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Incorporation of Chimeric Gag Protein into Retroviral Particles

ROBERT A. WELDON, JR.,[1] CHRISTINE R. ERDIE,[1] MARY G. OLIVER,[2] AND JOHN W. WILLS[1]*

Department of Biochemistry and Molecular Biology[1] and Department of Cellular Biology and Anatomy,[2] 1501 Kings Highway, P.O. Box 33932, Louisiana State University Medical Center-Shreveport, Shreveport, Louisiana 71130

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The product of the Rous sarcoma virus (RSV) *gag* gene, Pr76^{gag}, is a polyprotein precursor which is cleaved by the viral protease to yield the major structural proteins of the virion during particle assembly in avian host cells. We have recently shown that myristylated forms of the RSV Gag protein can induce particle formation with very high efficiency when expressed in mammalian cells (J. W. Wills, R. C. Craven, and J. A. Achacoso, J. Virol. 63:4331–4343, 1989). We made use of this mammalian system to examine the abilities of foreign antigens to be incorporated into particles when fused directly to the myristylated Gag protein. Our initial experiments showed that removal of various portions of the viral protease located at the carboxy terminus of the RSV Gag protein did not disrupt particle formation. We therefore chose this region for coupling of iso-1-cytochrome *c* from *Saccharomyces cerevisiae* to Gag. This was accomplished by constructing an in-frame fusion of the *CYC1* and *gag* coding sequences at a common restriction endonuclease site. Expression of the chimeric gene resulted in synthesis of the Gag-cytochrome fusion protein and its release into the cell culture medium. The chimeric particles were readily purified by simple centrifugation, and transmission electron microscopy of cells that produced them revealed a morphology similar to that of immature type C retrovirions.

The major structural proteins of retroviruses are encoded by the *gag* gene. The product of this gene, the Gag protein, is a polyprotein precursor and has the unique ability to drive particle formation in the absence of all other components of the virus (4, 5, 8, 35). Thus, assembly and release of noninfectious, virionlike particles do not require functions associated with the viral glycoproteins (*env* products), reverse transcriptase or integrase activity (*pol* products), nor packaging of the viral RNA genome which carries these genes (7, 9, 16, 20, 23). The ability of a single viral gene product to cause enveloped particle formation greatly simplifies studies of the budding process, and we have been investigating these events, using Rous sarcoma virus (RSV), a member of a group of closely related retroviruses (the avian sarcoma and leukosis virus group) that cause leukemias and other malignant diseases in several avian species (3, 33).

Like other type C retroviruses, RSV does not preassemble viral cores in the cytoplasm before their envelopment at the plasma membrane (29). Rather, the RSV Gag protein, Pr76^{gag}, morphologically appears first in a cell in crescent-shaped structures located immediately beneath the plasma membrane. As budding progresses at these sites, the viral envelope and core develop concurrently. Particles that have just been released from a cell contain an immature, electron-lucent core (13, 18, 37). Very rapidly after their release, the RSV Gag precursors contained in the immature particles are cleaved by viral protease to form the five major internal proteins of the virion (see Fig. 1A): MA (matrix protein), p10 (of unknown function), CA (capsid protein), NC (nucleocapsid protein), and PR (protease). These proteins were formerly named p19, p10, p27, p12, and p15, respectively (15). As a result of this proteolytic processing, the cores of the virion appear to collapse or condense and thereby become electron dense. Although processing of the Gag precursor is absolutely essential for infectivity, it is not a prerequisite for particle formation (13, 32, 35).

To simplify investigations of RSV assembly, we have recently modified the RSV Gag protein to enable it to function with full efficiency in mammalian cells (35). This was accomplished by creating a myristic acid addition site at the amino terminus of Pr76^{gag}. Expression of myristylated RSV Gag in mammalian cells results in a very rapid rate of particle formation ($t_{\frac{1}{2}}$, 30 min) which mimics that of the authentic protein in an RSV-infected avian cell. The particles that are released into the medium from mammalian cells contain only Gag cleavage products, and these have the same electrophoretic mobilities as authentic RSV. Furthermore, electron micrographs presented in this report show that the particles have an appearance that is indiscernible from that of RSV.

Because of the apparently simple requirements for assembly of retroviral particles, it seemed likely that components of the virion unnecessary for budding could be replaced with foreign proteins of interest without interference. Foreign proteins contained within such chimeric particles would be easy to collect from the growth medium of the cell cultures by simple centrifugation. Two distinct strategies for packaging of nonviral antigens into retroviral particles have been imagined (34), a process for which the term "retro-secretion" has been suggested. One strategy would be to attach the packaging signals found on the RSV glycoprotein onto a foreign glycoprotein of interest, thereby enabling it to be incorporated on the surface of the viral membrane. This approach is limited by an incomplete understanding of how retroviruses package their glycoproteins to the exclusion of those of the host. The other strategy for retro-secretion would be to attach the polypeptide of interest directly to the Gag protein, thereby allowing it to be directed to the site of assembly and packaged within the particles. We decided to test the internal packaging method by fusing iso-1-cytochrome *c* from *Saccharomyces cerevisiae* directly to the myristylated form of the RSV Gag protein. The experiments described here clearly demonstrate that Gag fusion proteins

* Corresponding author.

can be efficiently packaged and released from mammalian cells.

MATERIALS AND METHODS

DNAs and cell lines. Standard protocols were used for all DNA manipulations (21). The simian virus 40 (SV40)-based expression vectors, pSV.Myr1 and pSV.D37I, used to the express myristylated forms of the RSV Gag protein have been described previously (35). The *CYC1* gene of *S. cerevisiae* (26), contained in plasmid pAB16, and rabbit antiserum to purified iso-1-cytochrome *c* were generously provided by D. M. Hampsey (Louisiana State University Medical Center at Shreveport). Recombinant plasmids were propagated in *Escherichia coli* DH-1 (10) by using solid or liquid LB medium containing ampicillin (25 μg/ml). African green monkey kidney cells, CV-1 and COS-1, were propagated in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 3% fetal bovine serum and 7% bovine calf serum (Hyclone, Inc.).

Production of antibodies to RSV Gag proteins. Prague A and Prague C strains of RSV were prepared by transfection of molecularly cloned, infectious genomes (pJD100 and pATV-8, respectively) into turkey embryo fibroblasts. The virions were purified in two successive sucrose gradients (27). The purified virions were pelleted and suspended in phosphate-buffered saline, and equal portions of the two strains were combined at a final protein concentration of 2.8 mg/ml. The solution was then adjusted to 25 mM β-mercaptoethanol and incubated at room temperature for 15 min to encourage release of viral SU glycoprotein (gp85) and nonviral proteins bound to the external surface of the virion. The virions were layered onto 5% sucrose (in phosphate-buffered saline containing 20 mM β-mercaptoethanol) and pelleted by centrifugation at 45,000 rpm in a type 65 rotor for 45 min at 4°C. The pellet was suspended in 300 μl of TE buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA [pH 8.0]) and split into two equal portions. One half was adjusted to 1% sodium dodecyl sulfate (SDS), heated to 90°C for 2 min, and cooled on ice. The denatured proteins were then precipitated with 7 volumes of 100% ethanol, collected by microcentrifugation, washed with 80% ethanol, and air dried. The second half of the particles was solubilized with Triton X-100 (final concentration, 1%). The Triton X-100-solubilized fraction was then added directly to the SDS-denatured proteins, and the final volume was adjusted to 1.5 ml with phosphate-buffered saline. Insoluble material was dispersed by passing the sample several times through a 26-gauge needle, and 0.5 ml (containing approximately 700 μg of protein) was then mixed with 0.5 ml of Freund complete adjuvant and injected into a New Zealand White rabbit. Preimmune serum was shown to have no reactivity with RSV proteins when analyzed by immunoprecipitation or by immunoblotting procedures (data not shown). At 2-week intervals, the rabbit was boosted with 0.5 ml of the solubilized proteins mixed with 0.5 ml of Freund incomplete adjuvant. The resulting serum was found to react well with RSV Gag proteins on immunoblots and in immunoprecipitations.

Construction of carboxy-terminal truncations. Carboxy-terminal deletion mutants 3h and I9e were generated by removing portions of the 3′ *gag* sequence with exonuclease BAL 31 (New England BioLabs, Inc., Beverly, Mass.). These deletions originated from a unique site (*Eco*RV) located near the 3′ end of *gag* in the bacterial plasmid sequence of pSV.Myr1 (35). The digested DNA fragments were circularized by using T4 DNA ligase in the presence of *Xba*I linkers (5′-CTCTAGAG-3′). The endpoints of these deletions were determined by restriction enzyme analysis and double-stranded DNA sequencing as described below.

Another method used to construct carboxy-terminal deletions of $Pr76^{myr1}$ was to remove restriction endonuclease fragments spanning the 3′ end of *gag* in pSV.Myr1. The sticky ends produced by this method were made blunt, using the Klenow fragment of DNA polymerase I, and the DNA fragments were circularized, using T4 DNA ligase. The resulting plasmids, pSV.ΔEc-Bs, pSV.ΔBg-Bs, and pSV.ΔEs-Bs, lack *Eco*RI-*Bss*HII, *Bgl*II-*Bss*HII, and *Esp*I-*Bss*HII fragments, respectively.

Construction of chimeric plasmids. The method used to construct plasmids for expression of Gag fusion proteins is illustrated in Fig. 2. Plasmids pAB16 and pSV.Myr1 were digested with *Hin*dIII and *Bss*HII, respectively, and the resulting ends were made blunt, using mung bean nuclease. Both DNAs were then digested with *Eco*RI, and the appropriate fragments from each were purified and joined, using T4 DNA ligase to create pSV.MyCyE. The *Eco*RI-*Hin*dIII fragment from pAB16 contains all but the first 3 of the 108 codons of the iso-1-cytochrome *c*-coding region and includes 275 base pairs flanking the 3′ end of *CYC1*. Insertion of *CYC1* into pSV.Myr1 was confirmed by restriction endonuclease mapping and by dideoxy sequencing of the double-stranded DNA, using modified T7 DNA polymerase (Sequenase; United States Biochemical Co. Cleveland, Ohio) as suggested by the manufacturer. The iso-1-cytochrome *c*-coding sequence in pSV.MyCyE lies in the −1 reading frame with respect to *gag*. To put the two coding sequences in register, pSV.MyCyE was digested with *Eco*RI, treated with mung bean nuclease, and recircularized, using T4 DNA polymerase to create pSV.MyCY (see Fig. 2).

To insert an active-site mutation into the residual portion of RSV protease, the *Bgl*II-*Eco*RI fragment of *gag* in pSV.MyCyE was replaced with that of pSV.D37I (35). The latter plasmid is a derivative of pSV.Myr1 which substitutes isoleucine for aspartic acid at residue 37 of the protease. The plasmid obtained as a result of this fragment exchange, pSV.MyCyE.D37I, carries the iso-1-cytochrome *c* reading frame in the −1 reading frame with respect to *gag*. The two reading frames were put in register, using mung bean nuclease as described above to generate plasmid pSV.MyCY.D37I.

Transfection of mammalian cells and metabolic labeling. Before transfection, the bacterial plasmid sequence of the expression vectors was removed by digestion with *Xba*I (Fig. 2) and the DNA was ligated at a concentration of 25 μg/ml. This manipulation joined the 3′ end of the *gag* gene (or the *gag-CYC1* gene fusion) to the SV40 late polyadenylation signal. COS-1 cells were transfected, using the DEAE-dextran-chloroquine method as described previously (35, 36). The cells were labeled with L-[^{35}S]methionine (>1,000 Ci/mmol; ICN Pharmaceuticals, Irvine, Calif.) at 48 h post-transfection as described previously (35).

Purification of particles. The culture medium from transfected, radiolabeled COS-1 cells was transferred to a centrifuge tube, and cellular debris was removed by centrifugation at 4,500 × *g* for 20 min at 4°C. The supernatant was then layered onto a cushion of 5% (wt/wt) sucrose in TE buffer, and the particles were pelleted at 76,000 × *g* for 45 min at 4°C. The pellet was then suspended in 100 μl of TE buffer and stored at −80°C. Before electrophoresis, the particles were mixed with sample buffer (60 mM Tris hydrochloride [pH 6.8], 10% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.001% bromophenol blue) and heated at 90°C for 1 to 2 min.

Immunoprecipitation of Gag fusion proteins. Cell monolayers and particles in the growth medium were detergent disrupted in the presence of protease inhibitors (5 μg of pepstatin per ml, 500 μg of phenylmethylsulfonyl fluoride per ml, and 5 μg of leupeptin per ml) as described previously (35). All immunoprecipitations were performed overnight at 4°C with the indicated antisera. Antigen-antibody complexes were collected by using fixed *Staphylococcus aureus* cells prepared by standard protocols (11).

SDS-polyacrylamide gel electrophoresis. Proteins were separated by electrophoresis in SDS-polyacrylamide gels in a manner similar to that described before (35), except that a 29.2:0.8 ratio of acrylamide monomer and cross-linker (*N*,*N*′-methylenebisacrylamide) was used. The resolving gels contained 10 or 12% acrylamide, 0.1% SDS, and 400 mM Tris hydrochloride (pH 8.8), and the stacking gels contained 3% acrylamide, 0.1% SDS, and 60 mM Tris hydrochloride (pH 6.8). After electrophoresis, the gels were fixed and the radiolabeled proteins were detected by fluorography with Fluoro-Hance (Research Products International, Inc.) and Kodak X-Omat AR5 film at −80°C. Detection of [^{35}S]methionine-labeled proteins typically required overnight exposures.

Transmission electron microscopy. Monolayers of transfected cells were washed twice with phosphate-buffered saline and fixed with 2% paraformaldehyde–2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 3 h at room temperature. The cells were then scraped from the plate with a rubber policeman, rinsed three times with 0.1 M cacodylate buffer, and postfixed with 1% osmium tetroxide in 1% aqueous potassium ferrocyanide for 1 h at 4°C. The cells were then rinsed, stained en bloc with 1% aqueous uranyl acetate for 1 h at room temperature, dehydrated through 100% ethanol, and embedded in epoxy resins (Polybed 812-Araldite; Polysciences, Inc., Warrington, Pa.). Sections were cut at 70 to 100 nm with a diamond knife, stained with 1% uranyl acetate in 50% ethanol, and counterstained with 2% aqueous lead citrate. Samples were examined and photographed on a Philips CM 10 transmission electron microscope.

RESULTS

To test the feasibility of retro-secretion, we used the strategy of fusing foreign proteins to the retroviral protease domain which, in the case of RSV and its relatives, is included on the carboxy terminus of the Gag polyprotein precursor, Pr76^{gag} (14, 22). We decided to make fusions in this region because our previous work had shown that PR-mediated Gag processing is not a prerequisite for particle formation, at least in mammalian cells (35). However, because that finding was obtained using a mutant protease containing an amino acid substitution rather than a deletion, we could not predict with certainty whether molecular interactions between PR domains on the full-length Gag protein (e.g., to form dimers; 12, 17, 25) would be required for particle formation.

Carboxy-terminal deletions of the RSV Gag protein. To examine the need for the carboxy-terminal region of Pr76^{gag} in particle formation, various portions of the 3′ end of *gag* were removed either by deleting specific restriction endonuclease fragments or by using exonuclease BAL 31 (Fig. 1A). These deletions were introduced into pΔSV.Myr1, an SV40-based transient expression vector bearing the *myr1* allele of *gag* (Fig. 2). The full-length Myr1 protein is a derivative of Pr76^{gag} in which the first 10 amino acids have been replaced with those of pp60$^{v\text{-}src}$. This change allows addition of myristic acid to the amino terminus of the RSV Gag protein and enables its efficient packaging and release from mammalian cells in the form of membrane-enclosed particles (35). The presence of such particles in the culture medium can be readily assayed by specific immunoprecipitation of radiolabeled Gag proteins from the culture medium after solubilization of the viral membrane with detergents (see Materials and Methods). Electrophoresis of the proteins in an SDS-polyacrylamide gel showed that particle release was accompanied by complete processing of Pr76^{myr1} so that only the mature cleavage products were found in the medium (Fig. 1B, C, and D, lanes 2). Untransfected cells released no proteins that could be collected by immunoprecipitation with anti-RSV serum (Fig. 1B and C, lanes 1). Release of Gag proteins was also detected by pelleting the particles out of the medium and directly loading them onto the gel (see below).

Three restriction fragment deletions were constructed in pSV.Myr1 by removing the *Eco*RI-*Bss*HII, *Bgl*HII-*Bss*HII, or *Esp*I-*Bss*HII fragment of pSV.Myr1 (Fig. 1A). ΔEc-Bs, lacking the *Eco*RI-*Bss*HII fragment, is predicted to express a truncated protein containing the first 648 of the 701 amino acids of Pr76^{myr1} (including the first 71 of the 124 amino acids of PR) fused to four foreign residues (R-A-V-L). The ΔBg-Bs mutation extends into the CA-coding sequence and is predicted to result in a protein containing the first 418 amino acids of Pr76^{myr1} fused to 25 foreign residues (P-R-C-S-L-E-D-P-D-M-I-R-Y-I-D-E-F-G-Q-T-T-T-R-M-Q). The ΔEs-Bs mutation extends further into the CA-coding sequence and is predicted to result in a protein containing 348 amino acids fused to four foreign residues (R-A-V-L). The calculated molecular masses for the truncated proteins are 69 (ΔEc-Bs), 47 (ΔBg-Bs), and 37 (ΔEs-Bs) kilodaltons (kDa).

Two PR deletion mutants, 3h and I9e, were obtained by using BAL 31 (Fig. 1A). The product of the I9e allele is predicted to contain the first 673 amino acids of Pr76^{myr1} (including the first 96 residues of PR) fused to 22 foreign amino acids (S-L-E-D-P-D-M-I-R-Y-I-D-E-P-G-Q-T-T-T-R-M-Q). In the case of mutant 3h, the resulting 585-amino-acid protein is predicted to contain only seven amino acids of the protease and one foreign residue (L). The calculated molecular masses of the I9e and 3h proteins are 74 and 62 kDa, respectively.

To determine which of the truncated forms of Pr76^{myr1} were capable of inducing particle formation, COS-1 cells were transfected with pSV.3h, pSV.I9e, or two independently isolated clones of pSV.ΔEc-Bs, pSV.ΔBg-Bs and pΔSV.Es-Bs. After the transfected cells were labeled for 2.5 h with [^{35}S]methionine, the culture medium from each plate was harvested and cell lysates were prepared. RSV-specific proteins were collected by immunoprecipitation with an anti-RSV antibody, separated by SDS-polyacrylamide gel electrophoresis, and visualized by fluorography (Fig. 1). All of the mutants produced uncleaved Gag proteins because of removal of portions of PR. The relative mobilities of the truncated proteins in the gel were approximately those expected on the basis of the sizes of the deletions (i.e., Myr1 < I9e < Ec-Bs < 3h < Bg-Bs < Es-Bs). Some minor deviations from the calculated molecular masses were observed in the proteins of the two largest mutants (53 versus 47 kDa for ΔBg-Bs and 44 versus 37 kDa for ΔEs-Bs), but the independent isolates of each mutant behaved identically. Because we also observed unexpected gel mobilities when a single residue of PR was changed (D37I) and when four foreign amino acids were inserted into the p10 coding

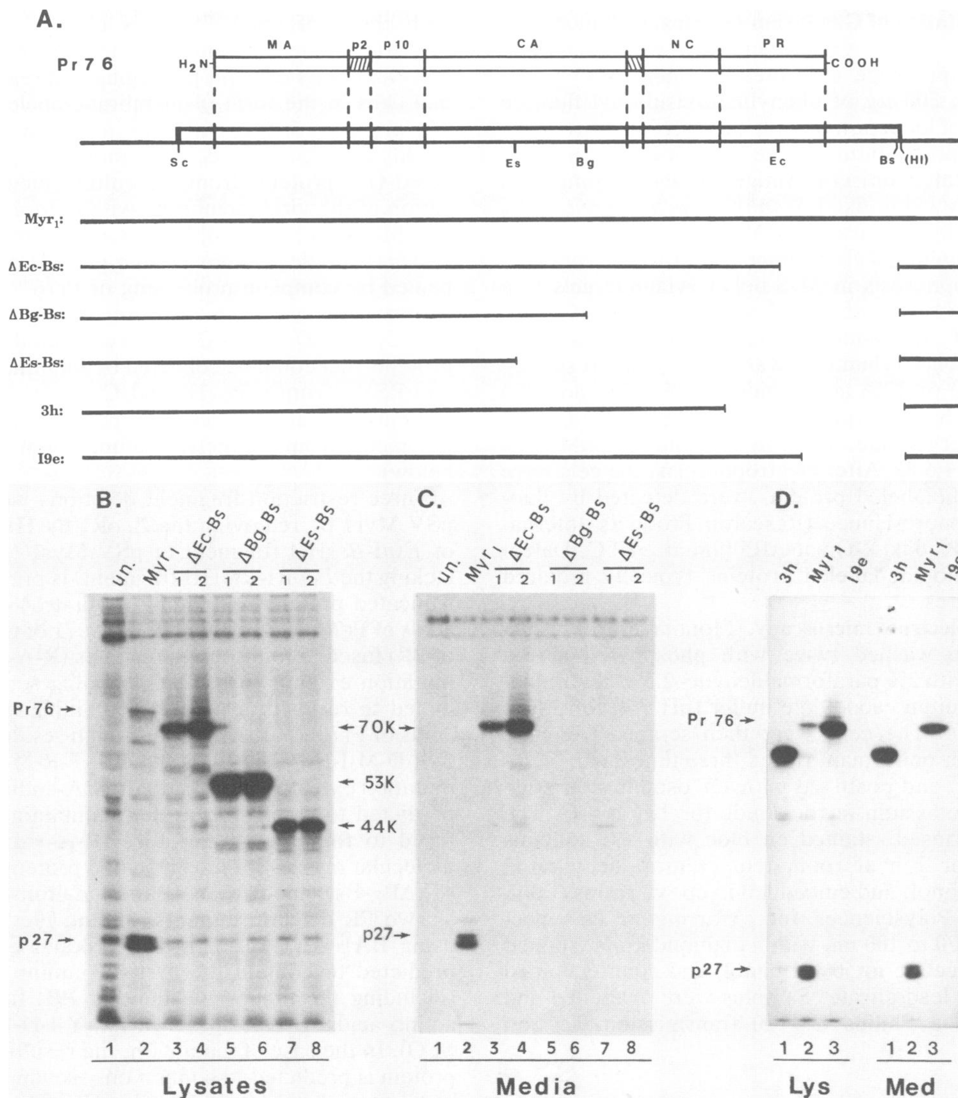

FIG. 1. Carboxy-terminal truncations of the Gag protein. (A) Schematic diagrams of the RSV Gag polyprotein, the *gag* coding sequence, and the various carboxy-terminal truncations. Sc, *Sac*I; Es, *Esp*I; Bg, *Bgl*II; Ec, *Eco*RI; Bs, *Bss*HII. (HI) represents a destroyed *Hpa*I site. (B and C) Some of the truncated Gag proteins were made as a result of removal of various restriction endonuclease fragments from the 3'-coding sequence. These were characterized by transfecting COS-1 cells with no DNA (lanes 1), pSV.Myrl (lanes 2), or two independently isolated clones each of deletion mutant plasmids ΔEc-Bs (lanes 3 and 4), ΔBg-Bs (lanes 5 and 6), and ΔEs-Bs (lanes 7 and 8). un., Untransfected. (D) Additional mutant plasmids were obtained using BAL 31 exonuclease. Two of these, 3h (lanes 1) and I93 (lanes 3), were transfected into COS-1 cells and compared with pSV.Myrl (lanes 2). After labeling for 2.5 h with [^{35}S]methionine, the culture media were harvested and the cells were lysed. Gag proteins were immunoprecipitated with anti-RSV serum, electrophoresed in SDS–12% polyacrylamide gels, and detected by fluorography. The bands near the top of each lane contained fibronectin, which binds directly to *S. aureus*.

sequence (unpublished data), we believe that the Gag protein maintains certain aspects of its secondary structure, unless altered by mutation, even in SDS-polyacrylamide gels.

The truncated proteins of the two mutants which lack PR, NC, and a portion of CA (ΔBg-Bs and ΔEs-Bs) were found to be trapped within cells (Fig. 1B and C, lanes 5 to 8). In contrast, the Gag proteins encoded by ΔEc-Bs, 3h, and I9e, which lack only portions of PR, were efficiently released as particles into the medium (Fig. 1B and C, lanes 3 and 4, and D, lanes 1 and 3, respectively). Because 3h lacks almost all of PR, we concluded that there are no functions located in this domain that are important for particle formation and decided to use the unique *Eco*RI site in the protease-coding sequence as the point of fusion in constructing chimeric *gag* genes.

Expression of chimeric Gag proteins. The foreign protein which we selected for packaging into particles was iso-1-cytochrome *c*, a mitochondrial protein from *S. cerevisiae*. This yeast protein was chosen for a number of reasons. (i) A plasmid (pAB16) containing the coding sequence (*CYC1*; Fig. 2) was locally available, as were antibodies to iso-1-cytochrome *c*. (ii) A convenient restriction endonuclease site (*Eco*RI; Fig. 2) is present near the 5' end of the *CYC1* coding sequence, which enabled almost the entire reading frame to be fused onto the *Eco*RI site in the PR-coding sequence (Fig. 2). We reasoned that coupling of only a portion of the cytochrome onto Pr76^{myr1} might interfere with the folding of both and obstruct particle production. (iii) Iso-1-cytochrome *c* is a small protein of only 108 residues. We wanted to use a small protein, since it had already been

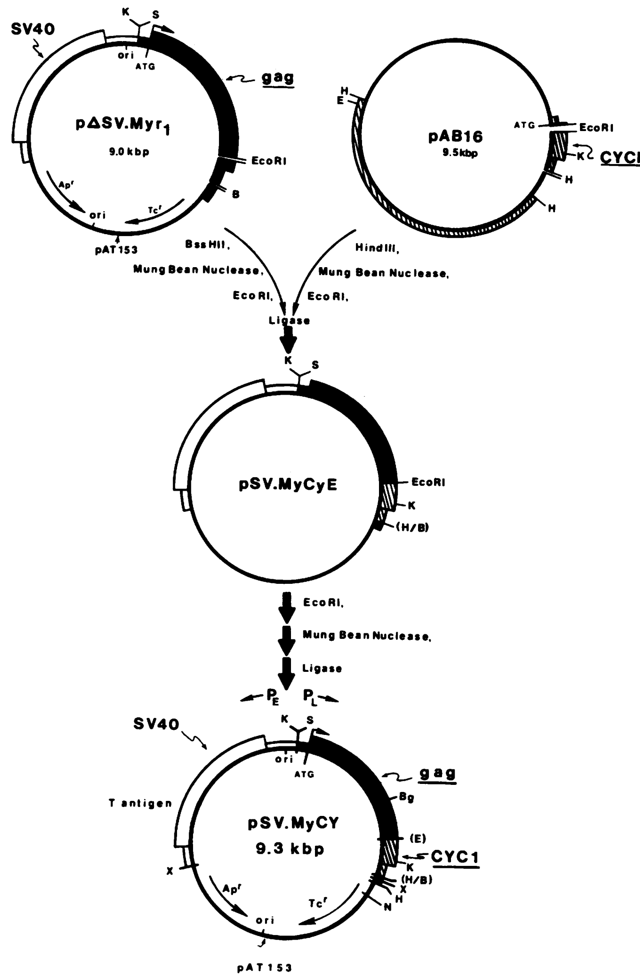

FIG. 2. Construction of chimeric genes. In pSV.Myrl, the solid black lines represent RSV *gag* sequences from the *SacI* site to the *HindIII* site. The large open lines represent SV40 sequences containing the early region, origin of replication (ori), and late promoter. The remaining sequences (thin lines) represent bacterial plasmid (pAT153) sequences carrying the bacterial origin, ampicillin resistance gene (Ap^r), and tetracycline resistance gene (Tc^r). In pAB16, the cross-hatched boxes represent *S. cerevisiae* DNA containing the *CYC1* coding sequence. In all of the plasmids, the thicker portions of the boxes denote coding regions of the respective genes, whereas the narrower regions are noncoding. K, *KpnI*; S, *SacI*; B, *BssHII*; H, *HindIII*; Bg, *BglII*; N, *NheI*; (E), destroyed *EcoRI* site; (H/B), destroyed *HindIII* and *BssHII* sites, respectively; P_E and P_L, SV40 early and late promoters, respectively. kbp, Kilobase pairs.

shown that large proteins, such as the *pol* product of murine leukemia virus, might destroy the budding process if fused to Gag protein all of the time instead of the 5% frequency that normally occurs (6). Whether a packaging limitation for proteins of larger size actually exists is unknown.

The strategy used to fuse the *gag* and *CYC1* reading frames is illustrated in Fig. 2 and explained in Materials and Methods. Replacement of the *EcoRI-BssHII* fragment of pSV.Myrl with the *EcoRI-HindIII* fragment from pAB16 to create pSV.MyCyE was expected to place the *CYC1* coding sequence into the −1 reading frame relative to that of *gag*; this was confirmed by DNA sequencing. The predicted product of this out-of-frame gene fusion is a protein of 69 kDa containing the first 646 amino acids of Pr76^{myr1} fused to only five foreign residues (Q-G-R-F-C). Thus, the MyCyE

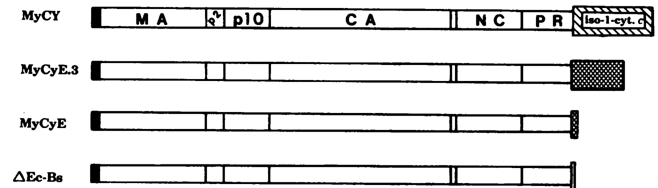

FIG. 3. Predicted Gag fusion proteins expressed from chimeric plasmids. The solid black box at the amino terminus of each polypeptide represents the first 10 amino acids of pp60$^{v\text{-}src}$. The five mature Gag proteins are indicated. The cross-hatched box in the MyCY polypeptide represents iso-1-cytochrome *c* fused to residue 70 of PR, the double-cross-hatched box in the MyCyE.3 polypeptide represents 68 residues predicted from *CYC1* open reading frame 3, and the checkered box in MyCyE represents six amino acids predicted from open reading frame 1. The open box in the ΔEc-Bs polypeptide represents the four amino acids predicted to be fused to residue 71 of PR.

protein would be very similar, but not quite identical, to the ΔEc-Bs product (Fig. 3). Consistent with this prediction, we found that COS-1 cells transfected with pSV.MyCyE expressed a protein of 69 kDa, designated p69^E, which was immunoprecipitated from the cell lysate and the detergent-treated medium with antibodies specific for RSV (Fig. 4A, lanes 5) but not with antibodies that recognize iso-1-cytochrome *c* (Fig. 4B, lanes 5). As expected, the anticytochrome antibody also did not react with Pr76^{myr1} or its cleavage products (Fig. 4B, lanes 2) but showed only nonspecific reactivity with proteins which were also present in the lysates from untransfected cells (Fig. 4B, lanes 1).

To align the *gag* and *CYC1* reading frames, pSV.MyCyE was cut at the unique *EcoRI* site and then the ends of the DNA were made blunt and rejoined by using mung bean nuclease and T4 DNA ligase, respectively. Presumptive clones of the desired plasmid, pSV.MyCY, were identified by screening for loss of the *EcoRI* site and then transfected into COS-1 cells to determine the sizes of the encoded proteins. The Gag-cytochrome fusion protein is predicted to have a molecular mass of 80 kDa and to contain the first 647 amino acids of Pr76^{myr1} linked to all but the first three of the 108 residues of iso-1-cytochrome *c* (Fig. 3). Two clones were obtained which indeed expressed an 80-kDa product, designated p80^{CY}, but two other clones were found to express a 76-kDa product. A protein of 76 kDa would be expected if the mung bean nuclease treatment had removed an extra base pair, causing the third reading frame of *CYC1* to be fused to *gag*. DNA sequencing confirmed that this had indeed happened, and clones of this type were designated pSV.MyCyE.3. The protein encoded by such clones, p76^{E3}, should contain the first 647 amino acids of Pr76^{myr1} and 68 foreign amino acids (Fig. 3). The long stretch of foreign, noncytochrome amino acids found on p76^{E3} appeared to impair particle release into the medium greatly (Fig. 4A, lanes 4), but low levels were detected in several other experiments (data not shown).

Although antibodies to RSV reacted well with all of the fusion proteins (Fig. 4A), only the products of pSV.MyCY reacted with anticytochrome antibodies (Fig. 4B); this confirmed that Gag-cytochrome fusion proteins had been made. Detection of p80^{CY} in the culture medium by using both antisera (Fig. 4, lanes 3) suggested that the fusion protein had been packaged into particles. This was confirmed by centrifuging the culture fluids under conditions suitable for pelleting of authentic RSV and by examination of the particles by electron microscopy (see below). Efficiency of par-

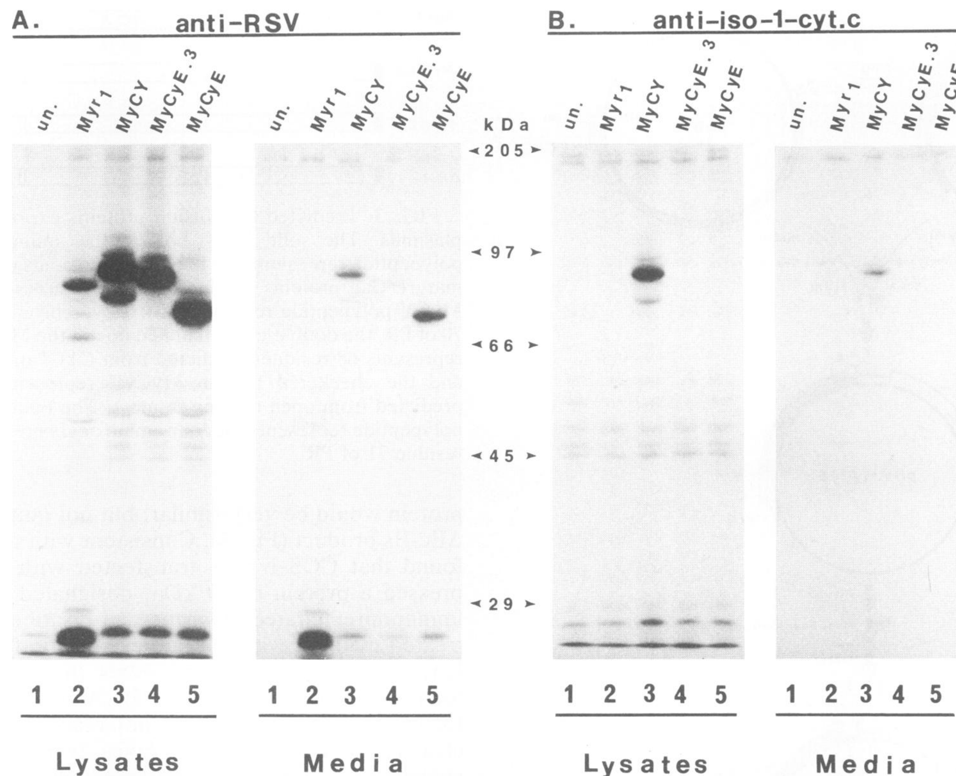

FIG. 4. Expression and release of chimeric Gag proteins from transfected cells. COS-1 cells were either untransfected (un., lanes 1) or transfected with pSV.Myrl (lanes 2), pSV.MyCY (lanes 3), pSV.MyCyE.3 (lanes 4), or pSV.MyCyE (lanes 5). After labeling for 2.5 h with [^{35}S]methionine, the culture media and cell monolayers were harvested. Gag- or cytochrome-specific proteins were collected from the samples by immunoprecipitations with antibodies to either RSV (A) or iso-1-cytochrome *c* (B). The labeled proteins were separated by electrophoresis in an SDS–10% polyacrylamide gel and detected by fluorography. The positions of the marker proteins are indicated.

ticle formation with the cytochrome fusion protein appeared to be somewhat impaired relative to that with Pr76^{myr1} in the experiment shown in Fig. 4, but many additional experiments (described below) demonstrated that this difference was actually small. Furthermore, pulse-chase experiments suggested that particles containing p80^{CY} were released with kinetics similar to those obtained with Pr76^{myr1} (data not shown).

Appearance of smaller Gag- and cytochrome-related polypeptides. An unexpected result obtained with all three chimeric genes was the appearance of proteins smaller than the predicted full-length fusion proteins. With pSV.MyCY, there were, in addition to p80^{CY}, products of 74 and 25 kDa which reacted with anti-RSV and anticytochrome antibodies (Fig. 4A, lanes 3). In our SDS–10% polyacrylamide gels, the 25-kDa protein ran very close to the dye front and comigrated with a cellular protein (lane 1) and with the RSV CA proteins (p27), but higher-concentration gels nicely resolved these proteins (see data presented below). Nevertheless, even a 10% gel showed that the p25, p74, and p80^{CY} were all released into the medium while the cellular protein was not. We are confident that p25 and p74 are not coimmunoprecipitated cellular proteins that stably interact with p80^{CY} because both were detected when first separated by gel electrophoresis and then assayed by immunoblotting with anti-RSV or anticytochrome serum (data not shown).

With pSV.MyCyE and pSV.MyCyE.3, a product of 25 kDa was also observed but one of 74 kDa was not. Immunoblotting experiments indicated that in both cases this small protein was also of noncellular origin (data not shown). Unlike the p25 of pSV.MyCY, these two species of p25 did not seem to react with the anticytochrome antibody, even though they reacted well with the anti-RSV antibody (compare medium samples in lanes 3 to 5 of Fig. 4A and B). Because this difference in reactivity was consistently observed, the p25 made by pSV.MyCY was named p25^{C} while that made by pSV.MyCyE and pSV.MyCyE.3 was named p25$^{E/3}$. We detected no differences between p25^{E} and p25^{3}.

One trivial explanation for the smaller polypeptides would be that they were generated from the Gag fusion proteins by cellular proteases when the transfected cells were lysed. This sort of nonspecific degradation of the wild-type RSV Gag protein has, in fact, been observed in certain mammalian cell types (30), but we have never before encountered problems of this type in our system. Indeed, all of our other Gag derivatives which bear carboxy-terminal truncations were well behaved and did not produce major bands of smaller proteins (Fig. 1). Even ΔEc-Bs, which is predicted to be identical to p69^{E} except for a slight difference in the foreign amino acids at the site of fusion, did not produce a p25 species. We routinely add a mixture of protease inhibitors (phenylmethylsulfonyl fluoride, pepstatin, and leupeptin) to our lysis buffers (see Materials and Methods), but even inclusion of *N*-ethylmaleimide, which was crucial for inhibition of nonspecific degradation in other mammalian cells (30), had no effect on the amounts of p25^{C} and p74 observed with pSV.MyCY (data not shown). Nevertheless, it could be argued that the fusion proteins happen to have a unique conformation that is susceptible to proteolysis by a

cellular protease that happens to be resistant to all four of these inhibitors.

One simple way to reduce drastically the opportunity for lysis-dependent degradation of viral proteins by cellular proteases is to purify the particles released from cells before disrupting them with detergents. Therefore, COS-1 cells were transfected with pSV.Myr1 or pSV.MyCY and metabolically labeled with [^{35}S]methionine for 12 h in serum-free medium. The labeling medium was then collected from the plate and centrifuged at low speed to remove any cellular debris that might be present, and the particles were sedimented through a cushion of 5% sucrose which occupied the bottom third of the centrifuge tube. The resulting pellet was immediately dissolved in sample buffer, and the proteins were loaded directly (without immunoprecipitation) onto an SDS-12% polyacrylamide gel.

The electrophoretic profile observed for the Myr1 cleavage products (Fig. 5, lane 2) was identical to that of authentic RSV Gag proteins (35). The major band of protein with a molecular mass of 27 kDa corresponds to the RSV CA protein, and the band running at 14 kDa contains the PR protein (p15). The NC (p12) and p10 proteins were difficult to detect because they each contain only one methionine residue for radiolabeling; moreover, NC tends to comigrate with PR. The mature MA protein (p19) appeared as a faint doublet of bands located between CA and NC-PR. An immature form of MA, still containing the spacer peptide (p2) which lies in between MA and p10 (Fig. 1; 19, 31, 35), appeared as a minor band located just below CA. Historically, this immature form of MA is called p23.

In the MyCY sample, p74 and p25^{C} were still observed, along with full-length fusion protein p80^{CY}, in spite of the purification procedure (Fig. 5, lane 1). Furthermore, the relative amounts of all three species were the same as those observed in other experiments. This result strongly argues that the two smaller proteins originate in vivo and are contained within the particles. Interestingly, p25^{C} (and p25$^{E/3}$ [data not shown]) appeared to comigrate with the immature form of MA, p23. This raises the possibility that p25^{C} might be derived from p80^{CY} by a proteolytic cleavage event during assembly of the chimeric particles. Detection of bands at positions intermediate to those of p74 and p25^{C} (Fig. 5) also appears to be consistent with the idea of precursor processing.

It seemed unlikely that cleavage of p80^{CY} could be mediated by retroviral protease, since nearly half of it had been removed during construction of the fusion proteins. Nevertheless, since the remaining portion of the protease domain contains the aspartic acid of the PR active site (residue 37), we decided that it was important to entertain the possibility of residual or reactivated PR activity. To address this notion, we introduced a mutation, D37I, into the remaining PR-coding sequence to replace aspartic acid with isoleucine. We have previously shown that this mutation abolishes PR-mediated cleavages in our mammalian cell system (35). The presence of the mutation was confirmed by screening for the presence of a new *Eco*RV site which is created by the mutation, and the resulting plasmid was called pSV.MyCY. D37I. COS-1 cells transfected with this or the parent construct were radiolabeled, and the Gag-related proteins were collected and analyzed as before with anti-RSV serum. The protease mutation had no effect on synthesis and release of p25^{C} (Fig. 6, lanes 4); similar results were obtained with multiple clones (data not shown). The only difference we observed with the D37I mutation was a consistently lower level of p74 in transfected cell lysates and culture media

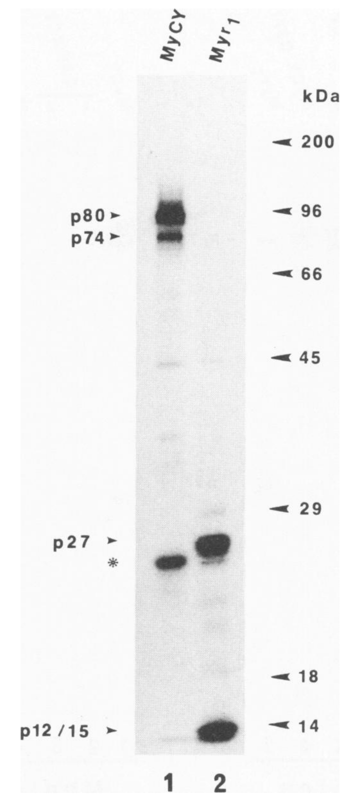

FIG. 5. Particles obtained by sedimentation. COS-1 cells were transfected with either pSV.MyCY or pSV.Myr1 DNA and later labeled with [^{35}S]methionine for 12 h. The medium from each culture was harvested and clarified, and the particles were collected by centrifugation through a cushion of 5% sucrose. A portion of the proteins contained in each pellet was analyzed without immunoprecipitation in an SDS-12% polyacrylamide gel, followed by fluorography. Lane 1 shows the protein profile of MyCY particle preparations; Lane 2, Myr1 particles. The asterisk indicates a comigrating polypeptide present in the MyCY and Myr1 particles.

relative to the control MyCY products (lanes 1). Thus, if p25^{C} is derived by cleavage of p80^{CY}, such an event cannot be PR mediated.

If any of the products of pSV.MyCY were indeed derived by some other assembly-related proteolytic processing event, then one might expect that mutations which disrupt particle formation would also disrupt the appearance of those products. To examine this possibility, we made use of an amino-terminally truncated form of the RSV Gag protein, Pr76^{gagX}, which is defective for particle formation (35). The protease domain contained within the GagX protein is fully wild type in sequence, but its activity is blocked, presumably because of its failure to be targeted to the site of assembly at the plasma membrane (unpublished data). As a result, the GagX protein remains essentially unprocessed and is found only associated with the cell lysate. Using the *gagX* and *CYC1* alleles, we truncated the amino terminus of p80^{CY} in a manner analogous to that of the GagX protein. This was accomplished by transferring an *Nhe*I-*Bgl*II fragment from pSV.MyCY (containing the fused *gag-CYC1* sequence; Fig. 2) into plasmid pΔSV.GAGX (35), which encodes Pr76^{gagX}. The desired clones of pSV.GAGXCY were identified by restriction endonuclease analysis, and two were analyzed as before after transfection into COS-1 cells.

As expected, placement of the GagX amino terminus onto

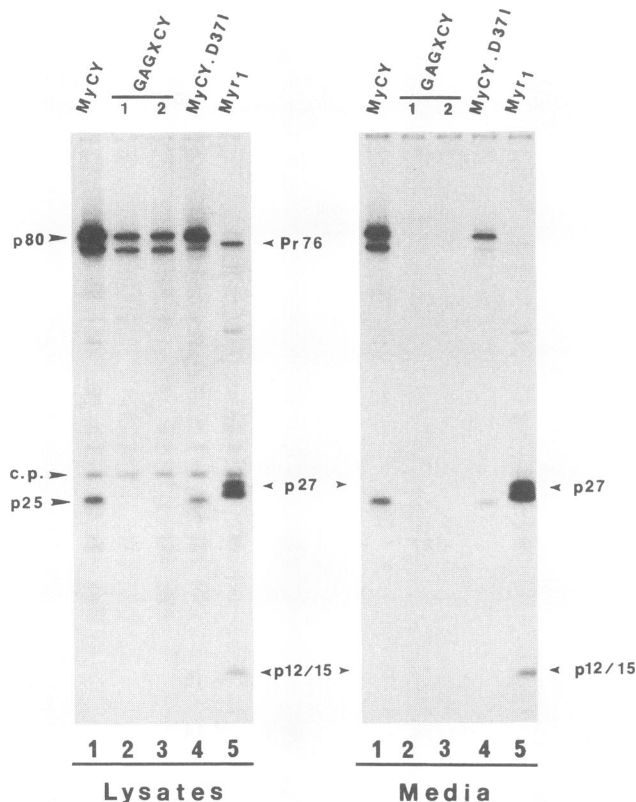

FIG. 6. Mutational analysis of the Gag-cytochrome fusion protein. COS-1 cells were transfected with pSV.MyCY (lanes 1), two independently isolated clones of pSV.GAGXCY (lanes 2 and 3), pSV.MyCY.D37I (lanes 4), or pSV.Myrl (lanes 5). After labeling with [^{35}S]methionine for 2.5 h, cell lysates and culture media were harvested and Gag-related proteins were collected by immunoprecipitation with anti-RSV antibodies. The resulting fluorogram obtained following electrophoresis of the proteins in an SDS–12% polyacrylamide gel is shown. A cellular protein which was nonspecifically precipitated is indicated (c.p.).

the fusion protein, creating GagXCY, abolished particle formation for both of the test clones (Fig. 6, media, lanes 2 and 3). Interestingly, examination of the cell lysates revealed that the GagX alteration totally abolished p25^{C} production, even though syntheses of p80^{CY} and p74 were unaffected (Fig. 6, lysates, lanes 2 and 3). Parallel immunoprecipitations using anticytochrome serum confirmed the presence of the cytochrome antigen on full-length p80^{CY}, as well as on p74, but also detected no polypeptides of 25 kDa. While these results are consistent with the idea that p25^{C} arises during particle assembly, an alternative hypothesis is that it is derived from a spliced mRNA whose synthesis or stability is affected by the sequence of the *gagX* allele. Resolution of these possibilities awaits further experimentation.

Morphology of pseudovirions. Having shown that foreign antigens can be internally packaged into retroviruslike particles when coupled to Pr76^{myr1}, we were curious about the appearance of the particles. Since the chimeric fusion proteins are not processed by the viral protease, the particles released from the cells might be expected to have a morphology similar to that of immature type C retroviruses. To examine the particles directly, transmission electron microscopy was performed. As a control, we first examined authentic RSV produced by infected turkey embryo fibroblasts. Both immature virions having electron-lucent cores (top particle) and mature virions having electron-dense cores (bottom particle) were detected (Fig. 7A). Additional examples of mature particles are shown in Fig. 7B. As expected, these virions ranged in diameter from approximately 90 to 120 nm and were surrounded by a double membrane (18, 29).

We also examined Myrl particles produced in COS-1 cells to determine their appearance relative to RSV. Like authentic Gag protein in RSV-infected avian cells, the Myrl protein is processed into mature structural proteins (MA, p10, CA, NC, and PR) and released in membrane-bound particles with a density nearly identical to that of RSV (35). On the basis of these observations, it was expected that Myrl particles would have morphologies similar, if not identical, to those of type C retroviruses, and they did (Fig. 7C and D). Because the half-time of Myrl processing and particle release are very fast (35), it was difficult to find immature Myrl particles. We therefore sought to accumulate such particles by destroying Pr76^{myr1} processing. COS-1 cells were transfected with a Myrl protease deletion mutant which does not exhibit Gag processing. The particles produced by this mutant assembled at the plasma membrane (Fig. 7E) and were released, but the immature cores never condensed (Fig. 7F). Over 30 particles were observed, and all had immature capsid morphology.

When the MyCY particles were examined, it was found that they generally resembled immature type C particles and had diameters of 90 to 110 nm. Their internal structures varied to a large extent, ranging from a very dense structure lying just under the envelope and circumscribing a very large electron-lucent area (Fig. 7G) to a more amorphous internal structure (Fig. 7H). Although we do not fully understand the structure of these pleomorphic particles, we hypothesize that the presence of the foreign polypeptides might disrupt some of the organized arrangement of Gag proteins normally found in mature virus particles.

DISCUSSION

Proteins secreted from eucaryotic cells are generally difficult to purify from the growth medium because of the high protein content of the serum usually required for growth. Likewise, nonsecreted proteins are difficult to purify because of the total intracellular protein mass. In contrast, viral structural proteins are generally easy to obtain simply because they are sequestered from other intracellular proteins and packaged into particles (virions) that can be readily collected and separated from nonviral proteins by centrifugation. The basic idea of retro-secretion is to confer upon proteins of interest the ability to be packaged into noninfectious, retroviruslike particles for easy concentration and purification from the cells. The experiments described here demonstrated that foreign antigens can be packaged into retrovirions of immature morphology if directly linked to the Gag protein.

Retroviruses have many features that make them particularly well suited for packaging of foreign proteins. As mentioned above, the Gag protein is the only viral product required for particle formation, and large portions of it are dispensable in our mammalian system (unpublished data). Also, most retroviruses do not kill host cells, making possible virion production for extended periods. For example, avian cells transformed by RSV can be maintained in roller bottle cultures for over 3 months without loss of yield in virion production (27; our unpublished data). Proteins contained within retrovirions are stable because of the lipid

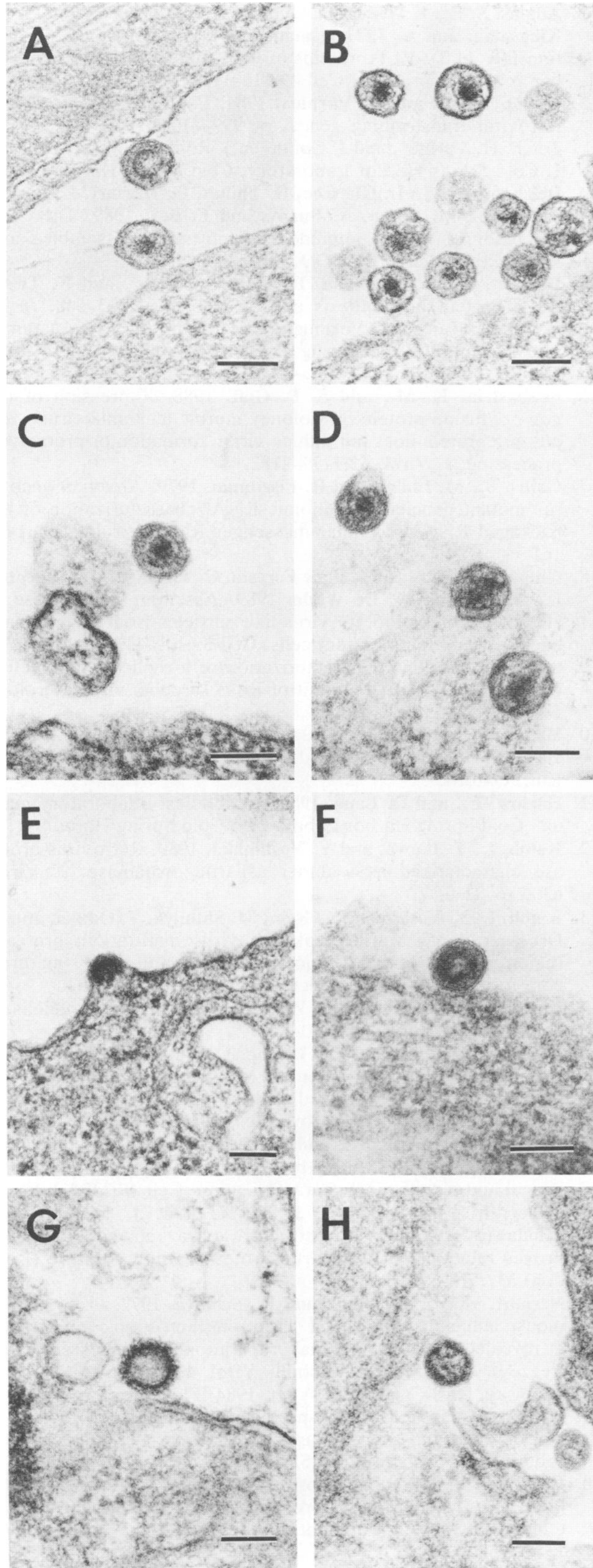

FIG. 7. Morphology of retrosecreted particles. Transmission electron micrographs of turkey embryo fibroblast cells infected with wild-type RSV reveal both immature (A, top particle) and mature (B) virions. COS-1 cells transfected with pSV.Myr1 expressed a full-length, myristylated Gag protein and produced particles (C and D) indistinguishable from those of authentic RSV. Particles produced by the transfected COS-1 cells were not found in the cytoplasm but seemed to assemble only at the plasma membrane in a manner typical for type C retroviruses (E). COS-1 cells expressing a protease mutant produced particles identical in appearance to immature RSV particles (F). COS-1 cells expressing the Gag-cytochrome fusion protein (G and H) produced particles which most resembled immature RSV particles. Bars, 100 nm.

bilayer that separates them from the surrounding environment, and because retroviruses are enveloped, it may eventually be possible to package foreign glycoproteins of interest onto their surfaces. Finally, the genomes of retroviruses are small, and the existence of a DNA intermediate allows chimeric genes to be easily constructed and expressed. These features appear to offer advantages over a related approach which makes use of the Ty retrotransposon of *S. cerevisiae* (1, 2). Ty particles are not enveloped and are not released from yeast cells, thus making it necessary to lyse the cells before purification.

Among retroviruses, RSV is a good choice for use in retro-secretion applications. Modification of its Gag protein to create a myristic acid addition site enables exceedingly rapid particle formation not only in simian cells (35) but also in murine and avian cells (unpublished data). RSV has never been associated with human disease in the 80 years since its discovery. Fully infectious RSV does not replicate in mammalian cells, even if it carries an allele for myristylated Gag protein (unpublished data), because of a variety of blocks to replication (33). Also, use of a modified RSV Gag protein in mammalian cells should be safe because the coding sequence does not contain significant homology to endogenous mammalian retroviruses which might otherwise be activated by recombination to produce unwanted infectious virions.

The limitations of retro-secretion are unknown. Experiments with murine leukemia virus have shown that its Gag protein no longer makes particles if fused to the Pol protein all of the time, rather than at the 5% level that normally occurs during murine leukemia virus infection (6). This might indicate that there are size restrictions on the amount of foreign protein that can be packaged into a retrovirion, but on the other hand, it may be that the *pol* product happens to interfere with folding and function with the Gag protein. If the latter proves to be the case, large-size foreign proteins potentially could be retro-secreted. If size limitations do exist, these may be relieved by removing the portions of the Gag protein that are unnecessary for particle formation. In this report, we have shown that the PR domain is dispensable for particle assembly in our mammalian system, a result which was suggested by previous studies of spontaneous, large deletion mutants expressed in avian cells (32). We have recently characterized a number of additional mutant Gag proteins which collectively demonstrate that over half of the Gag protein is not needed for assembly (unpublished data). Experiments are in progress to delineate the boundaries of the required domains.

Another potential limitation of retro-secretion is final separation of the foreign protein of interest from the Gag protein. We envision two possible solutions to this problem. One would be to insert a protease cleavage site at the junction of the two proteins. In this case, the chimeric particles would be solubilized to allow the fusion proteins to be cleaved in vitro by adding an exogenous protease capable of recognizing the cleavage site. The other approach would

be to replace an internal, nonessential region of Gag with the foreign protein of interest such that it is flanked by cleavage sites that are recognized by the viral protease PR. Inclusion of PR on the chimeric Gag protein would then allow in situ release of the foreign protein during particle formation.

Once the foreign protein is separated from the Gag protein, it would still reside in a mixture of Gag proteins, but of course, the composition of this mixture would be very simple. Indeed, we have been surprised by the apparent purity of retro-secreted proteins pelleted from medium by simple centrifugation. This report shows that very few, if any, cellular proteins are detected when the particles are labeled for an extended period before direct analysis in SDS-polyacrylamide gels. Absence of significant cellular protein contamination is typical of retroviruses (reviewed in references 24 and 28), but the mechanism(s) by which host proteins are excluded during particle assembly is not known. Although we cannot rule out the possibility that low levels of host proteins were present and escaped detection, retro-secretion nevertheless presents the opportunity for vast enrichment of foreign proteins before any in vitro purification steps are begun.

An unexpected result obtained upon expression of the in-frame *gag-CYC1* gene was the appearance of two smaller fusion proteins (p74 and $p25^{C}$) along with the expected, full-length Gag-cytochrome protein ($p80^{CY}$). These smaller polypeptides do not seem to arise by a trivial means (e.g., nonspecific degradation during cell lysis), but their origin is a mystery. They seem most likely to arise either by a specific proteolytic processing event or by mRNA splicing. We found that disruption of the budding process by truncation of the MA domain of the fusion protein (by making a recombinant with the *gagX* allele; 35) blocked $p25^{C}$ synthesis, suggesting that particle formation is a prerequisite for its appearance. This result is consistent with the idea of proteolytic processing, since retroviral proteases are activated only upon particle formation. However, if a proteolytic cleavage event does account for $p25^{C}$, the activity responsible could not involve the active site of RSV protease PR. Substitution of the aspartic acid normally situated in the PR active site, and retained on the fusion proteins, with an amino acid which destroys protease activity had no effect on $p25^{C}$ synthesis. To gain further insight into the smaller polypeptides and their importance to the process of retro-secretion, we are currently isolating and characterizing the mRNAs produced by the chimeric genes to determine whether spliced species exist. If the smaller proteins do arise by mRNA splicing, then it will be of interest to determine whether they are capable of forming particles on their own or are packaged only through molecular interactions with the full-length fusion protein.

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