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Synthesis and Assembly of Retrovirus Gag Precursors into Immature Capsids In Vitro

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The assembly of retroviral particles is mediated by the product of the *gag* gene; no other retroviral gene products are necessary for this process. While most retroviruses assemble their capsids at the plasma membrane, viruses of the type D class preassemble immature capsids within the cytoplasm of infected cells. This has allowed us to determine whether immature capsids of the prototypical type D retrovirus, Mason-Pfizer monkey virus (M-PMV), can assemble in a cell-free protein synthesis system. We report here that assembly of M-PMV Gag precursor proteins can occur in this in vitro system. Synthesized particles sediment in isopycnic gradients to the appropriate density and in thin-section electron micrographs have a size and appearance consistent with those of immature retrovirus capsids. The in vitro system described in this report appears to faithfully mimic the process of assembly which occurs in the host cell cytoplasm, since M-PMV *gag* mutants defective in in vivo assembly also fail to assemble in vitro. Likewise, the Gag precursor proteins of retroviruses that undergo type C morphogenesis, Rous sarcoma virus and human immunodeficiency virus, which do not preassemble capsids in vivo, fail to assemble particles in this system. Additionally, we demonstrate, with the use of anti-Gag antibodies, that this cell-free system can be utilized for analysis in vitro of potential inhibitors of retrovirus assembly.

Mason-Pfizer monkey virus (M-PMV) was first isolated from a spontaneous breast carcinoma of a rhesus monkey (5). Like mouse mammary tumor virus, a type B retrovirus, M-PMV forms intracytoplasmic capsids which make their way to the plasma membrane and bud. M-PMV, however, is categorized as type D to distinguish its extracellular morphology from those of type B particles (48). Thus, in contrast to the assembly of type C retroviruses, in which capsid assembly and budding occur concomitantly at the plasma membrane, type D and B retrovirus assembly is divided into two temporally and spatially separated stages (reviewed in references 13 and 16).

Extensive mutagenesis studies of the *gag* gene have revealed domains of the Gag polyprotein necessary for the proper formation of infectious virus particles. Analysis of the Rous sarcoma virus (RSV) Gag precursor has delineated three domains necessary for particle formation (52). These assembly domains are not necessarily equivalent to the domains defined by the mature core proteins of virions—matrix (MA), capsid (CA), and nucleocapsid (NC) (23). The mature proteins result from cleavage of the Gag precursor by the viral proteinase (PR). In addition to these major core proteins found in all retroviruses, many viruses contain additional proteins and/or peptides that are released upon proteolysis and which in the context of the precursor may function during assembly. The first of the assembly domains is the membrane-association domain which is located at the N terminus of Gag and which in mammalian retroviruses may in part consist of a myristic acid moiety (53). The second domain functions late during assembly and may be responsible for capsid release during budding (51). The third assembly domain appears to be located within the NC protein, is a region for interaction between Gag molecules, and may be responsible for the high packing density of Gag in the virus particle, perhaps through the association of the NC domain with RNA (49). Functional conservation of the assembly domains among retroviruses has been demonstrated by the ability of various chimeric Gag proteins to assemble and form particles and has led to the functional designations M, L, and I for membrane-binding, late, and interaction domains, respectively (2, 7, 30).

Since type D viruses preassemble capsids in the cytoplasm, they are thought to contain an element(s) which enables Gag to assemble in the absence of membranes. Type D and B viruses share an extra domain within Gag that is not found in type C viruses and which was hypothesized to confer the ability to assemble intracellularly upon Gag. Deletion analysis of this domain, p12, in M-PMV has determined that it is not essential for assembly itself, since capsids can still form in the cytoplasm when *gag* is overexpressed, but is required for intracytoplasmic assembly following lower levels of expression (43). Other domains which play crucial roles in the assembly process have been identified. These are the major homology region (MHR), the most highly conserved amino acid sequence among retrovirus Gag proteins (52), and the matrix (MA) domain. Point mutations within the MHR of M-PMV produce a range of effects from reduced infectivity, to altered processing of Gag, to a complete defect in capsid assembly (45). Similar effects upon assembly and infectivity of RSV and human immunodeficiency virus (HIV) were observed after mutation of the MHR (6, 25). The MA domain performs several assembly functions. MA plays a crucial role in the structural integrity of the Gag precursor, since deletion mutants display a pronounced instability and are thus inefficiently assembled (35). Several categories of phenotypes were observed in an analysis of random point mutants within MA (34, 36). Some mutations had no effect, while others resulted in loss of capsid stability. The remaining amino acid changes had profound effects upon assembly. Several of these display a defect in intracellular trans-

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port. Although these mutants can assemble capsids, they are not transported to the cell membrane (36). The most striking of the point mutants which alter transport is one in which the morphogenesis of M-PMV is altered from type D to type C (34). This final mutant, R55W, has helped to identify a potential signal, composed of an 18-amino-acid region which surrounds R55 and is highly conserved in MA among type B and D retroviruses, which may direct Gag to the intracellular site for assembly (34). Thus, cellular transport processes are likely dominant over assembly and may alter the site of capsid formation.

While mutational analyses in vivo has yielded an understanding of the role of domains of the Gag precursor in assembly, complete understanding of the mechanism of this process requires its analysis in an in vitro assembly system, in part because of the complicating aspect of intracellular transport of capsid precursors. Gag proteins produced in bacteria have been successfully assembled into capsid-like structures. Fragments of either the RSV or HIV Gag precursor equivalent to the CA plus NC domains can be assembled into rod-shaped coils when combined with RNA (3). Although the length of these rods is dependent upon the length of the RNA, the process appears to be independent of the RNA sequence. Studies of M-PMV *gag* expression in bacteria have demonstrated that capsids can assemble in *Escherichia coli*. Capsid-like structures can be seen by thin-section electron microscopy within cellular inclusion bodies. Most importantly, capsid structures can be reassembled from these inclusion bodies after solubilization in urea (22). Thus, a precedent exists for the cell-free assembly of retrovirus capsids.

We have further investigated the assembly of M-PMV immature capsids in a cell-free translation system. M-PMV Gag and Gag-Pro, when produced in a rabbit reticulocyte lysate, efficiently assemble into capsid-like structures indistinguishable in appearance from capsids produced in cells. In agreement with the value determined previously for capsids assembled in vitro from Gag produced in bacteria, capsids produced in in vitro translations sediment to a density of 1.2 g/ml. In contrast, we did not observe the production of capsid structures in lysates programmed with the *gag* genes of RSV and HIV, two retroviruses that undergo type C morphogenesis. Furthermore, by the use of antibodies directed against the MA domain of M-PMV Gag, we have demonstrated that this cell-free system can be utilized for analysis of potential inhibitors of retrovirus capsid assembly.

MATERIALS AND METHODS

DNA constructs. Plasmid pTZ-G was constructed by digesting plasmid pSHRM15 (32) with *Hha*I and then with mung bean nuclease to create blunt-ended fragments. The fragment containing the 5′ region of *gag* (nucleotides 484 to 1347; see reference 44 for numbering) was then inserted into the *Eco*RI site of plasmid pTZ18R (United States Biochemicals), which had been blunted with Klenow polymerase (thus recreating an *Eco*RI site; see below). A *Sac*I-to-*Xba*I fragment from pSHRM-X (a derivative of pSHRM15 with an *Xba*I site at position 5815) containing most of *gag* and all of *pro* and *pol* (nucleotides 1163 to 5815) was then inserted into the same sites of the resulting plasmid to reform the complete *gag*, *pro*, and *pol* genes positioned behind the T7 promoter of pTZ18R.

Plasmids pTZ-E154K and pTZ-L163H were constructed by replacing the *Sac*I-to-*Eco*72I fragment (nucleotides 1163 to 3277) from pTZ-G with the corresponding fragments from MHR mutants E154K and L163H, respectively. E154K and L163H are derivatives of pSHRM15 which each contain a different single point mutation within the MHR domain of CA (45). The presence of each mutation in the new T7 plasmids was confirmed by sequencing.

Plasmid pTF7CG was constructed by the following strategy. pTZ-G was digested with *Eco*RI and *Xho*I (nucleotides 484 to 5815); the resulting fragment was then inserted into the *Eco*RI and *Sal*I sites in the polylinker of pSP73 (Promega) to create plasmid pSP73GX. An *Nae*I site was introduced at the translational start codon of *gag* in pSP73GX by oligonucleotide-directed mutagenesis. The original sequence, ATGGGGC, was altered to AGCCGGC. The resulting plasmid was then digested with *Xho*I, treated with Klenow polymerase, and then digested with *Nae*I. The fragment containing M-PMV sequences was then ligated into vector pCITE-1 (Novagen), which had been digested with *Xba*I, treated with Klenow polymerase, and then digested with *Msc*I. pCITE-1 contains the encephalomyocarditis virus cap-independent translation enhancer (10). This pCITE-1-derived plasmid was then digested with *Eco*RI and *Sal*I and treated with Klenow polymerase. The fragment containing the cap-independent translation enhancer element and *gag-pro-pol* was then inserted into the Klenow polymerase-treated *Bam*HI site of plasmid pTF7.5 (12). The final resulting plasmid, pTF7CG, originally designed for the creation of a recombinant vaccinia virus, contains the encephalomyocarditis virus cap-independent translation enhancer element and M-PMV *gag*, *pro*, and *pol* flanked by a T7 promoter and a T7 terminator.

Plasmid pPRGagT7 was kindly provided by Volker M. Vogt of Cornell University. This plasmid is composed of the *Sac*I-to-*Hin*dIII fragment containing all of *gag* and part of *pol* (nucleotides 255 to 2870; see reference 42 for numbering) of Prague C RSV inserted into the multiple cloning site of phagemid pBS− (Stratagene). Plasmid pDAB72 was obtained through the AIDS Research and Reference Reagent Program, division of AIDS, National Institute of Allergy and Infectious Diseases, from Susan Erickson-Viitanen and contains the *gag* gene of HIV type 1 clone BH-10 positioned behind a T7 promoter (11).

Transcription and translation in vitro. Capped RNA transcripts were produced in vitro by using the mMESSAGE mMACHINE kit (Ambion) in accordance with the manufacturer's instructions. Plasmids were linearized before transcription at the *Eco*72I site in *pol* to produce *gag-pro* transcripts. Translation reactions were performed in the presence of [^{35}S]methionine (Amersham) in rabbit reticulocyte lysates (Promega) in accordance with the manufacturer's instructions. Alternatively, transcription and translation reactions were performed simultaneously with plasmid pTF7CG and the TNT Coupled Reticulocyte Lysate System (Promega).

Sucrose gradient analysis. Prior to fractionation on isopycnic sucrose gradients, translation reactions were incubated for 10 min on ice with 1% Triton X-100. Detergent-treated lysates were then diluted to a total volume of 100 μl with 30% (wt/wt) sucrose in 20 mM Tris (pH 8.0)–100 mM NaCl–100 μM dithiothreitol–0.1% Triton X-100 (gradient buffer) and loaded onto 2.2-ml continuous 30 to 55% (wt/wt) sucrose gradients in gradient buffer. Gradients were centrifuged in a TLS-55 rotor (Beckman Instruments) for 2 h at 55,000 rpm. Approximately 200-μl fractions were taken by hand with a Pipetman (Gilson) from the top of the gradient. The pellet was resuspended in 200 μl of 55% (wt/wt) sucrose in gradient buffer. Aliquots (5 or 10 μl) of each fraction were dissolved in sodium dodecyl sulfate (SDS) sample buffer and then loaded onto an SDS–12.5% polyacrylamide gel. After polyacrylamide gel electrophoresis (PAGE), radioactive bands were visualized by fluorography of sodium salicylate-impregnated gels. Quantitation of viral precursor proteins was performed by analysis of dried gels on an AMBIS radioanalytic imaging system (AMBIS Systems). Translational frameshifting efficiency was calculated after correction of radioactivity values for the number of methionines in each protein species.

Electron microscopy. For analysis of tissue culture cell lysates, COS-1 cells grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (Life Technologies), 1.0% vitamins, 20 mM L-glutamine, 100 U of penicillin G per ml, and 100 μg of streptomycin sulfate per ml were transfected by the DEAE-dextran method (40) with 1.5 μg of plasmid pSHRM15 (36). Forty-eight hours posttransfection, each 60-mm-diameter plate of cells was lysed in 1 ml of gradient buffer plus 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.15% Triton X-100 (0.25% total), and 0.25% sodium deoxycholate. The lysed cell suspension was then Dounce homogenized by 10 strokes on ice and cleared of nuclei by centrifugation in an Eppendorf microcentrifuge at 14,000 rpm for 1 min.

Particulate material present in either COS-1 cell lysates or in vitro translation reactions that had been prepared for comparison in parallel by dilution to 1 ml in the same detergent lysis buffer was then pelleted at 70,000 rpm for 30 min in a TLA-100.3 rotor (Beckman Instruments). For analysis of translation reactions alone, lysate was diluted twofold in gradient buffer before centrifugation. For analysis of sucrose density gradient material, fractions and the resuspended pellet were diluted twofold into gradient buffer and then loaded into Beckman microcentrifuge tubes containing a 20-μl plug of 0.8% agarose prepared in gradient buffer. Loaded tubes were centrifuged first at 30,000 rpm for 1 h and then immediately again without a stop at 70,000 rpm for 1 h in a TLA-100.3 rotor with microcentrifuge tube inserts (Beckman Instruments).

All pellets were fixed overnight in 1% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.0, at 4°C. After a rinse in PBS, pellets were postfixed in 1% buffered osmium tetroxide for 1 h. These pellets were rinsed once more and then dehydrated in a graded series of ethanol solutions beginning with 50% and ending with 100%. After dehydration, pellets were rinsed three times with propylene oxide and then embedded in Polybed. Ultrathin sections were acquired by using a Rechert-Jung Ultra Cut E ultramicrotome. After staining with uranyl acetate and lead citrate, sections were examined and photographed by using a Hitachi-7000 transmission electron microscope.

Production of monoclonal antibodies (MAbs). For MAb production, 8- to 12-week-old BALB/C mice were immunized with three monthly injections of M-PMV Gag polyprotein Pr78, which was produced in bacteria and renatured into capsid-like structures as described previously (22). Two weeks prior to the last injection, serum was collected and tested for high anti-Pr78 titer by radio-

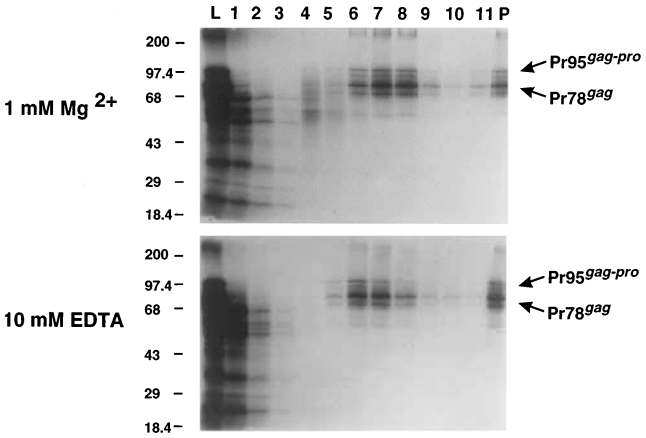

FIG. 1. Sucrose density gradient analysis of M-PMV *gag* and *gag-pro* translations. Aliquots of gradient fractions were electrophoresed on an SDS–10% polyacrylamide gel. Lane numbers indicate gradient fractions, beginning with the top fraction at number 1. Lanes L contained an equivalent aliquot of the translation reactions before gradient fractionation. Lanes P contained material remaining as a pellet in the tube after removal of the gradient. The numbers at the left indicate the positions of prestained molecular size standards; sizes are in kilodaltons. $Pr78^{gag}$ and $Pr95^{gag\text{-}pro}$ indicate the positions of the Gag and Gag-Pro precursor polypeptides of M-PMV, respectively. The upper and lower panels depict the fractionation of a translation reaction treated with 1 mM Mg^{2+} and 10 mM EDTA, respectively, as indicated.

immunoprecipitation and enzyme-linked immunosorbent assay (ELISA) (15). Three days after the last antigen injection, cells from the spleens and lymph nodes of high-titer mice were fused with mouse myeloma cell line P3X63-Ag8.653 in accordance with established methods (20). Fused cells were incubated in RPMI supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and hypoxanthine-aminopterin-thymidine (ICN-Flow Laboratories). Hybridomas were screened for antibodies to Pr78 by a modified ELISA. Briefly, 96-well plates were coated with polyclonal anti-Pr78 serum. The polyclonal anti-Pr78 serum was raised by injecting rabbits with Pr78 produced in bacteria (Lampire Biological Laboratories). The plates were blocked with 1% bovine serum albumin in PBS and then incubated with purified Pr78. After three washes with PBS, the plates were incubated with hybridoma medium. ELISAs were developed by standard methods (15). Positive cell lines were cloned by limiting dilution and adapted for growth in serum-free medium (Life Technologies). Antibodies from three cell clones (10.10, 118.1, and 120.18) were found to react with both Pr78 and the MA protein of purified M-PMV by immunoblot analysis (data not shown). The three anti-MA MAbs were purified from tissue culture supernatants by using a DEAE Memsep column (Millipore Waters) in accordance with established procedures (15) and then combined 1:1:1 by volume for use in assembly inhibition experiments.

RESULTS

M-PMV Gag and Gag-Pro precursor proteins form particulate structures when expressed in vitro. Expression of M-PMV precursor polyproteins in vitro was achieved by first producing their mRNA in vitro. Plasmid pTZ-G, which contains the *gag*, *pro*, and *pol* genes of M-PMV positioned downstream of a T7 promoter, was linearized for transcription at a convenient *Eco*72I site located immediately downstream of the *pro* reading frame. The resulting 3.0-kb RNA was used to program a rabbit reticulocyte lysate containing [^{35}S]methionine. Like the phylogenetically related type B retrovirus mouse mammary tumor virus (9), M-PMV produces three Gag-containing polyprotein precursors—Gag, Gag-Pro, and Gag-Pro-Pol—as the result of two ribosomal frameshift events (17, 18). Therefore, translation of this RNA should result in the synthesis of two polyproteins, Gag (Pr78) and Gag-Pro (Pr95). The third polyprotein, Gag-Pro-Pol (Pr180), cannot be produced because the *pol* sequence was not transcribed in vitro. As expected, both Pr78 and Pr95 were produced in reticulocyte lysates and were present in amounts that reflect a frameshifting efficiency of approximately 16% (data not shown), which is comparable to that previously found for mouse mammary tumor virus (18).

M-PMV capsids reassembled from Gag precursor protein produced in *E. coli* had previously been shown to sediment to a density of 1.2 g/ml (22). In addition, the expression of *gag* within COS-1 cells yields particles with a similar density (data

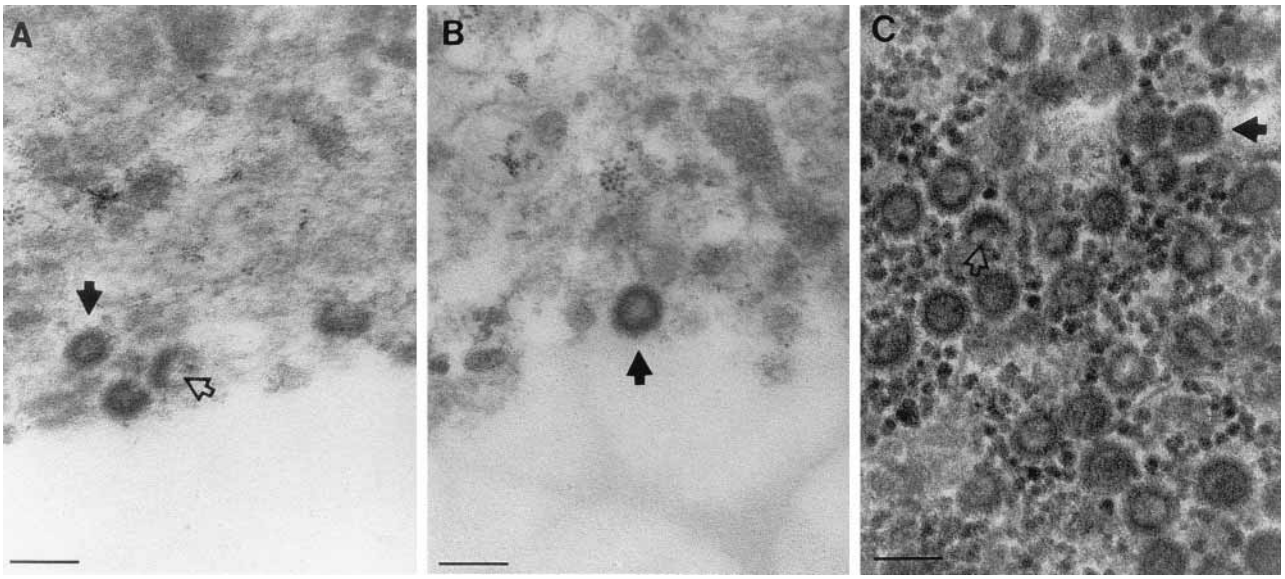

FIG. 2. Analysis of M-PMV translation reactions and transfected COS-1 cell lysates by thin-section electron microscopy. M-PMV Gag and Gag-Pro were translated in vitro as described in the legend to Fig. 1. COS-1 cells were transfected with pSHRM15, an infectious molecular clone of M-PMV. Lysates of transfected COS-1 cells and diluted translation reactions were centrifuged at high speed. The resulting pellets were fixed and processed for microscopy. The base of the pellet is evident as the line of transition between stained material and clear embedding medium in the section. (A and B) Pellet derived from reticulocyte lysate. (C) Pellet derived from transfected COS-1 cells. Solid arrows indicate apparently completed immature capsid structures. Open arrows indicate incompletely assembled capsids. Bars, approximately 100 nm.

not shown). Therefore, the possibility that this same precursor protein might assemble into capsids when synthesized in vitro was similarly tested by sedimentation of the translation lysate into a 30 to 55% (wt/wt) sucrose gradient. After fractionation of the gradient, aliquots of each fraction were analyzed by SDS-PAGE (Fig. 1). A significant portion of Gag and Gag-Pro sedimented as a population of molecules with a density of 1.19 to 1.21 g/ml in the gradient (Fig. 1, lanes 6 to 8), suggesting that this material is assembled into capsids. Note that as expected for capsids produced in an infected cell, both Gag and Gag-Pro were incorporated into this particulate material in approximately the same proportion as that in which they were synthesized.

In addition to the full-length precursor proteins, two additional minor bands of approximately 68 and 85 kDa were also found associated with Gag and Gag-Pro in the gradient. Gag-related proteins of similar sizes have been shown to be incorporated into immature capsids in vivo (33). The origin of these additional peptides is probably from initiation of translation from the second methionine codon at residue 99 in *gag*, which, if utilized, would result in precursor proteins approximately 10 kDa smaller than the full-length forms (44). Indeed, in vivo analysis of mutants with an altered codon at this position has confirmed that Pr68 and Pr85 arise from translational initiation at this second methionine codon (37).

In initial experiments, lysates were analyzed in gradients containing 1 mM $MgCl_2$ (Fig. 1, top). To rule out the possibility that the particulate material observed at a density of 1.19 to 1.21 g/ml was sedimented polysomes, lysates were treated with 10 mM EDTA to dissociate ribosomes (Fig. 1, bottom). Treatment with EDTA had no effect on the sedimentation of the Gag precursors; however, it did eliminate the diffuse band of material seen in the less dense regions of the gradient containing magnesium (compare lanes 4 of Fig. 1, top and bottom). This diffuse band is what one might expect to result from polysomes composed of randomly arrested nascent polypeptide chains. Thus, the Gag precursors banding around a density of 1.2 g/ml are not polysome associated.

Analysis of in vitro-synthesized Gag by electron microscopy. The results of gradient analyses of translation reactions strongly suggest that assembly of M-PMV capsids can occur in reticulocyte lysates; nevertheless, to provide visual proof of capsid assembly, a portion of lysates from experiments similar to that shown in Fig. 1 was centrifuged at high speed and the resulting pellets were processed for thin-section electron microscopy. For comparison of potential capsid-like structures produced in vitro with capsids produced in living cells, COS-1 cells transfected with pSHRM15, an infectious molecular clone of M-PMV, were processed for electron microscopy in parallel with the above-described translation reactions.

Structures consistent with the morphology of immature retrovirus capsids 70 to 90 nm in diameter were observed along the base of the pellet derived from the translation (Fig. 2A and B). These circular structures (solid arrows) clearly display the electron-dense ring characteristic of the intracytoplasmic particles of M-PMV (Fig. 2C) derived from transfected COS-1 cells. In addition, in both the reticulocyte lysate pellet and the COS-1 cell pellet, crescent-shaped structures (open arrows) were observed which display an electron-dense zone like that of the complete circular structures. It seems likely, therefore, that these crescents are incompletely assembled capsids. Thus, electron microscopy confirmed the evidence from gradient analyses that capsids were assembled in in vitro translation reactions.

Analysis of gradient material by electron microscopy. While capsid-like particles can be found in pelleted whole translation reactions, this observation does not provide information about the nature of the material sedimenting at different densities in sucrose gradients. We therefore attempted to analyze gradient fractions for the presence of capsids produced by the translation system described above but were unable to visualize particles in the fractions of the gradient that contained particulate material.

To increase the yield of assembled capsids for analysis of gradient fractions by electron microscopy, we utilized an M-PMV *gag-pro-pol* expression construct containing an internal ribosome entry site to enhance translation. This plasmid, pTF7CG, was used to program a coupled transcription-and-translation reaction. Gradient analyses followed by SDS-PAGE of these reactions produced results identical to those from simple translation reactions (data not shown). From an examination of unfractionated reactions by thin-section electron microscopy, we estimated that this enhanced expression system yielded a two- to threefold increase in the number of assembled capsids (data not shown).

Coupled transcription-and-translation reactions were then employed for analysis by electron microscopy of sedimented viral proteins. The material at a density of approximately 1.2 g/ml was diluted in detergent buffer and then centrifuged at high speed in a tube containing an agarose plug to stabilize the resulting tiny protein pellet. The combined protein and agarose pellet was then fixed and processed for thin-section electron microscopy. The small amount of stained material found in the pellet was composed of a large number of immature M-PMV capsid-like particles and partially assembled structures, as well as other material (Fig. 3A).We were unable to determine if the noncapsid material would also sediment to a density of 1.2 g/ml in the absence of capsid-like particles, since we found no stained material in agarose pellets prepared in parallel from control, DNA-negative reactions.

In addition to material in the gradient at the expected density for capsids, a significant portion of the viral polyproteins was found in the top fractions and in a pellet at the bottom of the gradient. The material that failed to sediment into gradients was presumably monomers of precursor proteins and was not analyzed further. To determine the nature of the material at the bottom of gradients, resuspended pellets were analyzed in parallel with the gradient fractions above. Samples derived from both DNA-positive and DNA-negative reactions contained ribosomes and other debris (Fig. 3B and C). However, in the DNA-positive sample we observed distinct structures. These appeared in a thin-section electron micrograph as long, continuous ribbons with a thickness of approximately 25 nm (Fig. 3B). The cross-sectional dimension of these structures is incompatible with membranes which are approximately 5 to 6 nm thick but is similar to that of the capsid shell wall (compare Fig. 3A with B). Therefore, a fraction of the Gag precursor protein may assemble into a dense, two-dimensional, sheet-like structure that pellets through sucrose gradients.

M-PMV point mutants fail to produce particulate viral proteins in vitro. If the mechanism of assembly of M-PMV capsids in this cell-free system is similar to that employed in the cytoplasm of infected cells, M-PMV mutants incapable of assembly in cells should fail to assemble in vitro. Two variants of M-PMV Gag with single amino acid substitutions within the MHR of the CA domain were tested for formation of particulate material in this system. Both of these mutants, E154K and L163H, are defective in capsid assembly in vivo (45). Particulate Gag of a discrete density was not produced in translations of either mutant (Fig. 4); the majority of these mutant precursors remained in the top fraction of the sucrose gradient. In contrast, most of the wild-type proteins sedi-

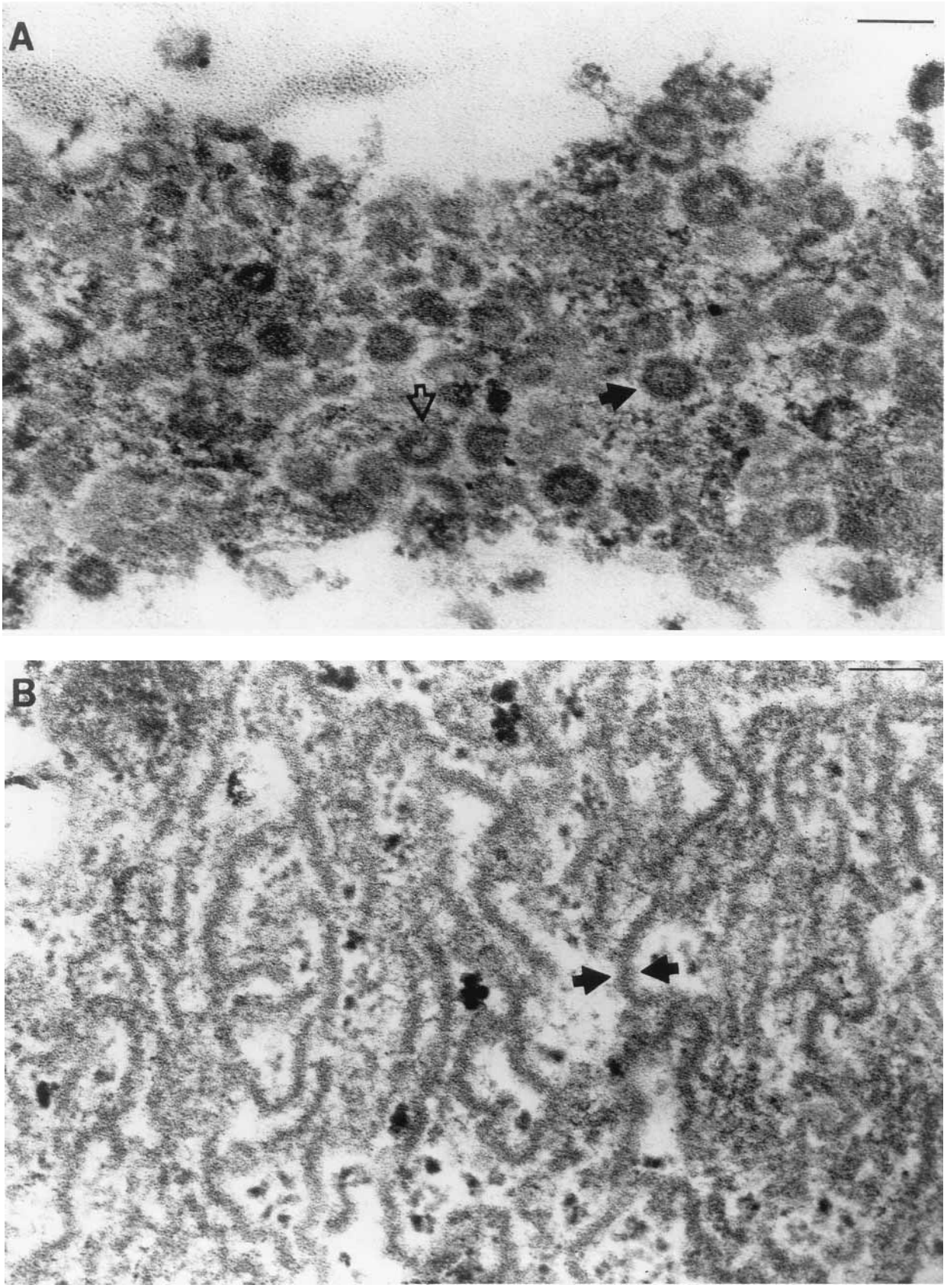

FIG. 3. Analysis of gradient-derived material by thin-section electron microscopy. Diluted gradient fractions or a sample of the resuspended pellet was centrifuged into an agarose plug. The resulting agarose-protein pellets were fixed and processed for microscopy. Bars, approximately 100 nm. (A) Material derived from the fraction corresponding to a density of 1.2 g/ml plus the two adjacent fractions. The solid arrow indicates an apparently completed capsid. The open arrow indicates an incompletely assembled structure. (B) Material resuspended from the gradient pellet. Solid arrows indicate structures unique to translation reactions containing synthesized M-PMV Gag and Gag-Pro. (C) Negative control. Material resuspended from the gradient pellet of a coupled transcription-and-translation reaction not programmed with DNA.

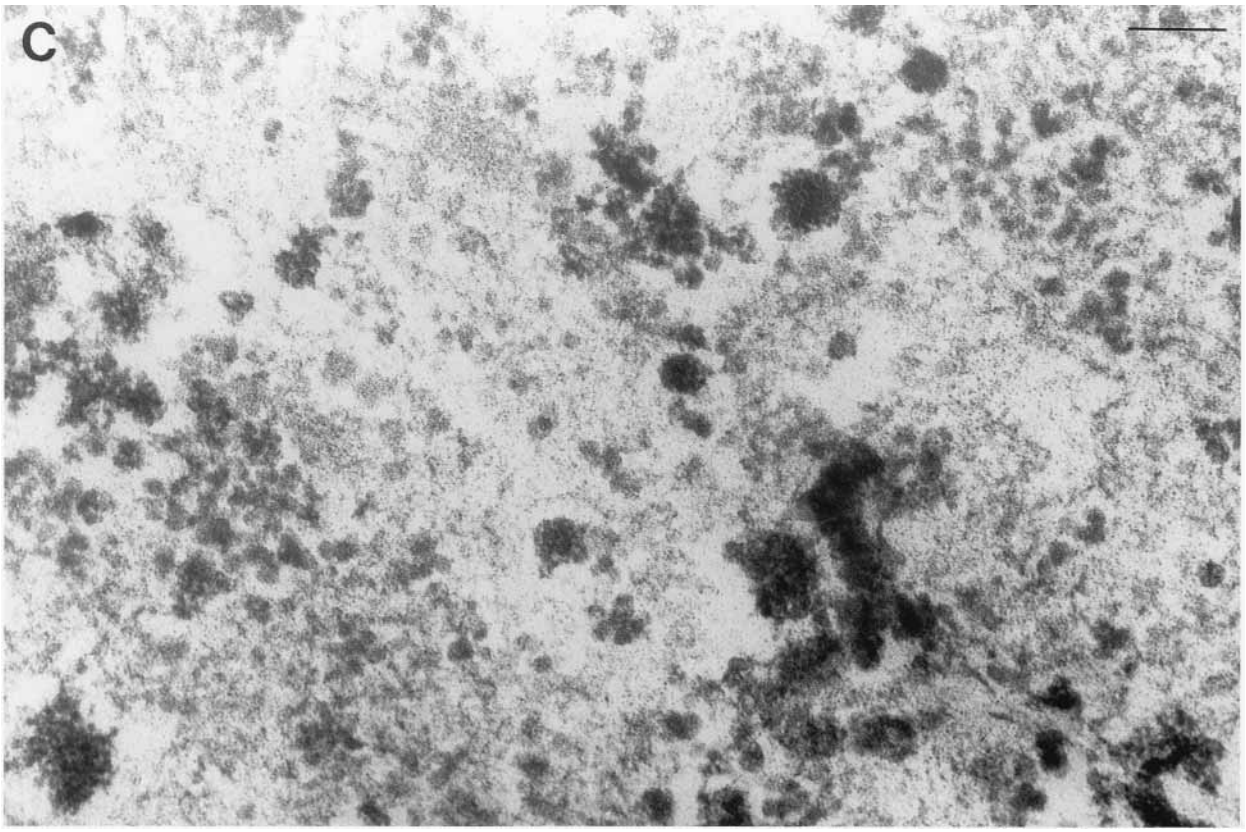

FIG. 3—*Continued.*

mented into the gradient. To further analyze these mutant Gag precursors, a portion of each of the lysates from the experiment shown in Fig. 4 was centrifuged at high speed and the resulting pellets were processed for thin-section electron microscopy. As before, immature capsid-like particles and partially assembled structures were found along the base of the pellet in the wild-type sample (not shown). Such structures were not seen in either of the MHR mutant samples or in the RNA-negative control (not shown). Only amorphous, darkly stained material that might represent aggregated or misfolded Gag was seen in samples of the two MHR mutants. Thus, the assembly of capsids in this in vitro system is sensitive to the same mutations that block assembly in vivo.

The retroviral Gag precursors of viruses with type C morphogenesis fail to assemble in vitro. Since type C retroviruses do not preassemble capsids in the cytoplasm of infected cells but concomitantly assemble and extrude from the plasma membrane, it was of interest to determine whether a type C retroviral Gag protein could also assemble in reticulocyte lysates. The RSV Gag precursor protein was examined in parallel with that of M-PMV. Gradient analysis showed that RSV Gag failed to form a discrete population of particulate matter similar to that formed by M-PMV Gag and produced a distribution of protein in gradients identical to that produced by the assembly-defective mutants of M-PMV (compare Fig. 4 and 5). Analysis of translation reactions by thin-section electron microscopy confirmed the gradient results in that no structures characteristic of a retroviral immature capsid were found in RSV samples (data not shown).

Although not formally classified as type C viruses, lentiviruses follow a similar assembly strategy, by which capsid formation and budding occur simultaneously at the plasma membrane of infected cells (47). Consequently, we hypothesized that HIV Gag would also fail to assemble into capsids in this in vitro expression system. Gradient analysis indicated that HIV Gag does not form a structure with a density similar to that of

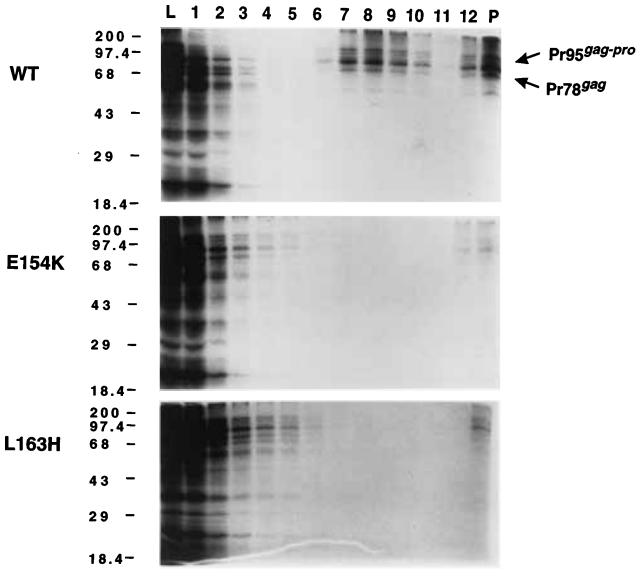

FIG. 4. Analysis of point mutants with mutations within the MHR of *gag*. Translation reactions were fractionated through gradients containing 5 mM EDTA. The experiment was performed and lane designations and molecular size standards are as described for Fig. 1. WT, E154K, and L163H indicate analyses of wild-type M-PMV, point mutant E154K, and point mutant L163H, respectively.

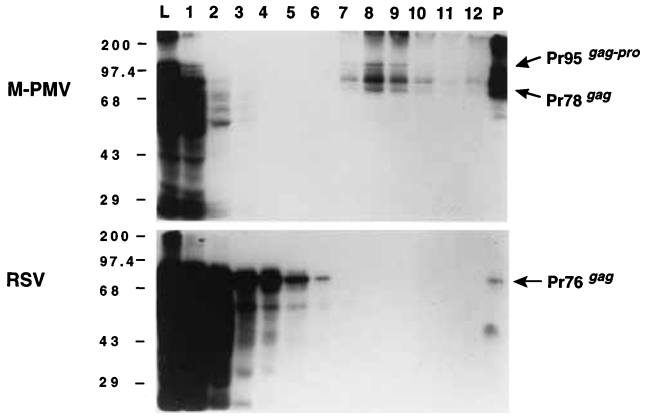

FIG. 5. Comparative analysis of in vitro-translated type D and type C retroviral Gag polyproteins by sucrose density gradient fractionation. The experiment was performed and lane designations and molecular size standards are as described for Fig. 1. Pr76^{gag} indicates the position of the Gag precursor polypeptide of RSV.

M-PMV capsids (Fig. 6). However, a reproducible peak of viral protein in the fraction corresponding to a density of approximately 1.15 to 1.16 g/ml was evident in gradients of HIV Gag (Fig. 6, lane 3). It is highly unlikely that this material represents immature HIV capsids, since it sedimented to a density expected for an enveloped retrovirus particle (8). To rule out this possibility, these HIV *gag* translations were also examined by electron microscopy but no capsid-like structures were observed (data not shown).

Antibodies against the MA domain of M-PMV Gag inhibit the in vitro assembly of capsids. A cell-free system for the production of capsids might prove useful for the analysis in vitro of potential inhibitors of retrovirus assembly. To test this concept, we utilized a cocktail of three partially purified MAbs that recognize the MA domain of M-PMV Gag. These antibodies had been raised against M-PMV Gag produced in bacteria and screened for reactivity to both the Gag precursor and the mature MA protein. We hypothesized that the attachment of an antibody molecule to Gag precursor monomers might block the Gag-Gag interactions that lead to capsid assembly.

In initial experiments, the anti-MA MAb cocktail was added to the reticulocyte lysate at the initiation of incubation for translation. Analysis of these reactions indicated that the antibody cocktail severely inhibited translation such that little Gag protein was produced (data not shown). To overcome this problem, a series of time course experiments were performed to determine a point at which sufficient precursor protein has been synthesized for capsid assembly to occur. In one series of experiments, the translation reaction was stopped at intervals of up to 1 h by freezing on dry ice. This revealed the time at which assembled Gag, as assayed by an increase in density, begins to be produced in the reaction. In a second series of experiments, cycloheximide to a concentration of 10 μg/ml was added at intervals to the translation reaction to inhibit protein synthesis, but the reaction was further incubated for a total of 2 h to allow the potential assembly of Gag precursors already synthesized. Sucrose gradient and SDS-PAGE analyses of these experiments indicated that after 30 min, sufficient Pr78 and Pr95 were produced to yield capsids but assembly had not yet occurred (data not shown). Thus, a point in time exists at which a potential inhibitor of assembly can be introduced into the reaction without concern for possible effects upon protein synthesis.

The ability of anti-MA MAbs to inhibit capsid assembly was then tested. Protein synthesis was allowed to proceed within the reticulocyte lysate for 30 min at 30°C. At that time, cycloheximide and the cocktail of anti-MA MAbs were added to the reaction. Alternatively, H_2O or control ascites fluid was added in place of the MAb cocktail. The lysate was then incubated for an additional 90 min, after which the reactions were analyzed by sucrose density gradient analysis and SDS-PAGE (Fig. 7). Addition of the anti-MA MAbs resulted in complete inhibition of the assembly of M-PMV Gag precursors (Fig. 7, center). In contrast, addition of H_2O or control ascites fluid had no effect upon assembly (Fig. 7, top and bottom, respectively). Thus, this cell-free capsid assembly system can be utilized to test potential inhibitors of retrovirus assembly.

DISCUSSION

We have described in this report an in vitro synthesis-and-assembly system for M-PMV capsids. M-PMV Gag and Gag-Pro precursor proteins were produced by translation in a rabbit reticulocyte lysate. These precursor proteins were observed to spontaneously assemble into particulate structures which appear by both density gradient and electron microscopy analysis to be immature capsids. Like capsids produced in the cytoplasm of infected cells the in vitro-produced capsid-like structures contain Gag and Gag-Pro in the same proportion as they are synthesized. Furthermore, they are stable and do not undergo viral protease-mediated maturation. The lack of protease activity in this circumstance is analogous to the situation observed with capsids that have assembled within the cell, where the protease is similarly inactive (36). As with most retroviruses, the M-PMV protease does not become activated until or subsequent to the processes of envelopment and budding (19).

The Gag proteins of two viruses, RSV and HIV, which follow the morphogenic pathway characteristic of type C viruses failed to form capsid-like structures in this system. Examination of RSV yielded results similar to those obtained with two M-PMV assembly-defective mutants in that no peak of Gag was observed in the buoyant fractions of density gradients. However, analysis of HIV Gag yielded a peak of material at a density of 1.15 to 1.16 g/ml in such gradients. We

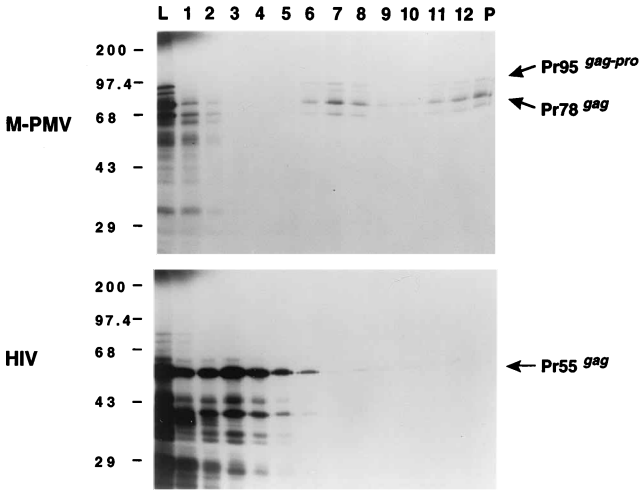

FIG. 6. Comparative analysis of in vitro-translated type D and lentivirus Gag polyproteins by sucrose density gradient fractionation. The experiment was performed and lane designations and molecular size standards are as described for Fig. 1. Pr55^{gag} indicates the position of the Gag precursor polypeptide of HIV.

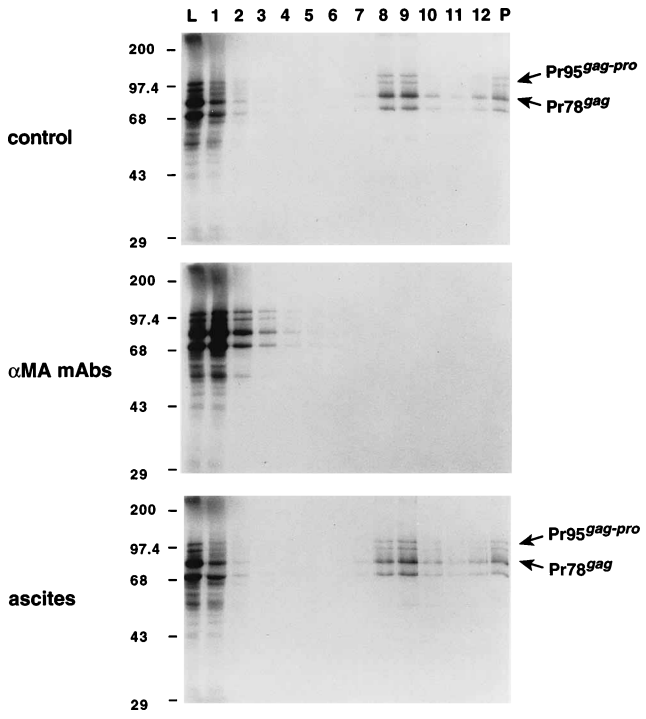

FIG. 7. Inhibition of M-PMV capsid assembly in vitro by anti-MA antibodies. Translation reactions were treated with cycloheximide either alone (top panel) or in combination with a cocktail of anti-MA antibodies (center panel) or ascites fluid (bottom panel) after 30 min of incubation. Reactions were further incubated for 90 min. M-PMV Gag polyproteins were analyzed by sucrose density gradient fractionation followed by SDS-PAGE. Lane designations and molecular size standards are as in Fig. 1.

predicted that this material would not represent assembled capsids since it is found at the same density as an enveloped retrovirus particle. One would expect naked capsids to have a greater density, like that of M-PMV, which is approximately 1.19 g/ml (22), and that of intracytoplasmic type A particles of mouse mammary tumor virus, which is 1.26 to 1.27 g/ml (46). Consistent with this prediction and despite the fact that both RSV and HIV Gag polyproteins were translated with an efficiency greater than that of M-PMV (Fig. 5 and 6), no immature capsid-like structures were found in either sample examined by electron microscopy.

This M-PMV Gag assembly system represents the first such system to both synthesize and assemble a retrovirus Gag protein in vitro. In this regard, it resembles that of hepatitis B virus, the core protein of which has also been shown to assemble into particles when synthesized in a wheat germ extract (24). Alphavirus core protein synthesized in vitro can be incorporated into assembling virus-like particles in the presence of purified core protein and RNA, but a reticulocyte lysate system, by itself, does not provide sufficient core protein for assembly to occur (50). A similar translation system derived from HeLa cells has also been developed for the cell-free replication of poliovirus (26, 27) and is capable of producing infectious virus (1). In vitro synthesis of M-PMV Gag, however, results in assembly of capsids without addition of exogenously produced Gag or production of the complete complement of viral components produced during replication.

The previously described in vitro assembly of M-PMV capsids utilized Gag protein expressed in and purified from bacteria (22). Similarly, baculovirus expression has been utilized to produce the components for assembly of polyomavirus (41), papillomavirus (21, 38), and herpesvirus (28) capsids in vitro. Interestingly, our results obtained with what is essentially a mammalian cytoplasmic lysate (31) parallel those of Klikova et al. with respect to the structures found assembled within bacteria (22). Apparently complete capsids, as well as incomplete and/or aberrantly assembled structures, were found in each case. Moreover, the sedimentation densities of capsids reconstituted from urea-solubilized inclusion bodies and those synthesized in the in vitro translation reactions are approximately the same at about 1.20 g/ml. This density is also similar to that determined for capsids purified from M-PMV-producing cells (data not shown). A surprising result of our investigation is that incompletely assembled capsids sedimented to the same density as apparently completely assembled spherical particles. This result suggests that the density of these particles is not merely the result of the envelopment of a certain volume by the capsid shell but is a property inherent in the shell itself, perhaps because the shell is a porous structure. If solvent can penetrate into the interior of a spherical capsid, then the density of such a sphere would be equivalent to that of fragments of the sphere.

Spontaneous assembly of M-PMV Gag within bacteria and reticulocyte lysates raises the possibility that cytosolic chaperonins play a role in the process. Such a role has been demonstrated for the assembly of bacteriophage T4 capsids in bacteria (14), and the involvement of a cytosolic chaperonin has been implicated in the assembly of hepatitis B virus capsids within a reticulocyte lysate system (24). In in vitro translations of HBV RNA, Lingappa et al. (24) found that HBV core protein found within the pellet of rate zonal sucrose gradients colocalized in a potential complex with CC60, a TCP-1-related chaperonin. By using isopycnic gradients, we also found M-PMV Gag protein within the pellet; however, we found that unlike the HBV core protein, which can be chased out of the potential CC60 complex and into a buoyant fraction that contains capsid structures indistinguishable from authentic HBV capsids, pelleted M-PMV Gag appears to be in an aberrant two-dimensional assembly. This aberrant structure is likely to be a dead-end assembly product, since it formed simultaneously with capsid-like structures in time course experiments (data not shown) and thus is unlikely to be an intermediate assembly complex with a chaperonin.

A two-dimensional assembly of Gag protein similar to that hypothesized above for M-PMV has been observed in studies of the assembly of a fragment of the RSV or HIV Gag protein consisting of CA plus NC expressed in and purified from bacteria (3). In the presence of RNA, CA-NC from either virus assembles into cylindrical particles, the length of which depends upon the length of the RNA. However, in the absence of RNA, CA-NC appears to assemble into two-dimensional sheets (3) which may provide a clue to the nature of the M-PMV Gag found at the bottom of gradients. These structures might represent multimerization of Gag precursors without RNA, while spherical capsid-like particles would result from coassembly of Gag with RNA. Direct analysis of these two Gag species is necessary to test this hypothesis.

If a CA-NC fragment of the RSV or HIV Gag precursor can coassemble with RNA in vitro, then why do the full Gag proteins fail to do so in translation reactions? This apparent inconsistency may reflect a need for a threshold concentration of Gag for assembly to occur. Campbell and Vogt (3) utilized a bacterial expression system to produce large quantities of protein, and assembly reactions were performed with a 1-mg/ml concentration of CA-NC. Such a concentration of Gag molecules is not possible from an in vitro translation reaction, and thus, in the absence of a cellular transport mechanism to con-

centrate the precursors and/or an auxiliary factor for assembly, such as an interaction with membranes, these type C Gag precursors may be unable to assemble. This possibility is supported by the observation that the high-level expression of fragments of HIV Gag or certain HIV Gag mutants by recombinant baculovirus can result in efficient intracellular assembly of capsid-like structures (4, 39). M-PMV Gag, on the other hand, is capable of efficient assembly within the cytosol even when expressed at a low level and thus may be capable of assembling into particles at low concentrations. Alternatively, M-PMV Gag may efficiently utilize a component in the reticulocyte lysate, such as a chaperonin, that assists in assembly.

The ability to conveniently produce a retrovirus immature capsid in vitro could facilitate the analysis of possible inhibitors of retrovirus replication targeted to capsid assembly. We have demonstrated the potential of this system by utilizing antibodies directed against the Gag precursor to block assembly. Moreover, we have shown that this system can differentiate between an effect of an inhibitor upon assembly and an effect upon protein synthesis. A further advantage of this system is its cell-free nature. Potential inhibitors can be tested without regard to availability within or toxic effects on a living cell. The identification of effective inhibitors and their targets could lead to the design of therapeutic antiretroviral agents for use in vivo. Capsid assembly is a viable target for inhibitory agents since it has been demonstrated that Gag-related peptides can disrupt the normal assembly of HIV and result in the release of immature, deformed particles (29).

The in vitro synthesis-and-assembly system described here for M-PMV capsids could provide a unique opportunity to investigate the involvement of cellular factors, in addition to chaperonins, that may play a role in capsid assembly. Furthermore, this cell-free system should prove invaluable for the delineation of Gag domains responsible for assembly versus those that interact with cellular transport mechanisms. Finally, the dissection of the molecular mechanisms involved in retrovirus assembly may facilitate the development of therapeutic inhibitors of replication for which this synthesis-and-assembly system provides a convenient assay.

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