A Multistep, ATP-dependent Pathway for Assembly of Human Immunodeficiency Virus Capsids in a Cell-free System

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Abstract. To understand the mechanism by which human immunodeficiency virus type 1 (HIV) capsids are formed, we have reconstituted the assembly of immature HIV capsids de novo in a cell-free system. Capsid authenticity is established by multiple biochemical and morphologic criteria. Known features of the assembly process are closely reproduced, indicating the fidelity of the cell-free reaction. Assembly is separated into co- and posttranslational phases, and three independent posttranslational requirements are demonstrated: (*a*) ATP, (*b*) a detergent-sensitive host factor, and (*c*) a detergent-insensitive host subcellular fraction that can be depleted and reconstituted. Assembly appears to proceed by way of multiple intermediates whose conversion to completed capsids can be blocked by either ATP depletion or treatment with nondenaturing detergent. Specific subsets of these intermediates accumulate upon expression of various assembly-defective Gag mutants in the cell-free system, suggesting that each mutant is blocked at a particular step in assembly. Furthermore, the accumulation of complexes of similar sizes in cells expressing the corresponding mutants suggests that comparable intermediates may exist in vivo. From these data, we propose a multi-step pathway for the biogenesis of HIV capsids, in which the assembly process can be disrupted at a number of discrete points.

THE protein shell of the human immunodeficiency virus type 1 (HIV)[1] virion, termed the HIV capsid or core, is composed of ~1,500 copies of the Pr55 Gag structural protein precursor (for review see Gelderblom, 1991). For proper assembly of the capsid to occur, Pr55 chains must undergo myristoylation (Gheysen et al., 1989; Gottlinger et al., 1989), an NH_2-terminal modification thought to occur co-translationally (Towler et al., 1988). The myristoylated chains are targeted to the host plasma membrane where assembly takes place concomitant with RNA encapsidation. As capsids are formed, they bud into the plasma membrane, which results in envelopment and subsequent release of viral particles from the cell. Coincident with these events, the immature viral particles undergo a maturation process, involving proteolytic processing of the precursor structural proteins and condensation of the capsids into collapsed, electron-dense cores (for review see Gelderblom, 1991; Wills and Craven, 1991).

Much of our knowledge of HIV capsid assembly comes from studies of viral particle formation in cultured cells transfected with viral genes. EM studies of these cells have indicated that the plasma membrane is the site of capsid assembly (Gelderblom, 1991; Wills and Craven, 1991). Analysis of various mutants of Pr55 have revealed key domains required for efficient capsid assembly and targeting to the plasma membrane (Gheysen et al., 1989; Gottlinger et al., 1989; Trono et al., 1989; Royer et al., 1991; Jowett et al., 1992; Facke et al., 1993; Wang and Barklis, 1993; Hockley et al., 1994; Spearman et al., 1994; Zhao et al., 1994). However, the actual mechanisms involved in coordinating the formation of an HIV capsid from 1,500 Gag monomers have not been elucidated. Many important questions about HIV capsid assembly remain unanswered, including whether assembly is an energy-dependent process, whether host proteins are required for assembly to take place, and whether assembly proceeds by way of discrete intermediates.

A major obstacle to addressing these questions experimentally has been the inherent difficulty of studying capsid assembly in cellular systems. In cells, many of the events in question proceed extremely rapidly and are not readily amenable to manipulation, making it difficult to identify *trans*-acting factors and energy substrates that may be required for assembly. Development of a cell-free system that recreates capsid biogenesis would greatly facilitate a biochemical dissection and mechanistic understanding of capsid formation.

In this study, we report the development of a cell-free

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1. *Abbreviations used in this paper*: HBV, Hepatitis B Virus; HIV, human immunodeficiency virus type 1; HSP, high speed pellet; HSS, high speed supernatant; HSP_d, detergent-treated high speed pellet; MCoA, myristoyl CoA.

system for the assembly of immature HIV capsids that appears to reproduce known features of capsid structure and biogenesis, including the phenotypes of a variety of assembly-competent and assembly-defective Gag mutants. We use this system to demonstrate that capsid formation can be dissociated into co- and posttranslational phases, each of which has distinct requirements. The reactions that occur during the posttranslational phase are dependent on ATP and at least two independent host factors, distinguished by their sensitivity to detergent. In addition, the posttranslational phase appears to proceed by way of previously unrecognized assembly intermediates. Expression of Gag mutants that are defective for assembly results in accumulation of these assembly intermediates in the cell-free system. Similar findings are obtained in cellular systems. Together, these data indicate that HIV capsid formation is a regulated, energy-dependent, multi-step process involving *trans*-acting host factors.

Materials and Methods

Plasmid Constructions

All plasmid constructions for cell-free transcription were made using PCR and other standard nucleic acid techniques (Sambrook et al., 1989). Plasmid vectors were derived from SP64 (Promega, Heidelberg, Germany) into which the 5′ untranslated region of *Xenopus laevis* globin had been inserted at the HindIII site (Melton et al., 1984). The gag ORF from HIV genomic DNA (a kind gift of Jay Levy, University of California, San Francisco, CA) was introduced downstream from the SP6 promoter and the globin untranslated region. The GΔA mutation was made by changing glycine at position 2 of Gag to alanine using PCR. The Pr46 mutant was made by introducing a stop codon after gly 435 which removes p6; Pr41 has a stop codon after arg 363 (in the COOH-terminal region of p24). These truncation mutants are comparable to those described by Jowett et al., 1992. To make the D_2 mutant, amino acids from gly 250 through val 260 were deleted (Hockley et al., 1994; Zhao et al., 1994). All changes engineered by PCR were verified by DNA sequencing.

Cell-free Reactions

Transcription with SP6 polymerase and translation of the transcription products in wheat germ extract containing [^{35}S]methionine (ICN Biochemicals, Irvine, CA) were performed as previously described (Lingappa et al., 1994), except for modifications noted below. Improved assembly was obtained when wheat germ extract, prepared as described before (Erickson and Blobel, 1983; Lingappa et al., 1994), was subjected to ultracentrifugation at 50,000 rpm (TLA 100 rotor; Beckman Instruments, Palo Alto, CA) for 15 min at 4°C and the supernatant used for in vitro translation (Lingappa, J.R., unpublished observations). 10 μM myristol CoA (MCoA; Sigma Chemical Co., St. Louis, MO) was added at the start of translation when indicated. Translation reactions ranged in volume from 20 to 100 μl and were incubated at 25°C for 150 min. Some reactions were adjusted to a final concentration of the following agents at times indicated in figure legends: 0.2 μM emetine (Sigma Chemical Co); 1.0 U apyrase (Sigma Chemical Co.) per μl translation; 0.002, 0.1, or 1.0% Nikkol. Results of some cell-free translation and assembly reactions were repeated using rabbit reticulocyte lysate that was prepared as described previously (Merrick, 1983). In pulse–chase experiments, translation reactions contained [^{35}S]cysteine (Amersham Corp., Arlington Heights, IL) for radiolabeling. 4 min into translation, 3 mM unlabeled cysteine was added, and the reaction was continued at 25°C for variable chase times, as indicated.

A standard curve for quantity of translation product was established by immunoblotting a cell-free translation product and standards of known concentration (Lingappa, J.R., unpublished observations). The amount of Pr55 was determined by interpolation on this curve.

Gradient Analysis

Cell-free reactions were diluted into ice cold NP40 buffer (containing 10 mM TrisAc, pH 7.4, 100 mM NaCl, 4 mM MgAc, 50 mM KAc, and 1% NP-40) and layered onto the following gradients. All sucrose solutions were made using the same buffer.

2 ml Sucrose Gradients. After centrifugation (TLS55 rotor; Beckman Instruments), 200 μl fractions were collected serially from the top. Sucrose step gradients for batch separation of soluble and particulate material (used in Figs. 1, 2, and 4) contained 600 μl of 66% sucrose, 900 μl of 40% sucrose, and 500 μl of 20% sucrose, and were centrifuged at 30,000 rpm for 35 min at 4°C. Particles of 500–750-S partition into fractions 4–6, which were collected for further analysis. Linear 10–50% sucrose gradients (used in Fig. 1) were centrifuged at 30,000 rpm for 35 min at 4°C.

2 ml CsCl Gradients. Equilibrium centrifugation was performed using 2 ml isopycnic CsCl gradients (used in Fig. 1) (402.6 μg/ml) centrifuged at 50,000 rpm for 26 h at 25°C, and 200 μl fractions were collected.

13 ml Sucrose Gradients. 13 ml 15–60% linear sucrose gradients were centrifuged (SW40 Ti rotor; Beckman Instruments) for 75 (Fig. 5) or 95 min (Fig. 3, 6, and 7) at 35,000 rpm and 5°C. Fractions of 350 μl were collected from the top of gradients using a Haake-Buchler gradient fractionator and were processed immediately. Either aliquots of fractions were loaded directly into SDS-PAGE buffer or the entire fraction was precipitated with TCA and washed with ethanol ether as previously described (Lingappa et al., 1994) before SDS-PAGE analysis. SDS-PAGE was performed using 15% gels, and some gels were fluorographed using Enhance (Dupont Co., Willmington, DE). Radiolabeled products of cell-free translations were visualized by autoradiography.

S-value Determinations

Estimates of S-values of Gag-containing complexes seen on 13 ml sucrose gradients were determined by the method of McEwen (1967) using the following formula: $S = \Delta I / \omega^2 t$ where S is the sedimentation coefficient of the particle in Svedberg units, ΔI is the time integral for sucrose at the separated zone minus the time integral for sucrose at the meniscus of the gradient, ω is rotor speed in radians/sec, and t is time in sec.

Values for I were determined for particles of a density of 1.3 g/cm^3 and for a temperature of 5°C, according to tables published by McEwen (1967). Calculated S-values for different fractions in the gradients are labeled as markers above each gradient tracing (Figs. 5–7). Markers such as BSA (5-S), macroglobulin (20-S), Hepatitis B Virus capsids (100-S), ribosomal subunits (40-S and 60-S), and polysomes (>100-S) were used to calibrate the gradients and to confirm the calculated S-values. However, it should be noted that the S-value assignments for each Gag-containing complex are meant to be very approximate estimates that allow a general description of the complex and should not be interpreted as constituting a detailed biophysical analysis.

Preparation of HSS, HSP, and HSP_d

Where indicated, wheat germ extract was centrifuged at either 50,000 rpm for 21 min or 100,000 rpm for 30 min (TLA 100 rotor; Beckman Instruments). The supernatant (HSS) of the 50,000 rpm spin was used for cell-free translation and assembly reactions. The pellet of the 100,000 rpm spin (HSP) was resuspended at a 5× concentration in buffer (25 mM Hepes, pH 7.4, 4 mM MgAc, 100 mM KAc, 0.25 M sucrose). Wheat germ extract adjusted to 0.5% Nikkol was subjected to the same ultracentrifugation in parallel to generate the detergent treated high speed pellet (HSP_d). This pellet was washed twice with 200 μl of the above nondetergent buffer to remove traces of detergent, and then resuspended. After treatment with emetine for 50 min, 1.8 μl of HSP or HSP_d was added to the 18 μl cell-free reactions programmed with HSS. Control reactions were treated with the same volume of buffer at the same time. At the end of the 150 min incubation, reactions were separated into soluble and particulate fractions and analyzed as described above.

Transfections and Production of Authentic Capsids

Cos-1 cells were transfected by the method of Forsayeth and Garcia (1994) using plasmids pSVGagRRE-R and pSVRev (Smith et al., 1993) that were a kind gift of D. Rekosh (University of Virginia, Charlottesville, VA). 4 d after transfection, immature HIV particles were purified from the culture medium by sedimentation through a 4 ml 20% sucrose cushion in an SW 40 rotor at 29,000 rpm for 120 min (Mergener et al., 1992). The pellet was harvested, stored in aliquots at −80°C, and treated with 1% NP-40 buffer just before use to remove envelopes. These de-enveloped, authentic immature HIV capsids were used as standards and analyzed in parallel with the products of cell-free reactions by a variety of methods,

including velocity sedimentation, equilibrium centrifugation, and electron microscopy.

Lysate of transfected Cos cells was prepared by solubilizing transfected cells on 60-mm plates in 700 μl 1% NP-40 buffer. This detergent lysate was passaged 20 times through a 20-gauge needle, and clarified by centrifugation for 10 min at 2000 *g*, and 150 μl of this supernatent was loaded onto 13 ml gradients for analysis, as described. Gag polypeptide present in the fractions was visualized by immunoblotting with a monoclonal antibody to Gag (Dako, Santa Barbara, CA). Bound antibody was detected using an enhanced chemiluminescence system (Amersham Intl., Amersham, UK). Band density was determined as described under image analysis below, and relative band densities were confirmed by quantitating films representing different exposure times.

Electron Microscopy

Cell-free reactions as well as authentic capsid standards were rapidly separated into soluble and particulate fractions on 2 ml 20–80% linear sucrose gradients by centrifugation for 10 min at 30,000 rpm. Under these conditions, authentic capsids partition into fractions 3–5, as determined in advance by immunoblotting of standards with Gag antibody. EM grids were glow discharged, and a drop of the Gag antibody was placed on each grid and allowed to air dry. Grids were inserted into eppendorf tubes containing particulate material from sucrose gradients and incubated for 10 min at 4°C to allow antibodies to bind to Gag-containing particles. Grids were then rinsed in water, and negative staining with uranyl acetate was performed as described (Dubochet et al., 1971). The following negative controls were analyzed in parallel: control transfections, in which Cos cells were transfected with lac Z-containing plasmids; and mock cell-free reactions, which were generated by programming in vitro transcriptions with buffer instead of gag cDNA and using this mock transcript for cell-free translation and assembly reactions. Experiments were repeated three additional times in a single blind fashion. In each of these experiments, the microscopist was able to correctly assign the identity of each sample based on the presence or absence of particles.

Image Analysis

Autoradiographs were digitized using an AGFA Focus Color Plus scanner and Adobe Photoshop software (Adobe Systems Incorporated, Mountain View, CA). Mean band densities were determined and adjusted for band size and background.

Results

Reconstitution of Immature HIV Capsid Assembly in a Cell-free System

Since we were interested in dissecting the early steps of capsid assembly rather than the better understood late stage of capsid maturation, we chose to study formation of immature HIV capsids, which undergo all of the steps of capsid assembly except the final protease-dependent maturation event. Assembly of immature capsids in cells requires expression of only the HIV Pr55 protein (Gheysen et al., 1989; Smith et al., 1993). However, we and others (Sakalian et al., 1996) have found that standard in vitro translation systems, consisting of a cytosolic extract, amino acids, an ATP regenerating system, and in vitro synthesized transcript coding for Pr55 Gag, fail to support assembly of HIV capsids (data not shown). Since assembly of HIV capsids, as well as assembly of other retroviral capsids, is known to require myristoylation of Gag (Gheysen et al., 1989; Gottlinger et al., 1989) as well as targeting of Gag to membranes (Jacobs et al., 1989; Bryant and Ratner, 1990; Rhee and Hunter, 1990; Wang and Barklis, 1993; Platt and Haffar, 1994; Hockley et al., 1994; Spearman et al., 1994), we modified the standard system to ensure that these events take place. MCoA was added to the reaction, and cellular extracts were fractionated to optimize capsid assembly. Translation of transcript encoding Gag in this cell-free system resulted in the synthesis of ~2 ng of Pr55 protein/μl translation reaction (data not shown).

We expected some of these newly synthesized Pr55 chains to become myristoylated, target to membrane vesicles present in the cellular extract, and assemble into stable capsids. Other Pr55 chains might achieve some but not all of the steps in capsid assembly, and would therefore be detectable as heterogeneous assembly intermediates. To quantify the amount of assembly, an efficient method of separating assembled capsids from the remainder of the translation reaction was required. This was achieved in two steps. First, after completion of the assembly reaction, membrane vesicles were solubilized using the nonionic detergent NP-40. This treatment, which removes membranes enveloping capsids but does not affect the integrity of fully assembled capsids (Yu et al., 1993; Spearman et al., 1994), should solubilize unassembled Pr55 chains that may be anchored to membranes. Secondly, the detergent-treated, completed reaction was size fractionated on a sucrose step gradient into a soluble fraction (containing material of <500-S) and a particulate fraction (containing material between 500- and 1,000-S) that would be expected to contain completed immature capsids (Royer et al., 1991). Thus, while our standard translation and assembly reaction was carried out in the absence of detergent to allow association with membrane vesicles, analysis of the reaction product was performed in the presence of detergent, allowing the assembly status of the naked proteinacious capsid to be assessed.

We found that the particulate fraction from a typical translation and assembly reaction contained between 15 and 40% of the total Pr55 synthesized (see Fig. 2). To assess whether this fraction contained properly assembled immature capsids, it was further analyzed by velocity sedimentation on linear sucrose gradients, equilibrium centrifugation in CsCl, and EM. For comparison, authentic immature HIV capsids produced in transfected cultured COS-1 cells were analyzed in parallel. We found that both detergent-treated capsids generated in the cell-free system and detergent-treated (de-enveloped) authentic capsids behaved as a relatively homogenous population of particles of ~750-S (Fig. 1, *A* and *B*), with a buoyant density of 1.36 g cm^{-3} (Fig. 1, *C* and *D*). Additionally, cell-free assembled capsids and the authentic standard were identical in size as judged by gel filtration (data not shown). When examined by EM, we found that capsids made in the cell-free system (Fig. 1 *E*, *1–4*) were morphologically similar to authentic capsids released from transfected cells (Fig. 1 *E*, *5* and *6*) and had the expected diameter of ~100 nm (Gelderblom, 1991). Thus, on average, 25% of the radiolabeled Pr55 protein synthesized in the cell-free system assembles into particles that closely resemble authentic immature HIV capsids generated in transfected cells, as judged by EM appearance as well as the biochemical criteria of size, sedimentation coefficient, and buoyant density.

Fidelity of Cell-free HIV Capsid Assembly

To determine whether the process of capsid assembly in

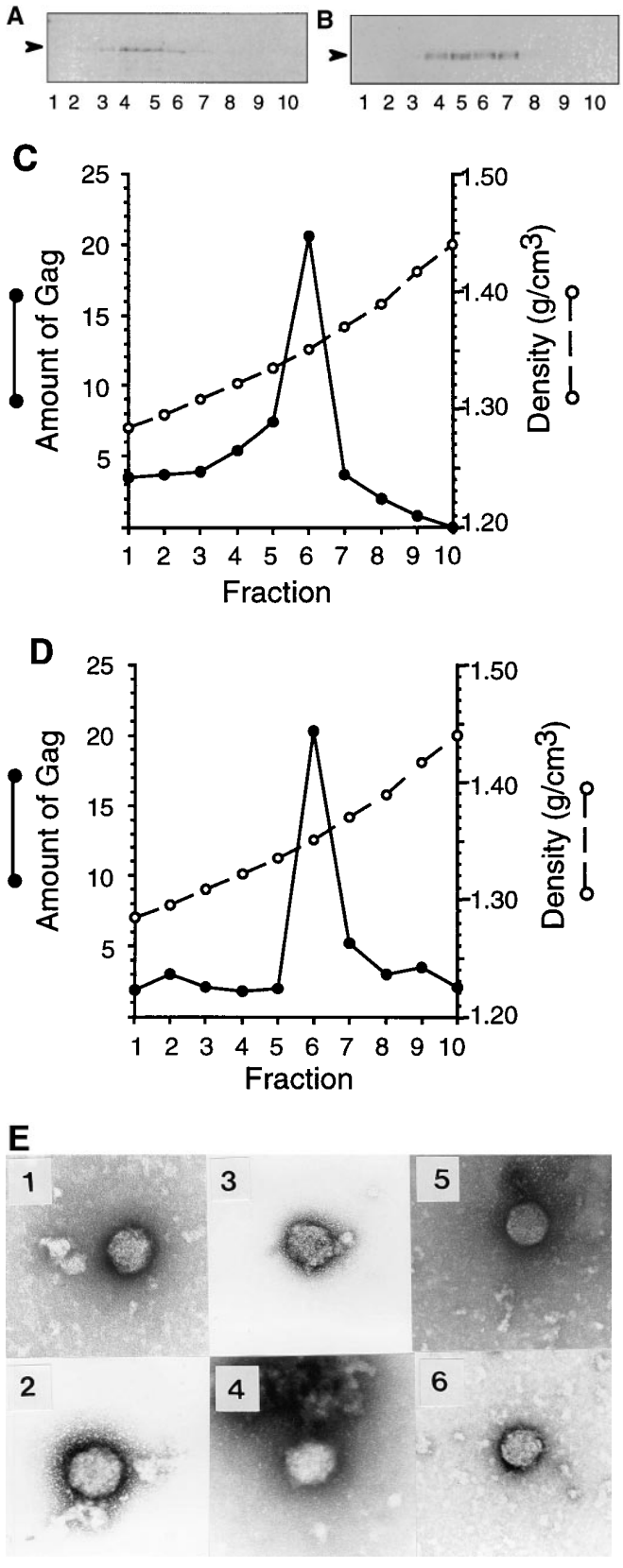

Figure 1. Immature HIV capsids synthesized in the cell-free system resemble authentic capsids by biochemical and morphologic criteria. (*A* and *B*) Migration of capsids on velocity sedimentation gradients. (*A*) Cell-free translation and assembly reactions containing 10 μM MCoA and ^{35}S methionine were programmed with HIV Gag transcript and performed as described in Materials and Methods. At the end of the reaction, samples were diluted into buffer containing 1% NP-40 and separated into soluble and particulate fractions on sucrose step gradients. Material in the particulate fraction (>500-S) was further analyzed by a variety of methods. Shown here is an analysis of the particulate fraction by velocity sedimentation on a linear sucrose gradient. Radiolabeled Pr55 protein in the gradient fractions was visualized by SDS-PAGE and autoradiography. The autoradiograph shows 10 consecutive fractions of a gradient with the top of the gradient at the left side. (*B*) Cell culture medium was collected from transfected Cos cells that were releasing immature HIV particles. Virus particles were harvested by ultracentrifugation through a 20% sucrose cushion, as described in Materials and Methods. Authentic immature capsids were generated by treating these harvested particles with detergent to remove their envelopes. These authentic capsids were sedimented on a velocity sedimentation gradient, in parallel with the particulate fraction of the cell-free reaction shown in *A*. Pr55 present in these fractions was visualized by immunoblotting. Fractions are displayed in the same order described for *A*. (*C* and *D*) Examination of buoyant density. The particulate fraction of the cell-free reaction (*C*) and of authentic immature capsids (*D*) was analyzed in parallel by equilibrium centrifugation on CsCl. Particle density was obtained by measuring the refractive index of each fraction and plotted on the right ordinate (*open circles*). Gag polypeptide in the fractions was visualized, and the amount of protein was determined by densitometry of bands and plotted on the left ordinate (*closed circles*). (*E*) Examination of electron microscopic appearance. The particulate fraction of the cell-free reaction (*1–4*) and authentic capsids (*5* and *6*) were incubated on EM grids that were coated with antibody to HIV Gag. 100-nm particles were visualized by negative staining.

the cell-free system is the same as that which occurs in cells, we examined whether known characteristics of capsid assembly were also reconstituted. Studies in cellular systems have demonstrated that myristoylation of Gag is required for proper capsid assembly (Gheysen, 1989; Gottlinger, 1989). This NH_2-terminal modification is thought to occur co-translationally (Wilcox et al., 1987). To assess the role of myristoylation in the cell-free system we omitted MCoA from the assembly reaction, or added it posttranslationally at 90 min into the reaction (when >90% of Pr55 chains have finished synthesis; data not shown). In both cases, a dramatic decrease in capsid assembly was observed (Fig. 2 *A*). Consistent with these data, we found that a Gag mutant that fails to become myristoylated (GΔA) also does not assemble in the cell-free system (see Fig. 3 *B*).

In cells, assembly of HIV capsids takes place at the inner surface of the host cell plasma membrane (Gelderblom, 1991; Wills and Craven, 1991). Membrane targeting of Pr55 chains is directed by the NH_2-terminal myristate moiety and by internal domains of the Pr55 polypeptide (Jacobs et al., 1989; Bryant and Ratner, 1990; Wang and Barklis, 1993; Platt and Haffar, 1994; Hockley et al., 1994; Spearman et al., 1994). Studies using Pr55 mutations have shown that abolishing membrane targeting results in inefficient capsid assembly and failure of capsid release (Jacobs et al., 1989; Bryant and Ratner 1990). For this reason, targeting to plasma membranes is thought to facilitate the nucleation of Gag chains at a site within the cell where assembly, envelopment, and budding of the newly formed capsids can occur. If immature HIV capsid assembly in the

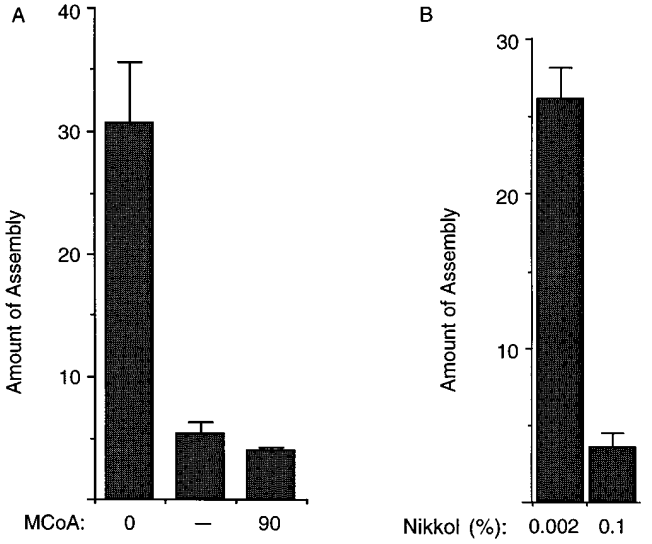

Figure 2. Characteristics of HIV capsid assembly are reproduced in the cell-free system. (*A*) Co-translational myristoylation is required for capsid assembly. Cell-free translation and assembly reactions were programmed with Gag transcript in the absence of added MCoA (−), or with 10 μM MCoA added either at the start of the reaction (*0*) or at 90 min into the reaction when translation is completed (*90*). The detergent-treated products of the cell-free reactions were separated into soluble and particulate fractions by centrifugation on step gradients, and radiolabeled protein in each fraction was visualized by SDS-PAGE and autoradiography. The amount of radiolabeled Pr55 in the particulate fraction (which contains assembled capsids) was determined by densitometry of bands and is expressed as a percentage of total Gag protein synthesized. The presence of MCoA had no effect on the total amount of Pr55 synthesized. Values shown are the average of three independent experiments, and error bars indicate standard error. (*B*) Effect of detergent on capsid assembly. Cell-free translation and assembly reactions containing 10 μM MCoA were programmed with Gag transcript. Nikkol, a nonionic detergent that does not affect protein synthesis, was added at the start of the translation reaction to a final concentration of 0.002 or 0.1%, as indicated. At the end of the incubation, the reactions were analyzed for amount of assembly as in Fig. 2 *A*. Addition of detergent had no effect on the total amount of Pr55 synthesized. Values shown are the average of three independent experiments, and error bars indicate standard error.

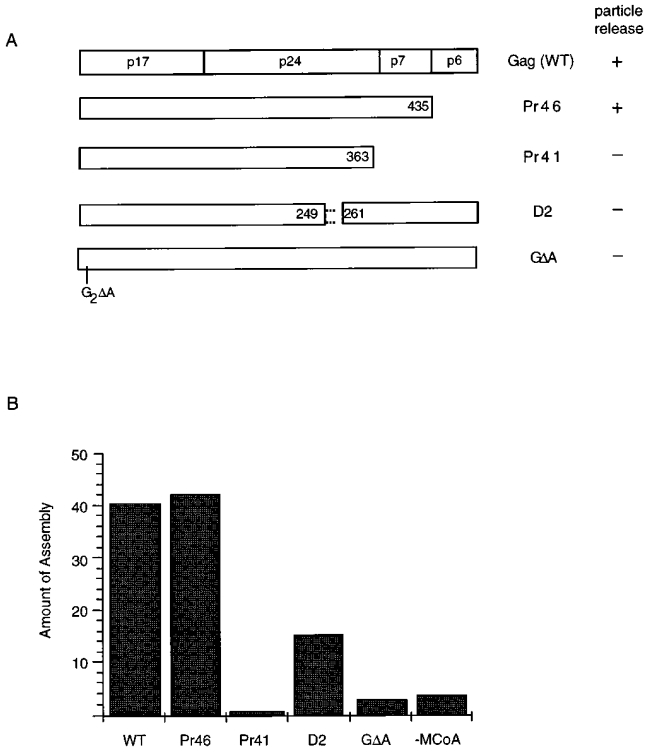

Figure 3. Phenotypes of known mutations in Gag appear to be reproduced in the cell-free system. (*A*) Diagram of mutations within Gag. Gag is a polyprotein precursor that consists of four domains, referred to as p17, p24, p7, and p6. The Pr46 and Pr41 mutants were constructed by introducing a stop codon truncation at amino acid 435 or at amino acid 363, respectively. In the D2 mutation, amino acids 250 to 260 are deleted. In the GΔA mutation, the glycine at amino acid 2 is substituted with an alanine, thereby blocking myristoylation. The known phenotypes with respect to particle release from cells expressing each of these mutants is indicated to the right. (*B*) Capsid assembly in cell-free reactions programmed with Gag mutants. Cell-free translation and assembly reactions were programmed with transcript coding for each of the Gag mutants described above, as well as transcript coding for wild-type Gag in the presence or absence of MCoA (labeled *WT* and *−MCoA*, respectively). At the end of the reaction period, each sample was detergent treated, fractionated by velocity sedimentation on 13 ml sucrose gradients, and analyzed by SDS-PAGE and autoradiography. The amount of radiolabeled translation product in the position of completed 750-S capsids was quantitated by densitometry and expressed for each reaction as a percentage of total synthesis. The total amount of translation was approximately equal in all reactions.

cell-free system requires membrane fragments (which are present in the wheat germ extracts used), it should be sensitive to addition of detergent at concentrations that solubilize membranes. Thus, as a final criterion of authenticity of the cell-free capsid assembly reaction, we investigated the sensitivity of cell-free capsid assembly to the presence of detergent during the translation and assembly reaction. We treated the assembly reaction with a gentle, nonionic detergent that does not inhibit any of the enzymatic reactions involved in protein synthesis (Walter and Blobel, 1980) and thus would not be likely to affect protein interactions involved in assembly. As expected, the detergent Nikkol added to a concentration of 0.1% at the beginning of the translation reaction had no effect on Gag polypeptide synthesis (data not shown). However, as shown in Fig. 2 *B*, Nikkol at this concentration largely abolished capsid assembly. This effect was not observed when Nikkol was used at a concentration of 0.002%, which is below that required to disrupt lipid bilayers (Walter and Blobel, 1980). Furthermore, we found that Nikkol added after the completion of the 150 min assembly reaction did not diminish the amount of assembly, even when added to a concentration of 1.0% (data not shown). Thus, it appears that whereas the completed capsid particle itself is not sensitive to Nikkol (even at high concentrations), assembly of this structure is inhibited by concentrations of Nikkol that are sufficient to solubilize membranes. These data are consistent with the idea that membranes are required for newly synthesized Pr55 to be efficiently assembled into capsids in the cell-free system. Thus, the dependence of assembly on

cotranslational myristoylation and the apparent requirement for membranes indicate that capsid assembly in the cell-free system faithfully reproduces characteristics of capsid assembly that have been described in cellular systems.

Studies of capsid assembly in cell culture model systems have revealed that certain mutations within the Gag coding region disrupt immature HIV capsid assembly. As a third criterion for fidelity, we examined the assembly phenotypes in the cell-free system of four previously described mutations in Gag (Fig. 3 *A*): (*a*) the Pr46 mutant, in which the COOH-terminal p6 domain of Gag is deleted (Royer et al., 1991; Jowett et al., 1992; Hockley et al., 1994; Spearman et al., 1994); (*b*) the Pr41 mutant, in which the deleted domains include p6, p7, and the distal end of p24 containing the p24–p7 protease cleavage site (Gheysen et al., 1989; Jowett et al., 1992; Hockley et al., 1994); (*c*) the D2 mutation, in which 10 amino acids of the p24 domain of Gag (upstream of the p24-p7 protease cleavage site) are deleted (Hockley et al., 1994; Zhao et al., 1994); and (*d*) the GΔA mutation, an NH_2-terminal single amino acid substitution that abolishes myristoylation of Gag (Gottlinger et al., 1989; Bryant and Ratner, 1990). Upon expression in cells, only the Pr46 mutant was capable of producing viral particles indistinguishable from those produced by expression of wild-type Gag (Royer et al., 1991; Jowett et al., 1992; Hockley et al., 1994; Spearman et al., 1994). Each of the other three mutants fails to result in proper viral particle production and release (Gheysen et al., 1989; Gottlinger et al., 1989; Bryant and Ratner, 1990; Jowett et al., 1992; Hockley et al., 1994; Zhao et al., 1994).

Assembly reactions were programmed with transcript encoding each of these mutations and the amount of immature capsid produced was quantitated as described in the legend of Fig. 3. As is shown in Fig. 3 *B*, the Pr41 and GΔA mutants failed to assemble into completed capsids, while ~40% of the total translation product of the assembly-competent Pr46 mutant and wild-type Gag assembled into completed capsids. The D2 mutant appeared to have generated a small amount of material in the region of completed capsids, but further analysis of this material revealed it to be the trail of a large Gag complex (of ~400–500 S) that does not comigrate with completed capsid (see Fig. 6 *E*). Thus, like Pr41 and GΔA, D2 did not assemble into the 750-S completed capsid. Together, these data indicate that the cell-free system appears to faithfully reproduce phenotypes of a variety of assembly-defective and -competent mutations in Gag.

Posttranslational Requirements for Capsid Assembly

For optimum assembly to occur, the cell-free reaction must be incubated at 25°C for at least 150 min (data not shown). We observed that whereas the majority of Pr55 synthesis occurs during the first hour of this incubation, significant capsid formation does not take place until the final 90 min of the reaction. Thus, an aliquot of the reaction incubated for only 50 min contains ~60% of the full length Pr55 chains that are present in an aliquot incubated for the standard 150 min (data not shown). However, essentially none of the chains present at the 50 min time point has assembled into capsids (data not shown), while at 150 min 25% have completed the assembly process (Fig. 4 *A*). This indicated

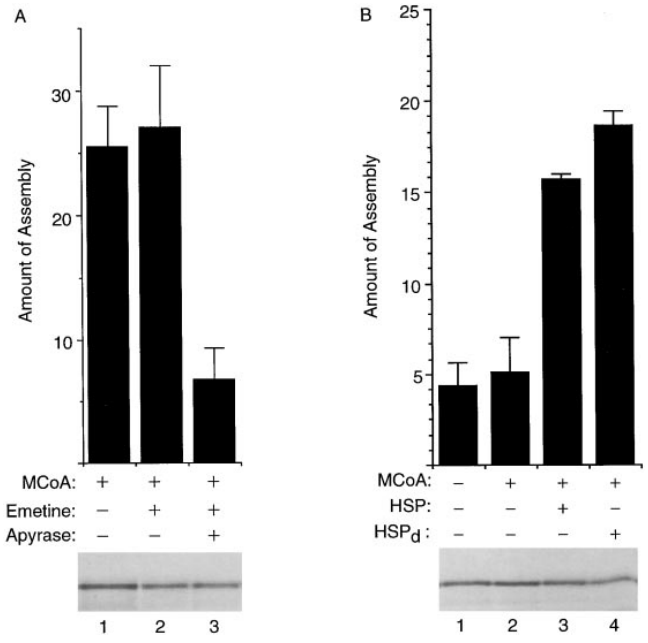

Figure 4. Both ATP and a subcellular fraction of the cell lysate are required for capsid assembly. (*A*) Effect of ATP hydrolysis on the posttranslational phase of capsid assembly. As in Fig. 2 *B*, cell-free translation and assembly reactions were programmed with Pr55 in the presence of 10 μM MCoA. At 50 min into translation, 0.2 μM emetine, a protein synthesis inhibitor, was added to some reactions as indicated. Immediately after emetine treatment, apyrase, an enzyme that hydrolyzes ATP, was added at a concentration of 1 U/μl to one of the emetine-treated reactions. At the end of the incubation (150 min), 1 μl of each reaction was analyzed directly by SDS-PAGE (autoradiographs are shown below *bar graph*). The remainder of the products was analyzed for amount of assembly as described in Fig. 2. Shown in the bar graph is the amount of Pr55 assembled as a percentage of total Pr55 synthesized in each reaction. Values in the bar graph are the average of three independent experiments, and error bars indicate the standard error. (*B*) Effect on assembly of depletion and reconstitution of a subcellular fraction. Wheat germ extract was subjected to ultracentrifugation as described in Materials and Methods to generate the HSS, HSP, and HSP_d. The HSS was used to program cell-free translation and assembly reactions in the presence or absence of 10 μM MCoA (as indicated). Each of these reactions was treated with the protein synthesis inhibitor emetine for 50 min. After this, the HSP or HSP_d was added to aliquots of the reaction as indicated below the bar graph. All reactions were incubated for a total of 150 min. A 1 μl aliquot was removed and analyzed directly by SDS-PAGE (shown below *bar graph*). The remainder of each reaction was analyzed for amount of assembly as described in Fig. 2 and plotted as a percentage of total Pr55 present in each reaction. The values shown in the bar graph are the average of three independent experiments, and error bars indicate the standard error.

to us that it might be possible to completely separate the translation and assembly phases of the reaction. To confirm this, a reaction was split into two aliquots at 50 min into the incubation. To one aliquot we added emetine, which blocks translation by inhibiting chain elongation, and then both aliquots were incubated to 150 min. While total Pr55 synthesis in the emetine-treated reaction was 60% of the control (Fig. 4 *A*, compare gel lanes *1* and *2*), the proportion of capsid assembly in this treated reaction was compa-

rable to that of the untreated control (Fig. 4 *A*, *bar graph*), indicating that assembly takes place even when translation is halted. Based on these data, one would expect that manipulations performed after emetine treatment would have effects on only the posttranslational phase of assembly and should not affect Pr55 synthesis, which is already completed.

Having established a means of analyzing events specific to assembly, we then examined whether the previously described detergent sensitivity of HIV capsid assembly (Fig. 2 *B*) was due to an effect on co- or posttranslational events. We treated Pr55 reactions with emetine and 0.1% Nikkol at 50 min into the reaction, continued the incubation to 150 min, and then determined the amount of assembly as previously described. Under these conditions, assembly was dramatically reduced (data not shown), just as it had been when Nikkol was added at the start of translation (Fig. 2 *B*). Therefore, we conclude that the detergent sensitive step(s) in HIV capsid assembly occurs posttranslationally.

Next we examined whether posttranslational events in capsid assembly are dependent on the presence of ATP. Translation of Gag was allowed to proceed for 50 min, at which point further protein synthesis was inhibited with emetine. The sample was divided, and one aliquot was depleted of ATP by the addition of apyrase, an enzyme that hydrolyzes free ATP. After completion of the assembly reaction, samples were analyzed as previously described for the amount of assembly that had occurred. We found that depletion of free ATP from the assembly reaction resulted in a dramatic reduction in capsid assembly (Fig. 4 *A*, *bar graph*). The effect of ATP depletion was not reversed by addition of the nonhydrolyzable analogues, AMP-PNP or GTPγ-S (data not shown), suggesting that ATP hydrolysis, and not just ATP binding, is required. As expected, addition of apyrase did not change the total amount of Pr55 synthesis (Fig. 4 *A*, compare gel lanes *2* and *3*). Furthermore, adding apyrase to the reaction after capsid assembly was completed had no effect on the amount of assembly (data not shown), ruling out the possibility of an effect of apyrase or ATP depletion on capsid stability. These data indicate that there is a requirement for ATP in the capsid assembly process, and that this ATP dependence is distinct from the energy requirements of protein synthesis.

A Subcellular Fraction Is Required for Capsid Assembly

Given that the Gag protein itself is not known to bind or hydrolyze ATP, the requirement for this energy substrate suggests that a host protein or proteins are involved in the assembly reaction. Demonstrating that such a host factor is required would involve showing that assembly fails to occur when this factor is depleted from the cellular extracts used in the assembly reaction. Furthermore, adding back the depleted fraction to the reaction should restore assembly. Thus, we sought to fractionate the translation extract in a manner that would leave intact all of the components needed for translation but remove at least one component required for assembly. The cellular extract used for translation and assembly was subfractionated by ultracentrifugation into two portions: HSS and HSP. The HSS, depleted of components that were 90-S or greater, supported Pr55 translation but not its assembly (Fig. 4 *B*). This indicates that the HSP likely contains an assembly specific host factor(s). This was demonstrated directly by showing that addition of the HSP posttranslationally (after emetine treatment) to unassembled Gag chains synthesized in the HSS resulted in a considerable restoration of particle assembly (Fig. 4 *B*). As expected, total synthesis of Pr55 was unaltered by addition of the HSP (Fig. 4 *B*, *gel*). Together, these data indicate that a subcellular fraction of the eukaryotic cell lysate is required for posttranslational events in capsid assembly to take place.

We had previously observed that detergent also disrupts the posttranslational phase of capsid assembly (data not shown). We examined, therefore, whether the HSP contained the detergent-sensitive component that is required for capsid formation. HSP was prepared from a cell extract treated with detergent (0.5% Nikkol). The resulting HSP was washed with detergent-free buffer and added posttranslationally to an assembly reaction. We found that the HSP from the detergent-treated extract was as active in promoting posttranslational capsid formation as the control HSP (Fig. 4 *B*, *bar graph*). Thus, separate detergent-sensitive and -insensitive host factors appear to be involved in the posttranslational phase of HIV capsid assembly. Furthermore, the detergent-insensitive host factor can be depleted by ultracentrifugation and then reconstituted by posttranslational addition.

Intermediates in the Assembly of HIV Capsids

The existence of multiple specific requirements for HIV capsid biogenesis, including host factors and ATP, suggests that discrete biochemical intermediates may exist during the assembly process. To date, such intermediates in capsid assembly have not been described, most likely due to the difficulty of detection in cellular systems where they would be present only transiently and in minute quantities. Since the cell-free system is less efficient at forming completed capsids than are cellular systems, it might constitute a better system for detecting such assembly intermediates. Consistent with this possibility, we found that under conditions where only a small amount of 750-S capsid assembly occurs in the cell-free reaction, analysis of the reaction products on 13 ml velocity sedimentation gradients revealed the presence of a series of Gag-containing complexes, in addition to 750-S completed capsids (Fig. 5 *A*). These complexes, which have calculated sedimentation coefficients of ~10-S, 80-S, and 150-S, will henceforth be referred to as complexes A, B, and C, respectively.

If these complexes represent assembly intermediates, they would be expected to be present in large quantities at early time points, and to be diminished at later times in the reaction. To assess whether these complexes displayed this behavior, we used a pulse–chase analysis to follow a small cohort of radiolabeled Pr55 chains over time. The cell-free reaction was performed with radiolabeled cysteine. After the first 4 min of translation, an excess of unlabeled cysteine was added to abolish further labeling of subsequently synthesized Pr55 chains. We observed that the total amount of radiolabeled Pr55 was the same at 25 and 150 min into the pulse–chase reaction (Fig. 5 *B*), indicating

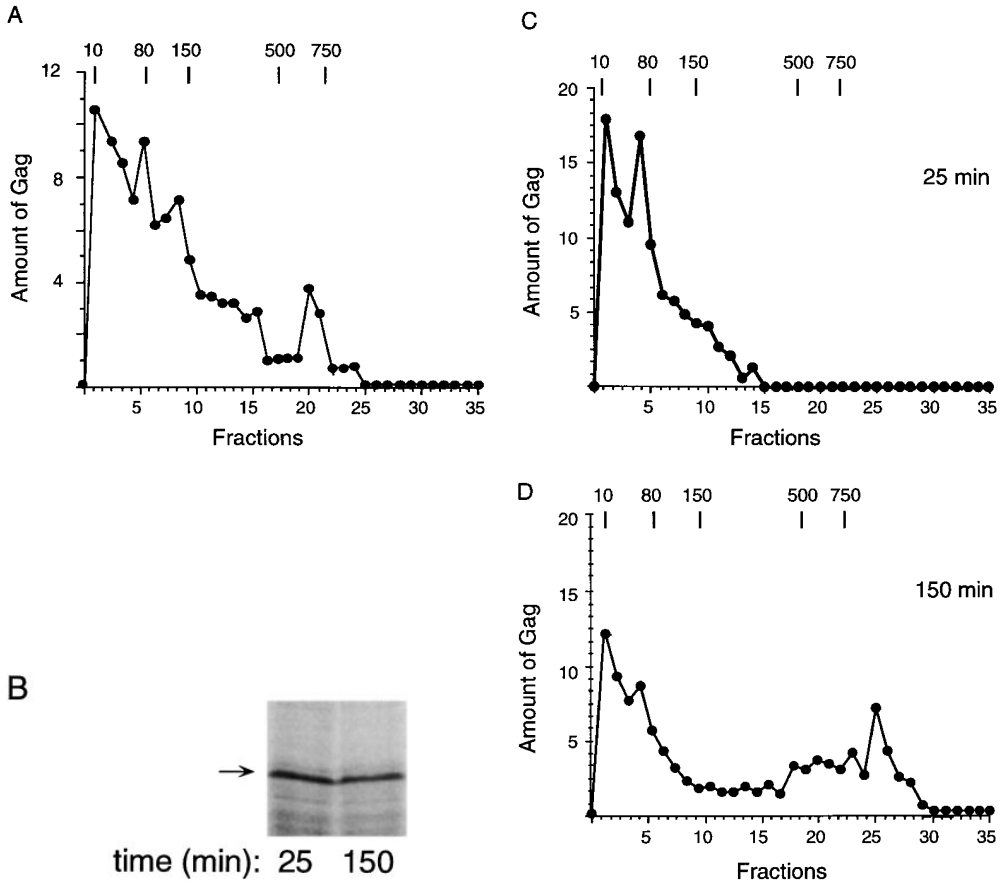

Figure 5. Pulse–chase analysis of HIV capsid assembly. (*A*) Analysis of a continuously labeled cell-free reaction by velocity sedimentation. Cell-free translation and assembly of Pr55 were performed as previously described. Upon completion of the cell-free reaction, the products were diluted in 1% NP-40 sample buffer on ice and were analyzed by velocity sedimentation on 13 ml 15–60% sucrose gradients. Fractions were collected from the top of each gradient as described in Materials and Methods, and the amount of radiolabeled Pr55 protein in each fraction was determined and expressed as a percentage of total Pr55 protein present in the reaction. The calculated positions of 10-, 80-, 150-, 500-, and 750-S complexes are indicated with markers above (see Materials and Methods). 750-S represents the position of authentic immature (de-enveloped) HIV capsids. (*B–D*) Analysis of a pulse–chase cell-free reaction by velocity sedimentation. Cell-free translation and assembly of Pr55 were performed as previously described, except that [^{35}S]cysteine was used for radiolabeling. At 4 min into translation, an excess of unlabeled cysteine was added to the reaction so that no further radiolabeling would occur. Aliquots of the reaction were collected 25 (*C*) and 150 min (*D*) into the reaction. 1 μl of each aliquot was analyzed by SDS-PAGE and autoradiography to reveal the total amount of radiolabeled Pr55 translation product (*B*, *arrow*) present at each chase time. The remainder of the aliquots was diluted into 1% NP-40 sample buffer on ice and analyzed by velocity sedimentation on 13 ml 15–60% sucrose gradients (5, *C* and *D*, respectively), in the manner described for 5 *A* above.

that neither further radiolabeling nor degradation of Pr55 had occurred after 25 min and confirming that the same population of Pr55 chains was being analyzed at both times. Aliquots of the cell-free reactions were removed at the times indicated, treated with detergent, and then analyzed on 13 ml velocity sedimentation gradients.

At 25 min into the reaction, all of the radiolabeled Pr55 was found in complexes A, B, and C (Fig. 5 *C*), with no radiolabeled Pr55 present in the region of completed 750-S capsids. While complexes A and B appear as peaks at ~ the 10-S and 80-S positions of the gradient, complex C appears as a less distinct shoulder in approximately the 150-S position. In marked contrast, examination of the assembly reaction at 150 min showed that a significant amount of radiolabeled Pr55 was assembled into completed capsids that migrated in the 750-S position (Fig. 5 *D*). Correspondingly, the amount of Pr55 in complexes A, B, and C was diminished by precisely the amount that was now found to be assembled, demonstrating that at least some of the material in complexes A, B, and C constitutes intermediates in the biogenesis of completed 750-S capsids.

At extremely short chase times (i.e., 13 min), when only some of the radiolabeled chains have completed synthesis, full length Pr55 chains were found exclusively in complex A on 13 ml sucrose gradients, while nascent chains that are not yet completed were in the form of polysomes of greater than 100-S (data not shown). Thus, polysome-associated nascent chains of Gag constitute the starting material in this pathway, and the 10-S complex A, which contains completed Gag chains, is likely to be the first intermediate in the formation of immature capsids. It is possible, therefore, that complexes B and C represent later assembly intermediates in the pathway of capsid formation.

If complexes A, B, and C do in fact constitute intermediates in assembly, one would predict that blockade of assembly would result in accumulation of Gag chains in the form of complexes with those S values. Furthermore, one would expect that blockade at different points along this pathway would result in accumulation of complexes A, B, and C in various combinations, as determined by the order of their appearance along the assembly pathway. For example, if an ordered pathway of intermediates exists, then blockade at early points in the pathway should result in accumulation of one or two Gag-containing complexes corresponding to early putative assembly intermediates, while blockade at a very late point in the pathway would result

in accumulation of all the putative assembly intermediates but not the final completed capsid product. To test these predictions, we blocked assembly in a variety of ways, both pharmacologically and through the use of mutations, and examined the Gag-containing complexes that were formed.

Pharmacological Blockade Results in Accumulation of Assembly Intermediates

Capsid assembly was disrupted by adding either apyrase posttranslationally (as in Fig. 3) or detergent co-translationally (as in Fig. 2), and the reaction products were analyzed by velocity sedimentation as described for Fig. 5. Material in fractions corresponding to the assembly intermediates and completed capsid were quantified and are presented in Table I. The untreated reaction contained Pr55 in complexes A, B, and C, as well as a peak in the final 750-S capsid position, while the treated reactions, as expected, contained no peak at the position of the final capsid product (Table I). Treatment with either apyrase or detergent resulted in accumulation of additional material in complexes B and C, but did not result in accumulation of additional material in complex A. This is consistent with the idea that complexes B and C are the more immediate precursors of the 750-S completed capsids, and that these interventions block the conversion of complexes B and C into the fully assembled capsid end product.

Assembly-defective Mutants Are Arrested at Specific Points in the Assembly Pathway

To determine whether the putative assembly intermediates accumulate when capsid assembly is blocked by specific mutations in Gag, cell-free reactions were programmed with each of the previously described assembly-competent and -defective Gag mutants (Fig. 3), incubated for 150 min, and then analyzed by velocity sedimentation. Reactions programmed with wild-type Gag (Fig. 6 *A*) or the assembly-competent Pr46 mutant (Fig. 6 *B*) were found to have nearly identical profiles, in which >30% of the radiolabeled Gag chains synthesized formed completed immature capsids (that migrate at 750-S), and the remainder was in the form of residual putative assembly intermediates A and B. Thus, these two assembly-competent forms of Gag appear to be indistinguishable in the cell-free system just as they are when expressed in cells (Royer et al., 1991; Jowett et al., 1992; Hockley et al., 1994; Spearman et al., 1994).

Table I. Effect of ATP Hydrolysis and Detergent Treatment on Assembly Intermediates

	A	B/C	Final capsid
Untreated	2,798	5,046	739
+ Apyrase	2,851	5,999	133
+ Detergent	2,656	6,130	189

Cell-free translation and assembly reactions were programmed with Pr55 in the presence of 10 μM MCoA. One reaction was treated with emetine for 50 min followed by apyrase (as described in Fig. 4), and a second reaction was treated with the detergent Nikkol to a final concentration of 0.1% added at the start of translation (as described in Fig. 2 *B*). The control reaction was untreated. Reactions were incubated for 150 min, diluted into 1% NP-40 sample buffer on ice, and analyzed in parallel by velocity sedimentation on 13-ml 15–60% sucrose gradients. Fractions were collected, and the amount of radiolabeled Pr55 protein present in the complexes A, B, and C and final completed capsid was determined. Note that material present in the 750-S region of the treated reactions consisted of a trail from a smaller complex.

Fig. 6 *C* shows the same analysis for the assembly defective Pr41 mutant. We found that all of the radiolabeled chains at the end of the Pr41 cell-free reaction were contained in a single complex that peaks at ~10-S and corresponds to complex A. Since the 10-S peak was very large and led to an irregular trail that could be masking 80- or 150-S peaks, we reanalyzed the products of a Pr41 reaction on a gradient that allowed high resolution in the 1–200-S size range (data not shown) and confirmed that all of the Pr41 translation product was present in complex A, which was ~10-S in size. Thus, in the cell-free system, it appears that Pr41 fails to progress beyond complex A, which is likely to represent the first intermediate in the assembly pathway.

Like Pr41, the myristoylation-incompetent GΔA mutant failed to assemble into 750-S capsids (Fig. 3 *B* and Fig. 6 *D*), but unlike Pr41, GΔA had distinct peaks in both the 10- and 80-S regions of the gradient (Fig. 6, compare *D* to *C*). These data indicate that the GΔA mutant, which contains the entire Gag coding region except for the myristoylation signal, is capable of forming complex A, which appeared to be the first assembly intermediate in the pulse–chase experiment, as well as complex B, but does not progress further towards forming completed capsids. These data suggest that complex B is likely to be the second assembly intermediate formed in the biogenesis of immature HIV capsids.

Previously, we had shown that in the absence of exogenously added MCoA, wild-type Gag failed to assemble in the cell-free system (Fig. 2 *A*), consistent with previous observations that myristoylation is required for proper capsid assembly to occur. Thus, a cell-free reaction programmed with wild-type Gag but performed in the absence of MCoA would be expected to be blocked at the same point in the assembly pathway as the GΔA mutant. Consistent with this, we found that assembly performed in the absence of MCoA resulted in formation of only complexes A and B (data not shown), and therefore closely resembled the GΔA mutant shown in Fig. 2 *D*.

Analysis of a cell-free reaction programmed with the D2 mutant is shown in Fig. 2 *E*. Unlike the previously described assembly-defective mutants, D2 was found to form a spectrum of Gag-containing complexes, including peaks corresponding to complexes A and B (at ~10- and 80-S), a shoulder corresponding to complex C (in the 150-S region), and an additional peak of ~400–500-S, that will henceforth be referred to as complex D. Note that complex D trails into the 750-S region, accounting for the appearance of a small amount of assembly in the simpler analysis presented in Fig. 1. However, the detailed analysis presented here makes it clear that in fact there is no discrete peak in the region of completed capsid (750-S). Thus, the D2 mutant appears to form a series of complexes corresponding in size to the assembly intermediates seen in the pulse–chase experiment (Fig. 5), as well as an additional complex of larger size, but fails to produce the completed 750-S product.

Gag-containing Complexes within Mammalian Cells

Having demonstrated by pulse–chase analysis, pharmacologic blockade, and analysis of mutants that assembly in-

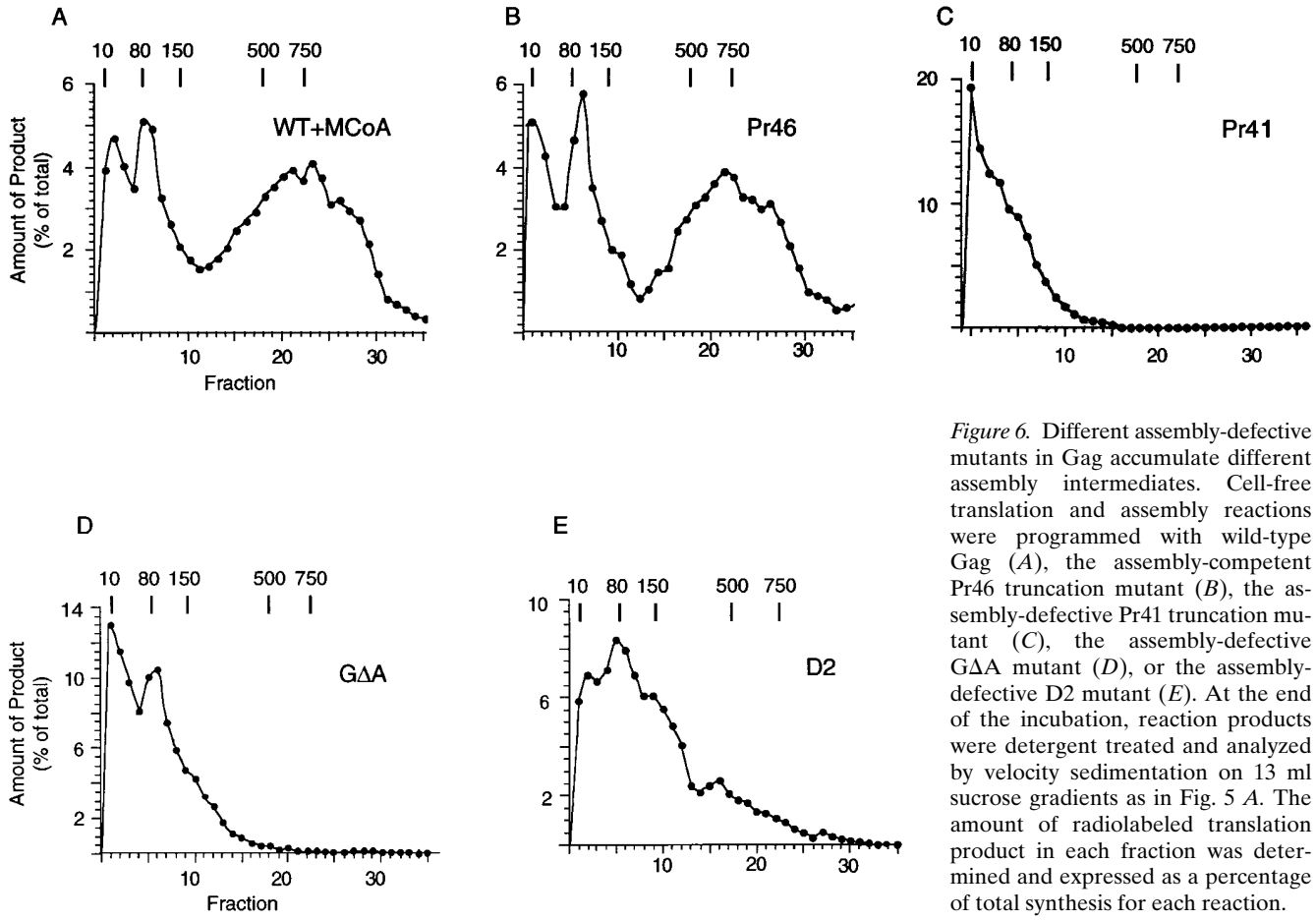

Figure 6. Different assembly-defective mutants in Gag accumulate different assembly intermediates. Cell-free translation and assembly reactions were programmed with wild-type Gag (*A*), the assembly-competent Pr46 truncation mutant (*B*), the assembly-defective Pr41 truncation mutant (*C*), the assembly-defective GΔA mutant (*D*), or the assembly-defective D2 mutant (*E*). At the end of the incubation, reaction products were detergent treated and analyzed by velocity sedimentation on 13 ml sucrose gradients as in Fig. 5 *A*. The amount of radiolabeled translation product in each fraction was determined and expressed as a percentage of total synthesis for each reaction.

termediates appear to exist in the cell-free system, we then examined whether complexes of the same sizes are present in mammalian cells that are expressing wild-type Gag or Gag mutants. The finding of such complexes would be consistent with the existence of similar transient intermediates in vivo.

Cos cells transfected with wild-type Gag were lysed in detergent, and the lysate was analyzed on 13 ml velocity sedimentation gradients, as in Figs. 5 and 6. Gradient fractions were immunoblotted with Gag antibody, and the relative amount of Gag polypeptide present in each fraction was quantitated as described in Materials and Methods (Fig. 7 *A*, *closed circles*). The cell lysate contained Pr55 in a spectrum of complexes ranging from 10- to ~200-S. Since cells efficiently release completed HIV capsids, most of the completed capsid would be expected to be in the medium at steady state. This was confirmed by the finding that a substantial amount of fully assembled 750-S capsid was present in the medium (Fig. 7 *A*, *open circles*), while the cell lysate contained no 750-S capsids (Fig. 7 *A*, *closed circles*). We conclude, therefore, that within cells, Pr55 is present in a heterogeneous mixture of complexes that have S values similar to complexes A, B, and C, the assembly intermediates found in the cell-free system.

If these complexes correspond to the assembly intermediates seen in the cell-free system, one would expect that different assembly-defective mutants would result in the accumulation of the same sized complexes in cellular systems as they do in the cell-free system. Thus, we examined whether the Pr41 mutant, which appears to be blocked after the first assembly intermediate in the cell-free system, and the D2 mutant, which appears to be blocked at the end of the assembly pathway in the cell-free system, resulted in corresponding patterns of Gag-containing complexes within cells. Cos cells were transfected with each of these mutants, and the medium as well as the lysate were examined by immunoblotting. As expected, medium from cells transfected with the assembly-defective Pr41 or D2 did not contain 750-S completed capsids (data not shown). The cell lysate of Cos cells transfected with the Pr41 mutant contained only material that peaked in the 10-S region of the velocity gradient (Fig. 7 *B*), resembling what had been found when the Pr41 mutant was expressed in the cell-free system (Fig. 6 *C*). The observation that the Pr41 reaction product migrated as a single complex that peaked in the 10-S region was confirmed by analysis on a variety of different velocity sedimentation gradients that allowed higher resolution in the 1–200-S size range (data not shown). In contrast, the cell lysate of Cos cells transfected with the D2 mutant contained a spectrum of immunoreactive complexes that ranged in size from 10–500-S (Fig. 7 *C*), resembling what had been found when D2 was expressed in the cell-free system (Fig. 6 *E*). Thus, the data from transfected cells suggests that the behavior of Gag mutants in the cell-free system reflects events in capsid assembly that occur in living cells.

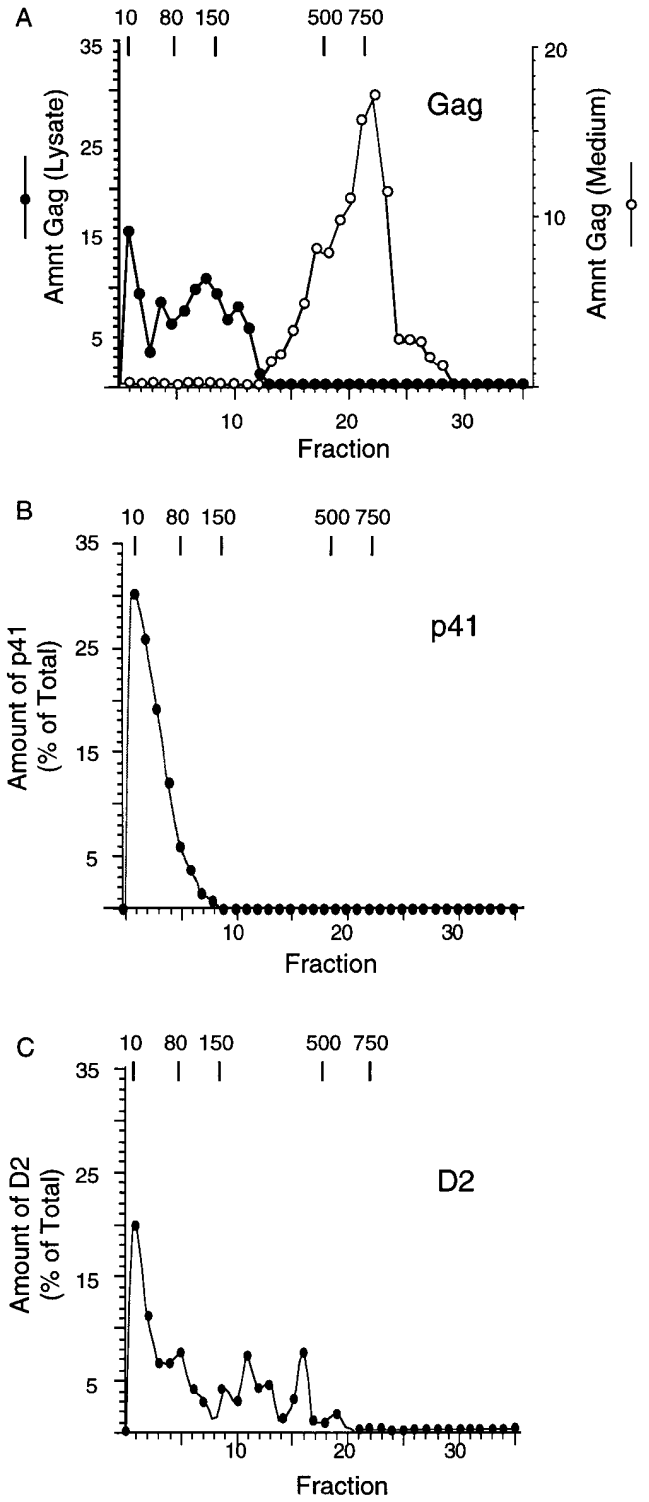

Figure 7. Gag-containing complexes in cells transfected with wild-type Gag or assembly-defective Gag mutants. (*A*) Cos-1 cells were transfected with a transfection vector encoding Pr55 cDNA, as described in Materials and Methods. 4 d later, the medium from the cells was collected. Viral particles in the medium were harvested by ultracentrifugation through a 20% sucrose cushion and then treated with detergent to remove envelopes. The transfected cells were solubilized in detergent to generate the cell lysate (see Materials and Methods). The particles from the medium (right ordinate, *open circles*) and the detergent lysate of the cells (left ordinate, *closed circles*) were analyzed in parallel by velocity sedimentation on 13 ml 15–60% sucrose gradients, as in Fig. 5. The amount of Pr55 protein in each fraction of these gradients was determined by immunoblotting and expressed as a percentage of total Pr55 protein present. The calculated positions of 10-, 80-,150-, 500-, and 750-S complexes are indicated with markers above (see Materials and Methods). 750-S represents the position of authentic, immature (de-enveloped) HIV capsids. (*B* and *C*) Cos-1 cells were transfected with a transfection vector encoding the Pr41 mutant (*B*) or the D2 mutant (*C*). Transfected cells were lysed in detergent, and the lysate was analyzed by velocity sedimentation on 13 ml sucrose gradients, as in *A* above. The amount of capsid protein in each fraction of these gradients was determined by immunoblotting with anti-Gag antibody, and was expressed as a percentage of total immunoreactive protein present in each reaction.

Discussion

In this paper, we first demonstrate that immature HIV capsids can be assembled in a cell-free system. We establish that the capsids produced closely resemble immature HIV capsids produced in cellular systems (Fig. 1) and that key known features of capsid assembly are faithfully reproduced in this cell-free system (Figs. 2 and 3). We then use it to dissect assembly into a number of steps. First, through the use of an inhibitor of protein synthesis, we demonstrate that assembly can be resolved into a co-translational phase, during which Pr55 polypeptides are synthesized, followed by a posttranslational phase, during which additional events necessary for assembly take place. Being able to separate these two phases allows us to analyze reactions specific to the assembly process, independent of other events such as protein synthesis.

By manipulating the posttranslational phase of assembly, we then demonstrate that it has at least three specific requirements (Fig. 4). First, ATP is necessary, since the enzyme apyrase is able to effectively block the posttranslational progression of completed Pr55 chains into fully assembled capsids. Second, this phase is sensitive to nonionic detergent, implying that membrane fragments are likely to be involved. Third, using a depletion and reconstitution approach, we demonstrate that a subcellular fraction of eukaryotic cell lysate is required for the posttranslational assembly events to take place.

In addition, multiple criteria are used to demonstrate that the posttranslational phase of assembly proceeds by way of a series of Gag-containing complexes that appear to constitute intermediates in the cell-free capsid assembly reaction (Figs. 5 and 6 and Table I). Finally, we show that complexes resembling assembly intermediates may also be present in cellular systems that are producing immature HIV particles, and that mutants in Gag have a similar behavior in both cellular and cell-free systems (Fig. 7).

Novel Requirements for HIV Capsid Assembly

To date, studies have not revealed whether HIV capsid assembly occurs by way of a passive process of Gag polypeptide multimerization, or whether assembly is an active, regulated process requiring Gag associations with other proteins, as well as Gag–Gag interactions. By demonstrating that the posttranslational phase of assembly requires ATP and a detergent-insensitive host cell component, our

data indicate that in fact HIV capsid assembly is an energy-dependent, coordinated process in which host proteins play a crucial role. Thus, the model of HIV capsid assembly supported by our data is conceptually very different from the view that many viral capsids are formed from capsid monomers by way of a passive process of "self assembly," requiring no components besides the individual capsid protein subunits (Klug, 1980). Indeed, while host factors have not previously been documented to play a role in the assembly of HIV capsids, the possibility of their existence has been raised by data demonstrating that yeast expressing Gag do not support HIV capsid assembly (Jacobs et al., 1989), while baculovirus and mammalian systems of expression do (for review see Boulanger and Jones, 1996). Such studies imply that yeast either contain a factor that inhibits HIV capsid assembly (that is absent in higher eukaryotes) or lack a factor necessary for assembly (that is present in higher eukaryotes). Our data support the latter possibility. Consistent with the notion that previously unrecognized host factors may be important at various points in the HIV life cycle is the recent demonstration that cyclophilins are associated with the HIV virus particle and are required for infectivity (Luban et al., 1993; Franke et al., 1994; Thali et al., 1994).

The finding that HIV capsid assembly requires ATP as well as host cell components suggests that the posttranslational phase of assembly may occur via a step-wise pathway of previously unrecognized assembly intermediates. We present three lines of evidence to support this view. First, using a pulse–chase analysis we demonstrate that a population of lower molecular weight Gag-containing complexes, referred to as complexes A, B, and C, is present at early time points in capsid assembly, and that Gag polypeptides from this population are converted into the final capsid product over time. Note that the possibility that these complexes represent polyribosomes has been ruled out by the demonstration that they are not sensitive to RNase A used at concentrations that result in complete digestion of messenger RNA (Lingappa, J.R., unpublished observations). At very short chase times, all of the radiolabeled Pr55 in the pulse–chase reaction is present in complex A, suggesting that this may be the first intermediate in the assembly reaction, appearing after completed Gag chains are released from ribosomes. It remains to be determined whether complex A constitutes either a monomer or dimer of Gag, or whether it is composed of a Gag chain associated with a host protein.

Secondly, we show that by blocking assembly pharmacologically, using either apyrase or detergent, material in two of these complexes (complexes B and C) accumulates, indicating that they are likely to be precursors to the completed 750-S capsid end product. Note that these data do not exclude the existence of other intermediates that might occur later in the pathway. Such intermediates would not be seen with apyrase or detergent blockade, since these agents appear to block assembly by preventing progression beyond the 150-S complex. The demonstration that both detergent and ATP depletion appear to result in the accumulation of the same assembly intermediates raises the possibility that this point in the assembly pathway may be a critical one, in which a variety of events necessary for assembly must take place in a coordinated fashion. Further work will be required to determine whether this represents an important point of regulation in the assembly pathway.

Finally, the analysis of three different Gag mutants that are known to be assembly defective lends further support to the presence of assembly intermediates. We find that each mutant results in the accumulation of different subsets of these assembly intermediates. Thus, a mutant that appears to be blocked after the first step in the assembly pathway results in the accumulation of complex A alone (Fig. 6 *C*), while one that appears to be blocked after the second step of the pathway results in accumulation of complex A and complex B (Fig. 6 *D*). A spectrum of complexes, including complexes A, B, and C, as well as the larger complex D, is seen upon expression of a mutant that appears to be blocked at a very late step in the assembly pathway (Fig. 6 *E*). Thus, analysis of these mutants supports the existence of intermediates in the assembly of HIV capsids, and also suggests an order to the appearance of such intermediates during the process of capsid assembly.

There are a number of caveats to our demonstration of assembly intermediates. First, one or more of these putative intermediates could in fact constitute a side reaction or dead end byproduct off the main pathway of capsid assembly. The most likely candidate for such a dead-end byproduct is complex D, which is only clearly apparent in the analysis of the assembly-defective D2 mutant (Fig. 6 *E*). Arguing against the possibility that complexes A, B, or C are simply nonproductive side reactions is the finding that the amount of each of these complexes varies inversely with the amount of completed, immature capsid formed. Such behavior would be expected for assembly intermediates but not for dead-end byproducts or side reactions. For example, the quantity of each of these complexes decreases over time in the pulse–chase experiment, during a period when completed capsids are appearing and the total number of radiolabeled Gag chains present is constant (Fig. 5). Likewise, complexes B and C increase in quantity when formation of capsids is blocked (Table I). Additionally, in an analysis of a mutant that is more efficient in capsid production than wild-type cells, we find that the amount of complex A drops to very low levels when very large amounts of capsid are being made (Hill, R.L., and J.R. Lingappa, unpublished observation). Thus, the simplest interpretation of our data is that the Gag complexes A, B, and C constitute true assembly intermediates, although definitive proof of this must await a functional examination of each of these intermediates in isolation.

A second caveat is that our analysis could be complicated by instability of Gag-containing complexes. Thus, lower molecular weight complexes could actually result from disassembly of a higher order complex. This seems unlikely to be a significant problem in our system, given that we have not observed any of the Gag-containing complexes to be labile in our studies. For example, in the pulse–chase experiments (Fig. 5), there is a clear-cut progression over time from complexes A, B, and C to completed immature capsids. Residual amounts of complexes A and B present at the end of the incubation are likely due to incomplete conversion resulting from depletion and inactivation of necessary factors over time.

A third caveat that should be noted is that there may ex-

ist additional intermediates in HIV capsid assembly that are not obvious from our current analysis. Finally, we wish to point out that the S-values assigned to each of the complexes described here are only approximate S-values, calculated from the migration of these complexes in velocity sedimentation gradients (see Materials and Methods). While one or more complexes may not appear to be distinct in a particular experiment or a particular gradient (i.e., the 150-S complex in Fig. 5 *C*), the specific complexes described here have been seen repeatedly in many different experiments and under a variety of gradient conditions and therefore are likely to represent discrete complexes that are difficult to resolve perfectly in every experiment given their transient and dynamic nature.

A Multi-step Pathway for the Assembly of HIV Capsids

Together, our results support a model of capsid assembly as a stepwise process consisting of discrete biochemical events with specific requirements for ATP and at least one detergent-insensitive component of eukaryotic cell lysate. The model in Fig. 8 presents the simplest scenario that unifies the data presented here. While further analysis of this system may reveal additional complexities, this scheme presents a biochemical framework against which hypotheses can now be tested.

While intermediates along such a pathway have not been demonstrated previously within cells, this could be a consequence of their transient nature and the difficulties involved in manipulating cellular systems. Further work will be required to determine to what extent the mechanism of cell-free HIV capsid assembly reflects biochemical events that occur in vivo. Here we show that, under steady state conditions, cells releasing immature HIV capsids contain Pr55 complexes that correspond in approximate size to complexes A, B, and C, the assembly intermediates observed in the cell-free system. In addition, assembly-defective mutants arrested at specific complexes in the cell-free system appear to be arrested in the form of similar sized complexes when they are expressed in cells (Fig. 7). While these data do not constitute proof that assembly intermediates exist in cells, they lend support to the idea that the process of HIV capsid assembly in the cell-free system is closely correlated to the capsid assembly process in cells. It should be noted that in other biological systems, transient intermediates that have been well documented in cell-free reactions have yet to be identified in cells. For example, in the case of targeting of nascent secretory proteins to the endoplasmic reticulum, signal recognition particle–bound nascent chains have been demonstrated biochemically only in cell-free systems (Walter and Blobel, 1983), even though genetic evidence confirms the importance of signal recognition particles in vivo (Hann and Walter, 1991).

A Mechanistic View of HIV Capsid Assembly

In addition to allowing us to identify novel steps in HIV capsid assembly, the cell-free system also allows us mecha-

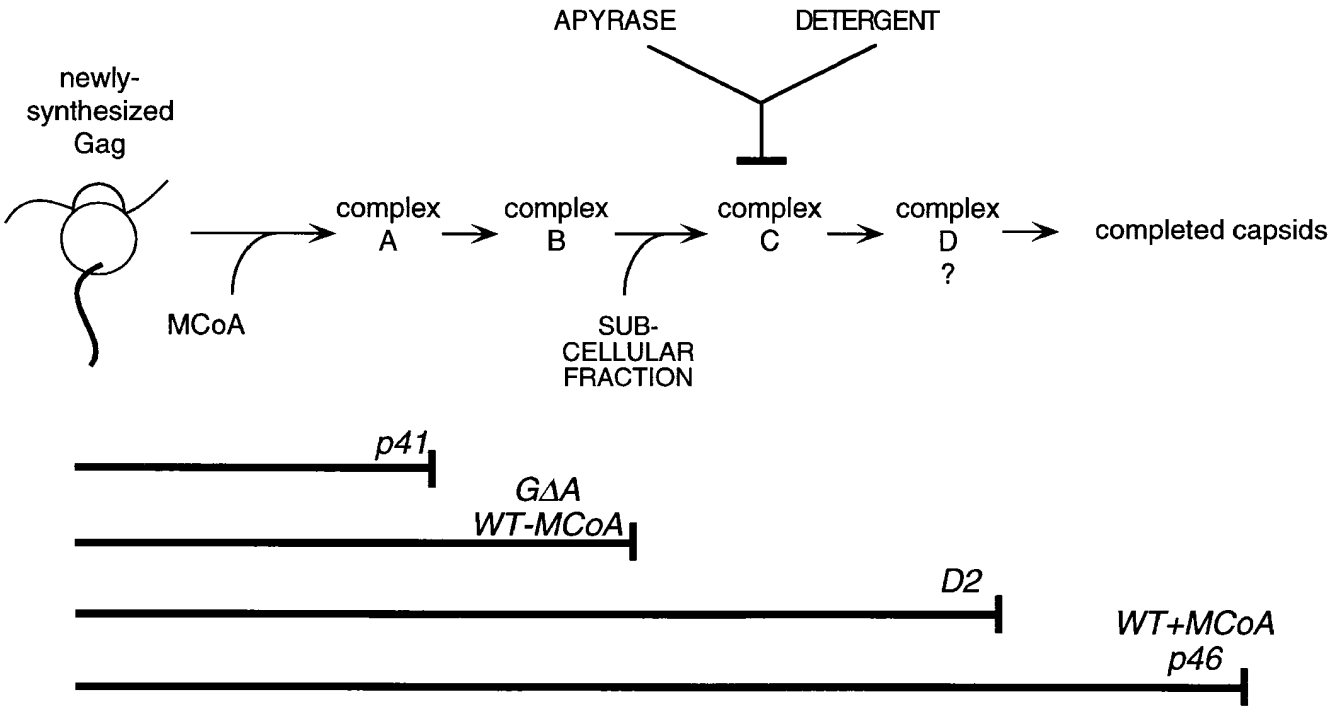

Figure 8. A model for the assembly of immature HIV capsids. This model is based on the simplest interpretation of the data presented. Newly synthesized Gag proteins are myristoylated cotranslationally. Nascent Gag polypeptides appear to chase into completed immature capsids by way of a series of Gag-containing complexes (complexes *A*, *B*, *C*, and *D*). Evidence presented in the text suggests that complexes A, B, and C may constitute assembly intermediates. It is less clear whether complex D constitutes an assembly intermediate or a side reaction. Both ATP and a subcellular fraction of eukaryotic cell extract are required for assembly to take place, while detergent and apyrase are proposed to disrupt assembly at the indicated position. The points along the pathway at which various mutants of Gag are arrested are indicated below the model.

nistic insights into known aspects of capsid assembly. For example, our data suggest that the reason that certain Gag mutants fail to assemble is because they lack domains of the Gag polypeptide that may be required, either directly or indirectly, for progression from one assembly intermediate to the next along the proposed pathway for HIV capsid assembly. Similarly, our results indicate that in the absence of the myristate moiety or in the absence of intact membranes, capsid assembly fails to progress beyond specific stages in the assembly process.

Altogether, we demonstrate that capsid assembly can be disrupted at different points in the proposed assembly pathway by five different means: (*a*) omitting MCoA during the co-translational phase of the reaction, (*b*) depleting ATP during the posttranslational phase, (*c*) treating with detergent either at the start of translation or posttranslationally, (*d*) removing the detergent-insensitive subcellular component(s) from the eukaryotic cell lysate used for the assembly reaction, and (*e*) programming the reaction with assembly-defective Gag mutants. While details of the mechanisms underlying these forms of blockade remain to be determined by further analysis, these data reveal the power of this approach for elucidating the biochemical events that underlie capsid assembly.

Cell-free Assembly of Other Viral Capsids

Cell-free systems have been used in the past to assemble simple viral particles whose formation is dependent only on cytosolic components of host cells (Molla et al., 1991; Lingappa et al., 1994; Sakalian et al., 1996). In the current study we demonstrate that such systems can also be used to reconstitute the assembly of much more complex viral capsids, such as HIV capsids, that require co-translational modifications as well as membrane targeting for their biogenesis. These data suggest that the cell-free system can be used to examine capsid assembly mechanisms of many different types of viruses.

Previously, we used a cell-free system to study the mechanism of assembly of a Hepatitis B Virus (HBV), a completely unrelated virus (Lingappa et al., 1994). Unlike HIV capsids, HBV capsids are formed free in the cytoplasm of the host cell (for review see Ganem, 1991). We found that these capsids assemble by way of an intermediate that is associated with at least one host protein. When this intermediate was isolated, manipulation of energy substrates resulted in release of the completed capsid end product. Thus, while cell-free HIV and HBV capsid assembly differ greatly in many details and in their complexity, they share some general mechanistic themes. Capsid formation in both systems involves assembly intermediates, the use of energy substrates, and association with host proteins. Thus, we propose that viruses of very different types may assemble their capsids via a step-wise pathway using host cell machinery. Whether the same or different host machinery is recruited by various viruses remains to be determined.

Implications of the Cell-free System for Viral Assembly

To allow an analysis of the earliest events in capsid biogenesis, we designed this cell-free system to assemble the simplest version of an HIV capsid, one that does not undergo maturation and lacks HIV genomic RNA and HIV-specific proteins (except for Gag). Now that the method has been established, it should be possible to supplement this system with such additional components in a manner that will extend the reconstitution and allow the roles of other components in capsid assembly to be determined. For example, using a modified version of this system, it should be possible to determine the requirements for encapsidation of viral RNA and viral specific enzymes. Note that although no HIV-specific RNA besides the gag transcript is included in the cell-free reactions described in this study, it is entirely possible that nonspecific RNA from the cellular extract becomes incorporated into the capsids that are made.

Recent studies demonstrating the highly dynamic nature of HIV (Ho et al., 1995; Wei et al., 1995) indicate that the only successful approach to combating this infection will be simultaneous, multi-drug therapy. Antiretroviral agents currently in use target only two aspects of the HIV life-cycle, namely reverse transcription and protease-dependent virion maturation. In order to develop novel targets for future antiretroviral therapy, a mechanistic analysis of other aspects of the HIV life cycle, such as capsid assembly, must be aggressively pursued. The work presented here establishes a means of carrying out such a biochemical analysis for assembly of the HIV capsid. Furthermore, these data indicate the existence of novel steps in viral assembly that appear to be amenable to blockade.

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