

# Efficient In Vivo and In Vitro Assembly of Retroviral Capsids from Gag Precursor Proteins Expressed in Bacteria

MICHAELA KLIKOVA,<sup>1</sup> SUNG S. RHEE,<sup>2</sup> ERIC HUNTER,<sup>2\*</sup> AND TOMAS RUML<sup>1</sup>

*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague, Czech Republic,<sup>1</sup> and Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294<sup>2</sup>*

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**The capsid precursor protein (Gag) of Mason-Pfizer monkey virus, the prototype type D retrovirus, has been expressed to high levels in bacteria under the control of the phage T7 promoter. Electron microscopic studies of induced cells revealed the assembly of capsid-like structures within inclusion bodies that formed at the poles of the cells 6 h after induction with isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). The inclusion bodies and enclosed capsid-like structures were solubilized completely in 8 M urea, but following renaturation, we observed assembly in vitro of capsid-like structures that demonstrated apparent icosahedral symmetry. These results demonstrate for the first time that retroviral capsid precursors have the propensity to self-assemble in vitro and point to new approaches for the analysis of retroviral assembly and structure.**

Retrovirus assembly, a key step in the viral replication cycle, involves a process in which distinct components of the virion are transported to an assembly site on the plasma membrane and associate there into a nascent virus particle. Unlike most other enveloped RNA viruses, in which the viral glycoproteins appear to catalyze virus particle formation, assembly and release of retrovirus particles occur when capsid proteins are produced in the absence of the other gene products. Studies have shown that expression of the *gag* gene alone in a number of systems results in the efficient assembly and release of membrane-enveloped virions (2, 3, 10, 12, 18, 28, 29, 35). Thus, the product of this gene has the structural information necessary to mediate intracellular transport, direct assembly of the capsid shell, and catalyze the process of membrane extrusion known as budding.

The *gag* gene product, a polyprotein precursor, is translated on free polyribosomes from an unspliced, genome length mRNA (5). Such precursors generally follow one of two pathways during the process of viral morphogenesis (8). In most retroviruses, the nascent Gag polyproteins are transported directly to the plasma membrane, where assembly of the capsid shell and membrane extrusion occur simultaneously. Viruses that undergo this form of morphogenesis include the type C avian and mammalian leukemia/sarcoma viruses (33) as well as the pathogenic viruses human T-cell leukemia virus type I and human immunodeficiency virus. In the second morphogenic class of viruses, the Gag precursors appear to be targeted to an intracytoplasmic site where capsid assembly occurs (23, 24). These assembled immature particles (which by analogy to non-enveloped viruses we define as capsids) are then transported to the plasma membrane, where they undergo budding and envelopment. Viruses that undergo this process of assembly and release include the type B mouse mammary tumor virus (MMTV) and the type D Mason-Pfizer monkey virus (M-PMV), as well as members of the spumavirus family (8, 33). Despite the different morphogenic pathways, the process by which Gag precursors assemble into capsids is probably similar for the type C and type B/D viruses, since a single amino acid change within the *gag* gene product of M-PMV was shown to divert it to the type C morphogenic pathway (23). Mutagenesis

has been used to define regions of the *gag* gene product that mediate the assembly process itself (6, 11, 15, 24, 29, 31, 34). In the case of Rous sarcoma virus, this yielded the striking finding that three assembly domains comprising less than 30% of the total Gag precursor were sufficient for assembly and release of particles (34). However, these in vivo studies are influenced by the requirement for capsid precursors to retain signals for intracellular transport and in some instances membrane association (36). Even with the type D viruses, in which assembly of capsids occurs prior to their transport to the plasma membrane, mutations which have no discernible effect on the assembly of capsids when Gag precursors are produced in high-level expression systems but which dramatically reduce this process when the same precursors are expressed at lower levels from an integrated viral genome have been described (29). Thus, a system in which assembly occurs in the absence of intracellular factors would greatly facilitate mechanistic studies of the assembly process.

In the papovaviruses, in vitro assembly of viral capsids has been reported for both polyomavirus (27) and human papillomavirus (16, 25) following high-level expression of the major capsid protein in bacteria and insect cells, respectively. In this report, we describe the high-level expression in bacteria of the Gag precursor protein of the primate retrovirus M-PMV and the assembly of viral capsid-like structures within inclusion bodies of the bacterial cells. Solubilization in 8 M urea and subsequent renaturation of the purified Gag polyprotein allowed, for the first time, efficient in vitro assembly of retroviral capsid-like structures with evidence of icosahedral symmetry.

## MATERIALS AND METHODS

**Construction of a vector for bacterial expression of the M-PMV Gag polyprotein.** To generate an *Nde*I restriction site in the 5' terminus of the M-PMV *gag* gene, oligonucleotide mutagenesis was performed. An oligonucleotide 22 residues long with a single base substitution was used to carry out the site-directed mutagenesis on single-stranded M13 viral DNA containing a 0.8-kb (*Nar*I-*Sac*I) fragment of M-PMV as previously described (22). The presence of the mutation was confirmed by dideoxy sequencing of the double-stranded DNA.

Following mutagenesis, a 0.7 kbp *Nde*I-*Sac*I fragment was excised from the replicative form of M13 and was cloned together with a *Sac*I-*Xho*I fragment containing the remainder of the M-PMV *gag-pro-pol* region into an *Nde*I-*Xho*I digested pGEMEX-2 bacterial expression vector (Promega) to yield plasmid pG10MNX. This places *gag* expression under transcriptional control of the T7 promoter. To express the Gag polyprotein only, pG10GAG was constructed by

\* Corresponding author.

deletion of *pro-pol* sequences from pG10MNX by partial digestion with *ApaI* (details of the plasmid construction can be obtained upon request).

**Expression and purification of Gag precursor and assembly of capsids in vitro.** For production of the M-PMV Gag polyprotein, plasmid pG10GAG was introduced into *Escherichia coli* BL21(DE3) and induced with isopropyl- $\beta$ -thiogalactopyranoside (IPTG) as described previously (32). The cells were harvested 6 h after induction and resuspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 150 mM NaCl, 1 mg of lysozyme per ml) and incubated for 30 min at room temperature. The lysis mixture was frozen at  $-70^{\circ}\text{C}$ , and the cells were disrupted by hydraulic press (LKB). The lysate was then incubated for 30 min at  $4^{\circ}\text{C}$  in the presence of sodium deoxycholate (0.1%), DNase I (10 mM) and RNase A (10  $\mu\text{g}/\text{ml}$ ) were then added, and the incubation continued for 30 min at  $37^{\circ}\text{C}$ . Following clarification, inclusion bodies were pelleted by centrifugation at  $12,000 \times g$  for 15 min and washed three times with 0.5% Triton X-100 and 10 mM EDTA. Proteins were solubilized for 1 h at room temperature in a solution, (5 mg [wet weight] of inclusion bodies per ml) containing 8 M urea, 0.2 M Tris (pH 8.0), 0.5% mercaptoethanol, 0.3 M NaCl, and 10 mM EDTA. The denatured solubilized proteins were refolded during three 8-h dialysis steps against dialysis buffer (50 mM Tris HCl [pH 8.8], 4 mM EDTA, 0.1% Triton X-100) containing decreasing amounts of urea (2.0, 1.0, and 0.0 M). Assembled capsids were pelleted by centrifugation at  $280,000 \times g$  for 10 min, resuspended in the starting volume of  $\text{H}_2\text{O}$ , and then analyzed by centrifugation through a 25 to 55% (wt/wt) linear sucrose gradient for 3 h at 40,000 rpm in an SW41 rotor.

**Western blot (immunoblot) analyses.** Samples were heated at  $100^{\circ}\text{C}$  for 5 min in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) protein sample buffer. Proteins were resolved on an SDS-polyacrylamide (12%) gel and transferred to nitrocellulose, using a Bio-Rad transblot apparatus as instructed by the manufacturer. Membranes were incubated in blotting solution (5% nonfat dry milk in phosphate-buffered saline [14]) with a 1:500 dilution of rabbit anti-p27 antiserum for 16 h at  $4^{\circ}\text{C}$ . After thorough rinsing in Tris-buffered saline solution, the membrane was incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Promega Corporation). The membranes were then washed and exposed to enhanced chemiluminescence reagent (DuPont NEN). Chemiluminescence was recorded on Kodak XAR-5 film.

## RESULTS

**Production of M-PMV structural proteins for assembly studies.** To obtain a high level of expression of the M-PMV Gag precursor protein, we constructed the bacterial expression vector described below. An *NdeI* restriction site was created by site-directed mutagenesis at the N terminus of the M-PMV *gag* gene in order to position the initiating methionine codon at an optimal distance from the Shine-Dalgarno sequence in the mRNA. The *NdeI-XhoI* 5.3-kbp fragment was cloned into the pGEMEX-2 vector under the transcriptional control of the T7 RNA polymerase promoter. The deletion of sequences downstream of the *gag* gene, accomplished by partial cleavage with *ApaI*, prevented synthesis of the M-PMV proteinase which we have found can be produced from *gag-pro* constructs and process the polyprotein precursor in bacterial cells (unpublished results). The T7-based *gag* gene expression system allows a high level of biosynthesis of the M-PMV Gag precursor, following induction with IPTG in *E. coli* BL21(DE3), which carries a chromosomal copy of the T7 polymerase gene under the control of the *lacUV5* promoter (Fig. 1). As with other heterologous proteins expressed in bacteria (32), the Gag polyprotein expressed at these high levels was localized terminally in the bacteria in the form of large inclusion bodies which could be visualized in a light microscope (data not shown).

**Assembly of M-PMV capsid-like structures in *E. coli*.** Thin sections of *E. coli* cells 6 h after induction with IPTG revealed intracytoplasmic inclusions that were devoid of ribosomes and that contained structures that had the appearance of incompletely and fully assembled M-PMV capsids (Fig. 2A and B). These structures were composed of electron-dense rings approximately 60 to 65 nm in diameter. In some cases, they appeared to be surrounded by an additional layer of material that expanded the structure to approximately 80 to 90 nm across (Fig. 2B, arrow). These dimensions are similar to those described for M-PMV and MMTV intracellular capsids assembled in mammalian cells (7, 20). Some inclusions containing

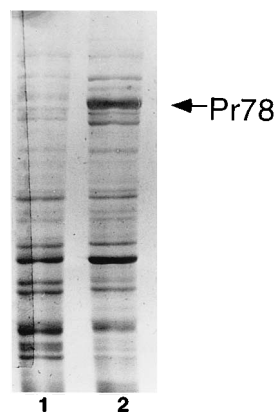


FIG. 1. Coomassie blue-stained SDS-polyacrylamide gel of equal amounts of bacterial lysates from cells carrying the *gag* gene expression vector pG10GAG. Lane 1, uninduced bacteria; lane 2, bacteria following induction for 6 h with 0.1 mM IPTG.

aberrantly assembled spiral-like structures could also be observed (Fig. 2B). No similar structures could be found in bacterial cells expressing the pGEMEX vector alone (data not shown). In experiments in which bacterial cultures were induced with IPTG for 30, 60, and 90 min prior to fixation and sectioning, increasing numbers of capsids could be observed in the forming inclusion bodies (data not shown), indicating that capsid assembly continued within the inclusion bodies during induction.

Inclusion bodies were purified from Gag-expressing bacteria by cell lysis followed by multiple washings of the pelleted inclusions in detergent-containing buffer. As can be seen from Fig. 2C, electron microscopic thin sections of these preparations revealed a more homogeneous pattern of assembled particles. The structures observed in these sections had a morphology indistinguishable from that of particles assembled in HeLa cells following expression of the same *gag* gene construct (29) and closely resembled particles which are assembled in virus-infected cells (Fig. 2D). As in virus-infected mammalian cells, these immature particles were approximately 90 nm in diameter and possessed an electron-lucent core surrounded by an electron-dense ring (approximately 60 to 70 nm in diameter) and a broader outer fringe (Fig. 2C and D). As with sections of intact bacteria, some of the capsid-like structures appeared to be assembled into spiral forms, indicative of occasional abnormal assembly processes. These studies confirm that capsid assembly occurs in the region of the inclusion bodies which also appears to contain large amounts of unassembled Gag protein (Fig. 2C).

**In vitro assembly of purified Gag precursors.** To purify Gag polyproteins, bacterial cells were lysed 6 h after induction. The lysate was centrifuged, and inclusion bodies were pelleted and then washed in detergent as described in Materials and Methods. These multiple washings resulted in a pellet composed of almost pure  $\text{Pr78}^{\text{gag}}$  which could be solubilized in 8 M urea in the presence of mercaptoethanol (0.5%). Solubilization was complete since following high-speed centrifugation ( $280,000 \times g$  for 10 min.), essentially all of the capsid precursor ( $\text{Pr78}^{\text{gag}}$ ) remained in the supernatant and undetectable amounts of protein were associated with the pellet (Fig. 3, lanes 1 and 2). Several approaches were examined to allow renaturation of the capsid precursors (16a), and it was found that the solubilized proteins could be refolded when the controlled dialysis conditions described in Materials and Methods were used. This

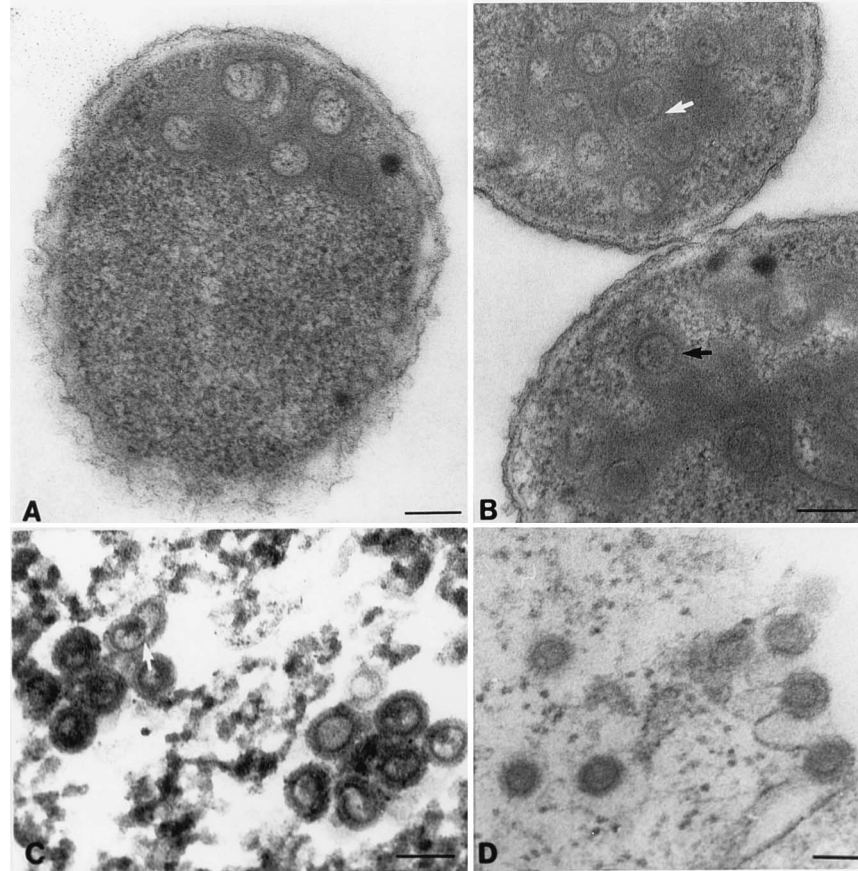


FIG. 2. Thin-section electron microscopy of bacterial cells 6 h after induction with IPTG (A and B) and of purified inclusion bodies (C). The outer layer of the assembled structures (B, black arrow) yields an estimated diameter of 80 to 90 nm. Aberrantly assembled capsids with a spiral-like structure are seen both in the bacterial sections (B, white arrow) and in purified inclusions (C, white arrow). (D) Thin section of HeLa cells infected with M-PMV. Bars represent 100 nm.

refolded material was not pelletable at low speed (Fig. 3, lanes 3 and 4), but approximately 50% could be pelleted following centrifugation at  $280,000 \times g$  for 10 min in a Beckman TL100 centrifuge (Fig. 3, lanes 5 and 6).

To investigate the size and homogeneity of the particulate material that assembled from the renatured proteins during

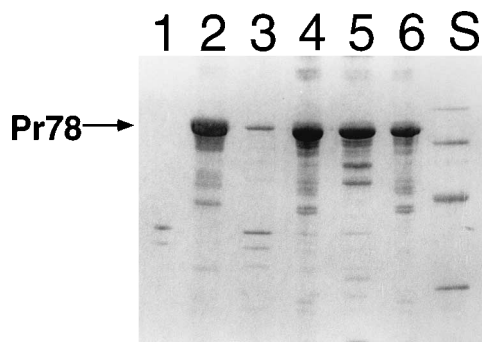


FIG. 3. Coomassie blue-stained SDS-polyacrylamide gel of urea-solubilized inclusion bodies. Proteins in inclusion bodies were solubilized with 8 M urea and were pelleted by high-speed centrifugation. Equal proportions of the pellet (lane 1) and supernatant (lane 2) fractions were separately analyzed by SDS-PAGE. Following renaturation dialysis, the samples were centrifuged at low speed ( $12,000 \times g$ ; lanes 3 [pellet] and 4 [supernatant]) and then at high speed ( $280,000 \times g$ ; lanes 5 [pellet] and 6 [supernatant]). Lane S shows protein standards of 95, 68, 41, and 31 kDa.

dialysis, the pelleted Gag protein was centrifuged through a 25 to 55% sucrose gradient in Triton X-100 lysis buffer as described in Materials and Methods. The proteins in collected fractions were detected by Western blotting after SDS-PAGE (Fig. 4). The results of this analysis showed that the bulk of the Gag precursor entered the gradient, with a peak of protein in fraction 6, at a density of approximately 1.19 g/ml. Additional, experiments in which identical gradients were centrifuged overnight to ensure equilibrium yielded similar results, with a

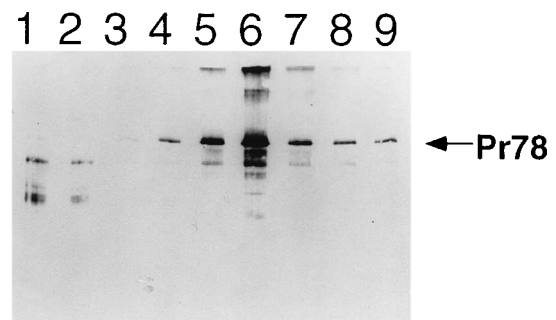


FIG. 4. Western blot of sucrose density gradient fractions. Particles pelleted from the renatured protein at high speed were subjected to centrifugation for 3 h at 31,000 rpm in a SW41 rotor. Aliquots from 0.5-ml fractions were analyzed by SDS-PAGE and Western blotting with a rabbit anti-M-PMV p27 antibody. Fractions 1 and 9 had densities of 1.07 and 1.22 g/ml, respectively.

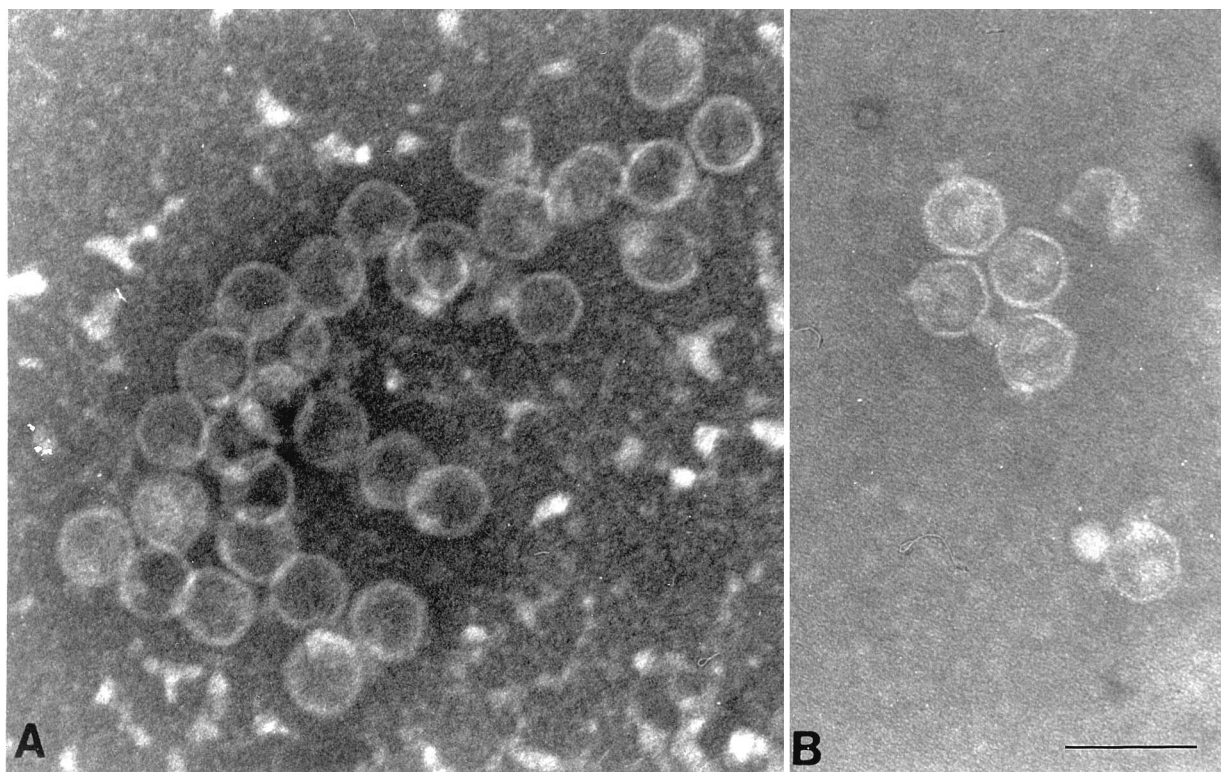


FIG. 5. Negative-stain electron microscopy of gradient-purified in vitro-assembled M-PMV capsids. Bar represents 100 nm.

peak of precursor at a density of 1.19 g/ml (data not shown). The material from fraction 6 of the experiment shown in Fig. 4, which contained the majority of reassembled proteins, was examined by negative-stain electron microscopy. As can be seen in Fig. 5, clusters of particles with a morphology similar to that described for intracytoplasmic capsids of MMTV (20) could be observed. These in vitro-assembled capsids exhibited a remarkably uniform size, with an estimated diameter of 60 to 70 nm. In some cases, the particles appeared to have an angular aspect suggestive of icosahedral symmetry. These data provide strong evidence that the M-PMV Gag polyprotein precursor is assembly competent in vitro and is able to form uniformly sized immature capsids, in the absence of viral RNA and detectable chaperonins, when suitable protein renaturation conditions are applied.

#### DISCUSSION

The process of retroviral capsid assembly requires a series of molecular interactions to occur between identical protein subunits that are encoded by the *gag* gene of the virus. In the type C viruses, this assembly process occurs inefficiently in the absence of membranes, which may provide a mechanism for concentrating the precursors to a critical level needed for assembly to occur. In contrast, the type B and D retroviruses efficiently assemble capsids in the hydrophilic environment of the cytoplasm, apparently by encoding signals within the Gag polyprotein to target or retain these precursors within a cytoplasmic compartment (23). We describe in this report the high-level biosynthesis of the M-PMV Gag polyprotein precursor in bacteria and show that while a majority of the protein accumulates in an apparently denatured form in polar inclusion bodies, some assembles into capsid-like structures. These uniformly sized, 80- to 90-nm particles are morphologically indis-

tinguishable from capsids that assemble in HeLa cells expressing the same protein from a similar T7-based vector. Similar structures have not been reported following bacterial expression of type C human immunodeficiency virus Gag precursors in prokaryotic cells (unpublished results and reference 17a). It is not clear whether the assembly of these capsid-like structures within *E. coli* cells requires the involvement of bacterial chaperonin molecules, as does the assembly of bacteriophage T4 capsids (9). However, such questions are amenable to genetic studies. In thin sections of both intact cells and isolated inclusion bodies, occasional aberrantly assembled, spiral-like structures could be observed. These resemble structures that we have observed in mammalian cells expressing mutant Gag precursors with alterations within the major homology region (31) and thus may result from the participation of incorrectly folded precursors in the assembly reaction.

A most striking observation presented here is the efficient in vitro assembly of capsid-like structures following complete solubilization of the bacterially expressed Gag polyprotein in 8 M urea and subsequent renaturation. A wide range of pH and salt conditions were explored in these studies (16a), but it was only under alkaline conditions (pH 8.8) and with low salt that efficient assembly of capsids occurred. In contrast, while bacterially expressed human immunodeficiency virus p24 (CA) protein was shown to assemble into higher-molecular-weight fibrous structures, no capsid-like structures were observed (4). This finding is consistent with the concept that a conformationally distinct precursor protein is the building block for assembly. Domains within the Gag precursor that are important for driving the assembly process may not reside within the boundaries of its mature cleavage products and may in fact span sites that are involved later in maturational cleavage of the capsids (13). As we and others have observed from in vivo

expression studies, the presence of the Gag-Pro-Pol precursors is not essential for the assembly process (10, 28, 29, 31, 35). The in vitro-assembled capsids isolated from the peak sucrose gradient fraction showed a remarkably uniform size of approximately 60 to 70 nm. This size is similar to that reported for MMTV particles isolated from mouse cell cytoplasmic fractions (20), although somewhat smaller than the 80- to 90-nm diameter reported for the latter. In contrast, the Ty1 retrotransposon capsids that are assembled in yeast cells appear to be heterogeneous in size and range from 15 to 39 nm in diameter (1). The in vitro-assembled M-PMV particles should therefore represent a potential substrate for structural studies.

In several of the negatively stained images, angular particles were seen, with hexagonal structures being most commonly observed (Fig. 5). Similar symmetrical forms could also be observed within thin sections of the bacterial cells, where an inner circular core was often surrounded by an angular layer of material (Fig. 2A and B). This is consistent with an icosahedral shape of the capsid. Angular shapes of various retroviruses in ultrathin sections of virions have been described by several investigators, and two recent studies of human and simian immunodeficiency viruses by Marx et al. (19) and Nermut et al. (21) have been interpreted to show the virion to be an icosadeltahedron with flat triangular facets similar to those of an adenovirus. The availability of large quantities of the in vitro-assembled M-PMV capsids should allow detailed cryoelectron microscopic studies of their structure. Moreover, the availability of an in vitro assembly system should facilitate analyses of the assembly process itself. In the case of icosahedral viral structures, only limited information exists on the polymerization process. For the T=3, RNA-containing plant viruses, capsid assembly appears to occur by sequential addition of coat protein dimers to an RNA-coat protein nucleation complex (30). In contrast, for the polyomaviruses, the capsid appears to be assembled by the direct association of 72 stable pentamers of coat protein with the double-stranded DNA (17, 27), and picornaviruses are also believed to be assembled by the association of pentameric structures (26). The lack of a discrete oligomer peak in preliminary centrifugation studies, as well as the existence of spiral-like structures, suggests that assembly of the M-PMV capsid may involve sequential addition of capsomers. However, more detailed studies will be required to establish the mechanism of nucleation and assembly of these capsids. Given the similarities between M-PMV capsid assembly and that of the pathogenic human retroviruses, the availability of an in vitro assembly system should open the way for a molecular dissection of the process and may provide new insights into novel approaches to interfere with the process.

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