989; Bryant and Ratner, 1990; Freed *et al.*, 1994; Chazal *et al.*, 1995; Facke *et al.*, 1993. Mutations that result in the formation of completed cell-free capsids or capsids that closely resemble wild-type in cells are indicated with a plus, while those mutations that completely block capsid formation are indicated with a minus. For truncations in the region around residue 390 (Pr41.9 or Pr 42), different results were obtained in cellular systems (indicated by  $\pm$ ), as described in detail in the text.

results in progressively more formation of the second (80S) assembly intermediate (Fig. 3) suggests that residues in NC proximal to the second cys-his box stabilize Gag–Gag interactions and increase the likelihood of progression from the 10S complex to the 80S complex and beyond. These findings are consistent with the results of Sandefur *et al.* (2000), which demonstrated that basic residues between residues 384 and 391, as well as residues in the first cys-his box and the basic linker region in NC, contribute to I domain function. Our results suggest that formation of the 80S intermediate may be a critical early step in assembly that is important for subsequent production of immature capsids of the correct size.

Another mutation that appears to block capsid forma-

tion at the first step in the assembly pathway is the 322  $\Delta$ S mutation, located in the distal region of CA, near NC. This mutation appears to produce a less-complete blockade than that produced by the truncation mutants Pr41 and Pr41.9, since a small amount of Gag accumulates in the position of the second (80S) assembly intermediate. These data suggest that both NC and the distal portion of CA play an important role in early events in Gag multimerization. It remains to be determined whether one or both of these regions assist in progression from the 10S to 80S assembly intermediate by binding to other Gag polypeptides, associating with a host factor necessary for capsid formation, binding to RNA, or performing a combination of these functions. Our demonstration that these regions are involved in Gag–Gag interactions that occur early in assembly will help to resolve these unanswered mechanistic questions.

The second category of defects in capsid assembly involves blockade of Gag multimerization after formation of the second (80S) assembly intermediate. The mutation that best exemplifies this category is the previously described 2G $\Delta$ A mutation (Lingappa *et al.*, 1997), since it produces large accumulations of only the 10S and 80S assembly intermediates. This mutation, in which the myristoylation signal is abolished by substituting the glycine at residue 2 of Gag with alanine, results in production of nonmyristoylated Gag chains (reviewed in Freed, 1998; Garnier *et al.*, 1998). Like regions of NC and the distal third of CA, the myristoylation domain appears to play a role, directly or indirectly, in early events in Gag multimerization (Lingappa *et al.*, 1997). Interestingly, the same blockade is seen when myristoyl CoA is omitted from the cell-free reaction programmed with wild-type Gag (Lingappa *et al.*, 1997), confirming that absence of the myristate moiety is the reason that 2G $\Delta$ A chains fail to progress beyond the 80S assembly intermediate. Note that the 322 $\Delta$ S mutation bears some resemblance to 2G $\Delta$ A, since it results in formation of a small amount of the 80S Gag-containing complex, albeit much less than is seen with 2G $\Delta$ A.

Blockade of multimerization after Gag chains have formed the first two assembly intermediates constitutes the third documented category of Gag multimerization defects. As shown in this study, a deletion in the region of the first cysteine residue in MA produces this defect, resulting in formation of Gag-containing complexes of greater than 80S but no completed 750S capsids. A region located in the middle of CA (residues 250–260) constitutes another domain in Gag that appears to be involved in late events in Gag multimerization in the cell-free system, as described previously (Lingappa *et al.*, 1997). Interestingly, neither point mutations around the second cysteine residue in MA nor deletion of residues 104–115 in MA has any effect on early or late events in Gag multimerization in the cell-free system. Others showed that mutations in these regions block particle

release by redirecting Gag chains to internal cellular membranes (Freed *et al.*, 1994; Chazal *et al.*, 1995). Our findings demonstrate that these mutations do not affect Gag multimerization and capsid formation per se.

The studies of numerous investigators (e.g., Gheysen *et al.*, 1989; Gottlinger *et al.*, 1989; Bennett *et al.*, 1993; Hong and Boulanger, 1993; Wang and Barklis, 1993; Dorfman *et al.*, 1994; Franke *et al.*, 1994; Spearman *et al.*, 1994; Zhang *et al.*, 1996; Borsetti *et al.*, 1998; Sandefur *et al.*, 1998, 2000; Accola *et al.*, 2000) support the idea that only a small number of domains in Gag are required for HIV-1 particle assembly. These include the myristate moiety (the M domain), the C-terminal third of CA, and a portion of NC (the I domains). Despite some discrepancies with regard to findings of various groups, there is general agreement that these domains play key roles in particle formation. Interestingly, our data in both this study and a previous study (Lingappa *et al.*, 1997) demonstrate that specific mutations in each of these regions results in accumulation of Gag-containing assembly intermediates of 80S or less, and therefore are involved in facilitating early events in Gag multimerization. Studies in cells suggest that the role of other regions of Gag in assembly is more complicated. Such regions include portions of MA distal to the M domain, and the proximal half of CA. For example, although point mutations in MA were previously found to alter particle production (e.g., Yuan *et al.*, 1993; Freed *et al.*, 1994; Chazal *et al.*, 1995; Ono *et al.*, 1997), large deletions reveal that residues in MA distal to the M domain are not necessary for particle formation or release (e.g., Wang *et al.*, 1993; Lee and Lineal, 1994; Borsetti *et al.*, 1998; Reil *et al.*, 1998; Accola *et al.*, 2000). Our findings to date suggest that mutations in such regions (including residues in MA distal to the M domain, and in the proximal portion of CA) either have no effect on Gag multimerization or have effects at relatively late times during capsid formation.

## MATERIALS AND METHODS

### Plasmid constructions

Plasmid vectors were derived from SP64 vector (Promega, Madison, WI) into which the 5' untranslated region of *Xenopus laevis* globin had been inserted at the *Hind*III site (Melton *et al.*, 1984). The gag ORF from SF2 HIV type 1 genomic DNA (a gift of Jay Levy) was introduced downstream from the SP6 promoter and the globin untranslated region as previously described (Lingappa *et al.*, 1997). Plasmids expressing mutations in Gag were constructed using standard nucleic acid techniques (Sambrook *et al.*, 1989). The Pr45, Pr44.3, Pr44.2, and Pr41.9 truncation mutants were made from the wild-type construct by using PCR to introduce stop codons (in bold) in Gag after the following residues: thr 429 (DNA sequence of mutant from bp 1280: AT TGC ACT **TAG TAA**), trp 416 (DNA sequence of mutant from bp1240:

GGC TGT TGG **TAG TAA**), gly 414 (DNA sequence of mutant from bp1235: AA AAG GGC **TAG TAA**), and lys 390 (DNA sequence of mutant from bp 1165 AGA AAG **TAG**). Deletions were made using a site-directed mutagenesis kit from Stratagene (La Jolla, CA). For the  $\Delta$ 56–68 construct, amino acids cys 57 through ser 67 were deleted (DNA sequence of mutant from bp 160: TCA GAA GGC CTT CAG ACA GGA TCA); for  $\Delta$ 104–115, amino acids glu 104 through lys 114 were deleted (DNA sequence of mutant from bp307: AAG ATT GCG CAG); and for  $\Delta$ 115–129, amino acids gln 116 through val 130 were deleted (DNA sequence of mutant from bp 330: A AGT AAG AAG AAG GCT AGC CAG AAT). Site-directed mutagenesis was also used to create the following single amino acid mutations: substitution of ile at position 59 with glu (59I $\Delta$ E) (DNA sequence of mutant from bp 170: GC CGC CAA GAA CTT GGA CAG); substitution of tyr at position 85 with gly (85Y $\Delta$ G) (DNA sequence of mutant from bp 250: ACC CTC GGG TGT GTA); substitution of val at position 87 with glu (87V $\Delta$ E) (DNA sequence of mutant from bp 259: TGT GAA CAT CAA); substitution of his at position 88 with gly (88H $\Delta$ G) (DNA sequence of mutant from bp 260: GT GTA GGT CAA); and substitution of leu with ser at position 322 (322L $\Delta$ S) (DNA sequence of mutant from bp 965: CC TTG AGC GTC CAA). All point mutations and deletions were confirmed by DNA sequencing.

### Cell-free reactions

Transcription in the cell-free system with SP6 polymerase was carried out at 40°C for 75 min. For Figs. 1, 2, 4, 5, and 6, translation of the transcription product was performed using wheat germ extract in the presence of <sup>35</sup>S-methionine at 26°C for 150 min as previously described (Lingappa *et al.*, 1997). For all cell-free reactions, the total amount of translated product was determined by analyzing a microliter of translation product by SDS-PAGE and autoradiography, followed by quantitation of the bands seen using densitometry. Translations were adjusted so that the amount of total translation product was the same for all reactions. Each mutation in Gag was expressed in the cell-free system and subjected to gradient analysis at least three independent times. All experiments included a wild-type Gag (WT) control. For analysis of the 80S intermediate in Fig. 3, incubations were performed for 45 min, adjusted to 10 mM EDTA, and analyzed on sucrose gradients as described below. For Fig. 7, cell-free reactions were performed as described above, and 10 aliquots were removed for gradient analysis at the standard time of 150 min as well as at 300 min, as indicated.

### Gradient analysis

Gradient solutions were made using sucrose dissolved in buffer containing 100 mM NaCl, 4 mM MgAc,

50 mM KAc, 1% NP-40, and 10 mM TrisAc at pH 7.4. By the use of a gradient maker, 13-ml continuous 15–60% sucrose gradients were prepared. A 10- $\mu$ l aliquot of translation product from each of the cell-free reactions was diluted into 100  $\mu$ l of NP-40 buffer and layered onto gradients. All samples in Figs. 1, 2, 4, 5, and 6 were centrifuged on these gradients at 35,000 rpm using a SW40 Ti rotor (Beckman Instruments, Fullerton, CA) for 95 min at 4°C, except for Spin 2 in Fig. 1B, which was centrifuged on these gradients at 40,000 rpm for 130 h in the SW40 Ti rotor. Samples in Fig. 7 were centrifuged on 5-ml linear 15–60% sucrose gradients at 45,000 rpm for 45 min in the MLS50 rotor. Fractions (350  $\mu$ l) were collected from the top of all gradients with a Haake-Buchler gradient fractionator. After fractionation, either the entire fraction was TCA-precipitated, washed with ethanol, and analyzed by SDS-PAGE, or an aliquot of the fraction was directly analyzed by SDS-PAGE. SDS-PAGE was performed using 15% acrylamide gels and the radiolabeled products were visualized by autoradiography.

For Fig. 2, discontinuous 2-ml 10–50% sucrose gradients were used to resolve 10S vs 80S assembly intermediates. A 10- $\mu$ l aliquot of the cell-free reaction product was diluted into 100  $\mu$ l of NP-40 buffer and layered onto gradients, which were centrifuged at 55,000 rpm in a Beckman TLS55 rotor for 1 h at 4°C. Ten fractions of 200  $\mu$ l were collected from the top of the gradient and analyzed by SDS-PAGE and autoradiography.

The methods of S-value determination (McEwen, 1967) and standardization using S-value markers were described previously (Lingappa *et al.*, 1997).

### Image analysis

Autoradiographs were digitized using Arcus II scanner (AGFA) and Adobe Photoshop software (Adobe Systems, Mountain View, CA). Mean band densities were obtained and the background was subtracted.

### ACKNOWLEDGMENTS

We acknowledge Vishwanath Lingappa for advice and support; Shannyn Riba, Peter Choi, Concepcion Zimmerman, Fred Calayag, and Jason Kucinzski for technical assistance; and Tom Bartee and colleagues at General Mills for providing wheat germ. J.R.L. is supported by National Institutes of Health R29 Grant AI41881.

### REFERENCES

Accola, M. A., Strack, B., and Gottlinger, H. G. (2000). Efficient particle production by minimal Gag constructs which retain the carboxy-terminal domain of human immunodeficiency virus type 1 capsid-p2 and a late assembly domain. *J. Virol.* **74**, 5395–5402.

Bennett, R. P., Nelle, T. D., and Wills, J. W. (1993). Functional chimeras of the Rous sarcoma virus and human immunodeficiency virus Gag proteins. *J. Virol.* **67**, 6487–6498.

Berkowitz, R., Fisher, J., and Goff, S. P. (1996). RNA packaging. *Curr. Top. Microbiol. Immunol.* **214**, 177–218.

Borsetti, A., Ohagen, A., and Gottlinger, H. G. (1998). The C-terminal half

of the human immunodeficiency virus type 1 Gag precursor is sufficient for efficient particle assembly. *J. Virol.* **72**, 9313–9317.

Boulanger, P., and Jones, I. (1996). Use of heterologous expression systems to study retroviral morphogenesis. *Curr. Top. Microbiol. Immunol.* **214**, 238–260.

Bryant, M., and Ratner, L. (1990). Myristoylation-dependent replication and assembly of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **87**, 523–527.

Chazal, N., Gay, B., Carriere, C., Tournier, J., and Boulanger, P. (1995). Human immunodeficiency virus type 1 MA deletion mutants expressed in baculovirus-infected cells: *cis* and *trans* effects on the Gag precursor assembly pathway. *J. Virol.* **69**, 365–375.

Dawson, L., and Yu, X.-F. (1998). The role of nucleocapsid of HIV-1 in virus assembly. *Virology* **251**, 141–157.

Dorfman, T., Bukovsky, A., Ohagen, A., Hoglund, S., and Gottlinger, H. G. (1994). Functional domains of the capsid protein of human immunodeficiency virus type 1. *J. Virol.* **68**, 8180–8187.

Facke, M., Janetzko, A., Shoeman, R. L., and Krausslich, H.-G. (1993). A large deletion in the matrix domain of the human immunodeficiency virus Gag gene redirects virus particle assembly from the plasma membrane to the endoplasmic reticulum. *J. Virol.* **67**, 4972–4980.

Franke, E. K., Yuan, H. E. H., Bossolt, K. L., Goff, S. P., and Luban, J. (1994). Specificity and sequence requirements for interactions between various retroviral Gag proteins. *J. Virol.* **66**, 5300–5305.

Freed, E. O. (1998). HIV-1 Gag proteins: Diverse functions in the virus life cycle. *Virology* **251**, 1–15.

Freed, E. O., Orenstein, J. M., Buckler-White, A. J., and Martin, M. (1994). Single amino acid changes in the human immunodeficiency virus type 1 matrix protein block virus particle production. *J. Virol.* **68**, 5311–5320.

Garnier, L., Bowzard, J. B., and Wills, J. W. (1998). Recent advances and remaining problems in HIV assembly. *AIDS* **12**(Suppl. A), S5–S16.

Gelderblom, H. R. (1991). Assembly and morphology of HIV: Potential effect of structure on viral function. *AIDS* **5**, 617–638.

Gheysen, D., Jacobs, E., de Foresta, F., Thiriart, C., Francotte, M., Thines, D., and de Wilde, M. (1989). Assembly and release of HIV-1 precursor Pr55Gag virus-like particles from recombinant baculovirus-infected insect cells. *Cell* **59**, 103–112.

Gottlinger, H. G., Sodroski, J. G., and Haseltine, W. A. (1989). Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **86**, 5781–5785.

Hockley, D. J., Nermut, M. V., Grief, C., Jowett, J. B. M., and Jones, I. M. (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.

Hong, S. S., and Boulanger, P. (1993). Assembly-defective point mutants of the human immunodeficiency virus type 1 Gag precursor phenotypically expressed in recombinant baculovirus-infected cells. *J. Virol.* **67**, 2787–2798.

Jowett, J. B. M., Hockley, D. J., Nermut, M., and Jones, I. M. (1992). Distinct signals in human immunodeficiency virus type 1 Pr55 necessary for RNA binding and particle formation. *J. Gen. Virol.* **73**, 3079–3086.

Krausslich, H.-G., and Welker, R. (1996). Intracellular transport of retroviral capsid components. *Curr. Top. Microbiol. Immunol.* **214**, 25–63.

Lee, P. P., and Linial, M. L. (1994). Efficient particle formation can occur if the matrix domain of human immunodeficiency virus type 1 Gag is substituted by a myristoylation signal. *J. Virol.* **68**, 6644–6654.

Lee, Y.-M., Liu, B., and Yu, X.-F. (1999). Formation of virus assembly intermediate complexes in the cytoplasm by wild-type and assembly-defective mutant human immunodeficiency virus type 1 and their association with membranes. *J. Virol.* **73**, 5654–5662.

Lee, Y.-M., and Yu, X.-F. (1998). Identification and characterization of virus assembly intermediate complexes in HIV-1-infected CD4+ cells. *Virology* **243**, 78–93.

Lingappa, J. R., Hill, R., Wong, M. L., and Hegde, R. S. (1997). A

- multi-step, ATP-dependent pathway for assembly of human immunodeficiency virus (HIV) capsids in a cell-free system. *J. Cell Biol.* **136**, 567–581.
- Lingappa, J. R., Martin, R. L., Wong, M. L., Ganem, D., Welch, W. J., and Lingappa, V. R. (1994). A eukaryotic cytosolic chaperonin is associated with a high molecular weight intermediate in the assembly of hepatitis B virus capsid, a multimeric particle. *J. Cell Biol.* **125**, 99–111.
- McEwen, C. R. (1967). Tables for estimating sedimentation through linear concentration gradients of sucrose solution. *Anal. Biochem.* **20**, 114–149.
- Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12**, 7035–7056.
- Ono, A., Huang, M., and Freed, E. O. (1997). Characterization of human immunodeficiency virus type 1 matrix revertants: Effects on virus assembly, gag processing, and env incorporation into virions. *J. Virol.* **71**, 4409–4418.
- Reil, H., Bukovsky, A. A., Gelderblom, H. R., and Gottlinger, H. G. (1998). Efficient HIV-1 replication can occur in the absence of the viral matrix protein. *EMBO J.* **17**, 2699–2708.
- Royer, M., Cerutti, M., Gay, B., Hong, S.-S., Devauchelle, G., and Boulanger, P. (1991). Functional domains of HIV-1 Gag-polyproteins expressed in baculovirus-infected cells. *Virology* **184**, 417–422.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sandefur, S., Smith, R. M., Varthakavi, V., and Spearman, P. (2000). Mapping and characterization of the N-terminal I domain of human immunodeficiency virus type 1 Pr55 Gag. *J. Virol.* **74**, 7238–7249.
- Sandefur, S., Varthakavi, V., and Spearman, P. (1998). The I domain is required for efficient plasma membrane binding of human immunodeficiency virus type 1 Pr55 Gag. *J. Virol.* **72**, 2723–2732.
- Spearman, P., and Ratner, L. (1996). Human immunodeficiency virus type 1 capsid formation in reticulocyte lysates. *J. Virol.* **70**, 8187–8194.
- Spearman, P., Wang, U.-J., Heyden, N. V., and Ratner, L. (1994). Identification of human immunodeficiency virus type 1 Gag protein domains essential to membrane binding and particle assembly. *J. Virol.* **68**, 3232–3242.
- Wang, C.-T., and Barklis, E. (1993). Assembly, processing, and infectivity of human immunodeficiency virus type 1 Gag mutants. *J. Virol.* **67**, 4264–4273.
- Wang, C.-T., Zhang, Y., McDermott, J., and Barklis, E. (1993). Conditional infectivity of a human immunodeficiency virus matrix domain deletion mutant. *J. Virol.* **67**, 7067–7076.
- Wills, J. W., and Craven, R. C. (1991). Form, function and use of retroviral Gag proteins. *AIDS* **5**, 639–654.
- Yuan, X., Yu, X., Lee, T.-H., and Essex, M. (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.
- Zhang, W. H., and Barklis, E. (1997). Effects of nucleocapsid mutations on human immunodeficiency virus assembly and RNA encapsidation. *J. Virol.* **71**, 6765–6776.
- Zhang, W. H., Hockley, D. J., Nermut, M. V., Morikawa, Y., and Jones, I. M. (1996). Gag–Gag interactions in the C-terminal domain of human immunodeficiency virus type 1 p24 capsid antigen are essential for Gag particle assembly. *J. Gen. Virol.* **77**, 743–751.
- Zhao, Y., Jones, I. A., Hockley, D., Nermut, M. V., and Roy, P. (1994). Complementation of human immunodeficiency virus type 1 (HIV-1) Gag particle formation. *Virology* **199**, 403–408.