JOURNAL OF VIROLOGY, Sept. 1993, p. 5550–5561
0022-538X/93/095550-12$02.00/0

Vol. 67, No. 9

Characterization of a Small (25-Kilodalton) Derivative of the Rous Sarcoma Virus Gag Protein Competent for Particle Release

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Received 15 April 1993/Accepted 15 June 1993

Retroviral Gag proteins have the ability to induce budding and particle release from the plasma membrane when expressed in the absence of all of the other virus-encoded components; however, the locations of the functional domains within the Gag protein that are important for this process are poorly understood. It was shown previously that the protease sequence of the Rous sarcoma virus (RSV) Gag protein can be replaced with a foreign polypeptide, iso-1-cytochrome *c* from a yeast, without disrupting particle assembly (R. A. Weldon, Jr., C. R. Erdie, M. G. Oliver, and J. W. Wills, J. Virol. 64:4169–4179, 1990). An unexpected product of the chimeric *gag* gene is a small, Gag-related protein named $p25^{C}$. This product was of interest because of its high efficiency of packaging into particles. The goal of the experiments described here was to determine the mechanism by which $p25^{C}$ is synthesized and packaged into particles. The results demonstrate that it is not the product of proteolytic processing of the Gag-cytochrome precursor but is derived from an unusual spliced mRNA. cDNA clones of the spliced mRNA were obtained, and each expressed a product of approximately 25 kDa, designated $p25^{MI}$, which was released into the growth medium in membrane-enclosed particles that were much lighter than authentic retrovirions as measured in sucrose density gradients. DNA sequencing revealed that the clones encode the first 180 of the 701 amino acids of the RSV Gag protein and no residues from iso-1-cytochrome *c*. This suggested that a domain in the carboxy-terminal half of Gag is important for the packaging of Gag proteins into dense arrays within the particles. In support of this hypothesis, particles of the correct density were obtained when a small segment from the carboxy terminus of the RSV Gag protein (residues 417 to 584) was included on the end of p25.

Rous sarcoma virus (RSV) is an avian retrovirus which exhibits morphogenic events typical of the vast majority of other retroviruses (37). In particular, the internal structural proteins of RSV do not assemble into visible cores in the cytoplasm prior to their envelopment at the plasma membrane. Rather, the first morphological sign of particle assembly is the appearance of discrete, electron-dense patches intimately associated with the inner face of the plasma membrane. As morphogenesis proceeds at these sites, a crescent-shaped structure emerges from the cell surface as the virion core and envelope develop concurrently. The only viral product needed for this process of budding is the Gag polyprotein, which in the case of RSV is known as $Pr76^{gag}$ (9, 38). The other components of the virus—reverse transcriptase (RT), integrase, glycoproteins (SU and TM), and genomic RNA—are all dispensable for budding.

During or soon after budding, Gag polyproteins are processed by the viral protease to release the major proteins found in the interior of the infectious virus. In the case of RSV, these cleavage products include (in the order in which they are linked in the precursor) matrix (MA), p2, p10, capsid (CA), nucleocapsid (NC), and protease (PR). MA, CA, and NC are products common to all retroviruses and are positioned at similar locations both in the Gag precursor and in the mature virion; however, they have little or no amino acid sequence homology among viruses from different species (37). Similarly, all retroviruses possess a PR, although in most retroviruses it is not encoded in the *gag* gene. Products p2 and p10 have unknown subviral positions and appear to be unique to RSV and its relatives.

In all retroviruses, including those that assemble their cores in the cytoplasm, proteolytic cleavage of the Gag protein brings about a dramatic reorganization of the internal structure of the virion. Whereas immature retrovirions have doughnut-shaped, electron-lucent cores, those of mature virions have dense centers, the exact shape of which is characteristic for each retrovirus. Cleavage of the Gag precursor serves at least two purposes (37). First, it destroys the ability of the molecule to direct the budding process (3, 19); thus, when the progeny virus enters the next host cell, budding cannot take place directly. Second, processing allows new functions to be elicited that will be needed for the successful infection of the next cell (for example, functions required during the synthesis and transport of the double-stranded proviral DNA to the nucleus).

Although Gag processing is prerequisite for core maturation and viral infectivity, it is not essential for the budding process. It has been shown that the PR sequence of the RSV Gag protein can be deleted or replaced with nonviral proteins without deleterious effects on budding (35). Thus, when iso-1-cytochrome *c* (the *CYC1* gene product of *Saccharomyces cerevisiae*) is fused directly to the RSV Gag protein in the place of PR, the chimeric protein, $p80^{CY}$, is released from the cell in virus-like particles.

In the course of the Gag-cytochrome experiments, it was surprising to find that the transfected cells produced particles containing not only the full-length fusion protein but also large amounts of two smaller, Gag-related proteins (p74 and $p25^{C}$). It was hypothesized that $p80^{CY}$ might be pro-

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cessed into the smaller polypeptides by a nonviral PR located at the site of budding. This idea was supported by two pieces of evidence (35). First, since PR is absent, the proteins could not have arisen by a PR-mediated event. Second, a deletion in the MA sequence that abolishes membrane binding, budding, and processing of the RSV Gag protein seemed to block $p25^C$ synthesis when introduced into $p80^{CY}$. Nevertheless, the processing idea seemed unlikely since no evidence for the involvement of a cellular PR in budding has been reported.

Another possible explanation for the appearance of the smaller polypeptides is that they are expressed independently from spliced mRNAs. If this mechanism were correct, then it would be interesting to learn how the smaller polypeptides become packaged into particles. In particular, it would be important to determine whether $p25^C$ is packaged by complementation (through an interaction with the full-length fusion protein) or is capable of directing the formation of particles in an independent manner. Both possibilities were intriguing because of the very small size of $p25^C$ relative to Gag. This report demonstrates that $p25^C$ is indeed expressed from a spliced mRNA and is independently capable of being released from cells in a membrane-enclosed, particulate form.

MATERIALS AND METHODS

Previous modifications of the RSV *gag* gene. The wild-type RSV *gag* gene was obtained from pATV-8, an infectious clone of the RSV Prague C genome, and all nucleotide numbers correspond to the pATV-8 numbering system (32). pSV.Myr0, pSV.Myr1, pSV.GagX, pSV.MyCY, pSV.MyCyE, and pSV.GagXCy are simian virus 40-based expression vectors containing the *myr0*, *myr1*, *gagx*, *mycy*, *mycye*, and *gagxcy* alleles of the RSV *gag* gene and have been previously described (35, 38). Two of the carboxy-terminal deletion mutants (3h and Bg-Bs) and two of the internal deletion mutants (MA1 and R-3J) are described elsewhere, as is a double mutant (DM1) in which the R-3J and 3h deletions have been combined (35, 36, 39).

All derivatives of the above plasmids were constructed by standard protocols for manipulating DNA fragments (31) as outlined in the sections below. Recombinant plasmids were propagated in *Escherichia coli* DH-1 (13) with Luria-Bertani medium containing ampicillin (25 μg/ml). All mutants were identified by restriction endonuclease mapping and confirmed by double-stranded DNA sequencing with modified T7 DNA polymerase (Sequenase; United States Biochemical Co., Cleveland, Ohio). Furthermore, two independently isolated clones of each of the plasmid constructs were chosen at random and analyzed in transfection experiments to rule out the possibility of unwanted mutations.

Construction of pSV.MyCY and pSV.Myr1 derivatives. The original MyCY protein was expressed with the first 10 amino acids of the oncoprotein $p60^{src}$ on its amino terminus. To replace this sequence with the wild-type Gag amino terminus, pSV.MoCY was constructed by replacing the *Bgl*II-*Nhe*I fragment of pSV.Myr0 (nucleotides [nt] 1630 to 2948) with that of pSV.MyCY. To search for intron sequences in the original *mycy* gene, a series of internal deletions and frameshift mutations were introduced into the *myr1-CYC1* coding region of pSV.MyCY as follows. Two internal deletion mutants, pSV.MA1CY and pSV.R3JCY, were constructed by replacing the *Sac*I-*Bgl*II fragment (nt 255 and 1630, respectively) of pSV.MyCY with that of pSV.MA1 or pSV.R-3J; these mutants have been previously described (36, 39). The resulting derivatives lack residues 9 to 84 (in pSV.MA1CY) or 195 to 363 (in pSV.R3JCY). A third derivative, pSV.3hCY, was constructed by fusing the *CYC1* open reading frame (starting at the fifth codon) to the 3′ end of a truncated *gag* gene (the 3h allele [35]) that lacks all of the PR coding sequence except that for the first seven amino acids. For this, pSV.3h was digested with *Xba*I (which cuts at the 3′ end of the 3h allele) and pSV.MyCyE was digested with *Eco*RI (which cuts in the 5′ end of the *CYC1* sequence). The resulting ends were made blunt with mung bean nuclease, and then both DNAs were digested with *Sac*I (which cuts upstream of the RSV *gag* sequence) and the appropriate fragments of each (i.e., the small fragment of pSV.3h and the large fragment of pSV.MyCyE) were purified and joined with T4 DNA ligase. Also, two +1 frameshift mutations were introduced into the *mycy* coding region. pSV.MyCYXf was constructed by digesting pSV.MyCY with *Xho*I (at nt 630, within codon 85), making the ends blunt with the Klenow fragment of *E. coli* DNA polymerase, and ligating the ends with T4 DNA ligase. Construction of the other frameshift mutation required a two-step cloning procedure. First, pSV.RNotCY was constructed by replacing the *Sac*I-*Bgl*II fragment of pSV.MyCY with that from pSV.RNot. pSV.RNot is a derivative of pSV.Myr1 which has an in-frame *Not*I linker inserted at the *Rsa*I site (nt 1071) of *gag* (36). In the second step, pSV.RNotCY was digested with *Not*I, treated with the Klenow fragment to make the ends blunt, and ligated with T4 DNA ligase to create pSV.MyCY.Nf. Another derivative, this one encoding a large carboxy-terminal deletion of the RSV Gag protein, was constructed by removing the *Not*I-*Bss*HII fragment of pSV.RNot (nt 1071 to 2724), treating the ends with Klenow fragment and ligating with T4 DNA ligase to create pSV.No-Bs.

In vitro transcription vector. pGEM.DM1 was constructed for the in vitro synthesis of ^{32}P-labeled, antisense *gag* RNA. To this end, pSV.DM1 was cut with *Mlu*I and *Xba*I and the 1,210-bp *gag* fragment was gel purified. Insertion of this fragment into the corresponding sites of the pGEM-7Zf(+) (Promega) polylinker (nt 96 and 31, respectively) placed the 3′ end of *gag* near the T7 promoter. RNA transcripts were made with T7 RNA polymerase (Promega) after digesting pGEM.DM1 with *Mlu*I.

RNA isolation, analysis, and cDNA synthesis. Total RNA was isolated from transfected COS-1 cells by the guanidinium thiocyanate-cesium chloride method (31). RNAs with poly(A) tails were isolated with oligo$(dT)_{20-30}$ by the Fast-track poly(A) isolation kit (Invitrogen Corp.). RNA molecules were separated in standard 1.2% agarose–formaldehyde (0.66 M) gels (31), transferred to Immobilon P membranes (Millipore), cross-linked in a Stratalinker (Stratagene) with 1,200 μJ of UV light, and detected by hybridization with 1×10^8 cpm of ^{32}P-labeled, antisense DM1 RNA.

cDNA was synthesized from *mycy* or *mocy* mRNA by using avian myeloblastosis virus (AMV) RT (AMV RT XL; Life Sciences, Inc.). Briefly, 1 μg of poly(A)-selected RNA was incubated in 25 μl of AMV RT buffer [50 mM Tris-HCl (pH 8.0); 100 mM KCl; 8 mM $MgCl_2$; 4 mM dithiothreitol; 0.8 μM (each) dATP, dTTP, dCTP, and dGTP; 20 mg of oligo$(dT)_{12-18}$ (Pharmacia) per ml; 1 U of RNasin (Promega) per ml; and 200 U of AMV RT XL per ml] for 1 h at 42°C.

PCR amplification, cloning, and nucleotide sequence analysis. The polymerase chain reaction (PCR) was used to amplify *mycy* and *mocy* cDNAs with a Perkin-Elmer Cetus DNA thermal cycler. The upstream PCR primer (MAS;

5′-CCAGGCGTGATTCTGG, nt 346 to 361) lies near the *gag* initiation codon, and the downstream primer (RVPCY; 5′-GTTGCGCGCGTACACGCGTCTGTACAG) includes nucleotides complementary to a sequence immediately adjacent to *CYC1* and a noncomplementary *Bss*HII restriction site for subsequent cloning steps. After 30 cycles of amplification (94°C for 1 min, 60°C for 2 min, and 72°C for 3 min) with Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus), the products were separated in a 1% agarose gel. Fragments with an apparent length of 1,000 bp were eluted from the gel and digested with *Sau*I (which cuts shortly after the initiation codon in *myr1*) and *Bss*HII. The DNA fragments were inserted into the place of the *Sau*I-*Bss*HII fragment of pSV.Myr1 and transformed into *E. coli* DH-1, and the resulting clones were screened for the presence of plasmids of the expected size. Clones that expressed the expected 25-kDa, Gag-related polypeptide were identified by transfecting COS-1 cells and analyzing the products with anti-RSV antibodies (see below). These were named pSV.25M1.

Construction of pSV.25M1 derivatives. The first 10 amino acids of the product encoded by pSV.25M1 correspond to those of the Src oncoprotein. To replace those with the wild-type sequence of the RSV Gag protein, pSV.Myr0 and pSV.25M1 (clone a) were digested with *Sac*I and *Xho*I. The small DNA fragment of the former and the large fragment of the latter were purified and ligated to create pSV.25M0. To construct pSV.25M1EX, the *gag* sequence in pSV.25M1 (clones a and c) was detached from the *CYC1* sequence by digestion with *Sac*I and *Dra*I (nt 255 and 918, respectively). The 663-bp fragment was purified and ligated to the 6,375-bp *Sac*I-*Eco*RV fragment of pSV.Myr1 in the presence of excess *Xba*I linkers (5′-CTAGTCTAGACTAG). Recombinants containing one of each of the three fragments were identified by restriction endonuclease analysis and DNA sequencing. To correct the single point mutation (G to A; see text) at nt 603 in pSV.25M1 (clone a), this plasmid and pSV.Myr1 were digested with *Xho*I and *Bss*HII. The small (592-bp) fragment from pSV.25M1 was ligated with the large (6,930-bp) fragment from pSV.Myr1 to generate pSV.25M1R. Plasmid pSV.25AD3 was constructed by first inserting the small *Sac*I-*Dra*I fragment of pSV.25M1 (clone a) between the *Sac*I and *Eco*RV sites of pSP72 (Promega) to create pSP.SD. In this intermediate plasmid, there is a unique *Bgl*II site located just after the 3′ end of the *gag* sequence. pSP.SD was digested with *Mlu*I (located just after the start codon in the *myr1* allele) and *Bgl*II to excise the *gag* sequence, and it was ligated with the large *Mlu*I-*Bgl*II fragment of pSV.3h. Linkage of the two *Bgl*II ends fuses *gag* codon 180 directly to codon 417, resulting in a 236-residue internal deletion.

Transfection of mammalian cells and metabolic labeling. Prior to transfection, the expression plasmids were digested with *Xba*I to remove the bacterial plasmid sequence and then incubated with T4 DNA ligase to join the 3′ end of the *gag* gene to the polyadenylation signal, as previously described (38). COS-1 cells were propagated, transfected, and labeled with L-[^{35}S]methionine as previously described (38). The labeling times were 2.5 h, except where noted.

Cell lysis, immunoprecipitations, and SDS-polyacrylamide gel electrophoresis. The labeling medium on 35-mm plates (400 μl) was transferred to a microcentrifuge tube, and the monolayers were washed once with an equal volume of ice-cold phosphate-buffered saline. This wash was added to the medium, and cell debris was removed by microcentrifugation for 1 min. The supernatant was mixed with detergents to disrupt the particles, and detergent lysates of the labeled cells were prepared as described before (35, 38). Proteins were immunoprecipitated with a rabbit antiserum against RSV (reactive with MA, CA, NC, and PR), electrophoresed in sodium dodecyl sulfate (SDS)–12% polyacrylamide gels, and detected by fluorography, as described before (35).

Treatment of particles with trypsin. Gag-related polypeptides released into the medium from pSV.Myr1- or pSV.25M1-transfected COS-1 cells were analyzed for their resistance to trypsin in the presence and absence of 1% Triton X-100 as previously described (38).

Sucrose gradient analysis. At 48 h posttransfection, cells (in 60-mm plates) were labeled in 0.5 ml (for velocity gradients) or 1.0 ml (for isopycnic gradients) of serum-free, methionine-free Dulbecco's medium supplemented with 100 μCi of [^{35}S]methionine. After a 7- to 8-h labeling period, the medium was transferred to a microcentrifuge tube and cellular debris was removed by centrifugation at 15,000 × *g* for 1 min. An aliquot of purified Moloney murine leukemia virus (Mo-MLV [a generous gift from Alan Rein, Frederick Cancer Research and Development Center, Frederick, Md.]) was added to each sample to provide an internal control. Each mixture was layered onto 10 to 30% sucrose (for velocity gradients) or 10 to 50% sucrose (for isopycnic gradients) and centrifuged at 82,500 × *g* at 4°C for 0.5 h (for velocity gradients) or 16 h (for isopycnic gradients), and then 0.6-ml fractions were collected through the bottom of each tube. Gag proteins in each fraction were immunoprecipitated and electrophoresed in an SDS–12% polyacrylamide gel, and the intensity of each of the bands on the resulting fluorogram was quantitated by laser densitometry. The fractions containing Mo-MLV particles were identified with previously described RT assays (5), except that the reaction buffer contained 6 mM $MnCl_2$ and 60 mM NaCl. The sucrose density of each fraction was measured on a refractometer.

RESULTS

Pr76^{myr1} is a myristylated derivative of the RSV Gag protein (Pr76^{gag}) that functions with full efficiency in cells from a variety of species, including simian, murine, and avian cells. Cells expressing this Gag protein release virus-like particles that contain the full complement of mature Gag proteins (MA, CA, NC, and PR) and have an internal structure that is morphologically indistinguishable from authentic RSV. Moreover, PR mutants of the Myr1 protein behave as expected—virus-like particles are produced even though proteolytic processing of Pr76^{myr1} does not occur (9, 35, 38). However, when the PR coding region of *myr1* is replaced with *CYC1* (the gene for iso-1-cytochrome *c* from *S. cerevisiae*; see Fig. 1A), two smaller Gag-related proteins are found in the particles, along with the full-length fusion protein, p80^{CY} (35). To begin our analysis of the mechanism by which these smaller products arise and are incorporated into particles, several mutations were introduced into the chimeric allele, *mycy*, carried within the expression vector pSV.MyCY.

Mutational analysis of the chimeric *mycy* gene. The first 10 residues of the Myr1 and MyCY proteins are from the oncoprotein p60^{src} and provide a site for the addition of myristic acid (Fig. 1A). These few residues direct membrane associations, apparently through an interaction with a cellular protein (the Src receptor) located on the inner face of the plasma membrane (17, 20, 28–30). Thus, the Myr1 and MyCY proteins may be targeted to this unique membrane location instead of the normal site of Gag-mediated budding (39). With this in mind, it seemed possible that the smaller

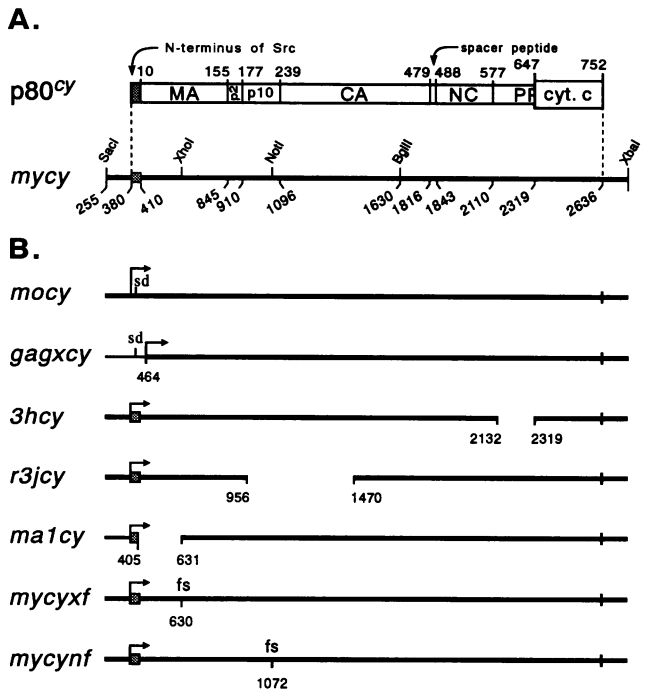

FIG. 1. Alleles of the *mycy* gene. (A) The MyCY fusion protein ($p80^{CY}$) is encoded by the chimeric *gag* gene (bottom). Iso-1-cytochrome *c* (cyt. c) is fused to the carboxy-terminal end of the Gag protein at amino acid residue 647 within the viral PR. MA, p2, p10, CA, spacer peptide, and NC are cleaved from the Gag protein when PR is active. The first 10 residues of $p60^{src}$ (small stippled box at the amino-terminal end) provide a site for the addition of myristic acid. (B) Derivatives of the *mycy* gene. Alleles *mycy*, *3hcy*, *r3jcy*, *ma1cy*, *mycyxf*, and *mycynf* contain the first 10 codons from v-*src*, while alleles *mocy* and *gagxcy* possess the wild-type, 5′ end of *gag*, including the 5′ splice site (sd). The arrows indicate the translation initiation site (nt 380). In the case of *gagxcy*, the exact initiation site is uncertain but is thought to occur at an internal AUG codon located 28 codons (nt 464) downstream of the normal initiation site. The deletion and frameshift (fs) mutations were introduced as described in Materials and Methods. The frameshift mutation in *mycyxf* was made at the *Xho*I site, while that in *mycynf* was made at the *Not*I site.

products of the *mycy* gene might arise from $p80^{CY}$ through the action of a host-encoded PR located near the Src receptor. To replace the Src residues with the wild-type, amino-terminal Gag sequence, pSV.MoCY was created (Fig. 1B). Monolayers of COS-1 cells were transfected with this recombinant to determine whether it directs the synthesis of products smaller than $p80^{CY}$. Parallel cultures were transfected with the parental plasmid (pSV.MyCY) or with pSV.GagXCY, the latter of which expresses a form of $p80^{CY}$ missing the first 28 residues of the Gag protein sequence and is incapable of budding (Fig. 1B [35]). Two days later, the cells were labeled with [^{35}S]methionine for 2.5 h, and the Gag proteins were immunoprecipitated from the cells and medium of each culture with RSV-specific antibodies. After electrophoresis in an SDS-polyacrylamide gel, the labeled proteins were visualized by fluorography.

Cells transfected with the parental plasmid released into the medium proteins with sizes of 80, 74, and 25 kDa (Fig. 2, lanes 2), but the pSV.MoCY-transfected cells appeared not to express p25 (lanes 3). Although this is consistent with the idea of processing, other observations are not. For example, small amounts of p25 were detected in many subsequent experiments with pSV.MoCY (not shown); thus, its synthesis was not completely blocked. In addition, p74 continued to be synthesized at high levels, not only when the wild-type amino terminus was present but also when the first few residues were deleted and budding was blocked, as is the case with the product of pSV.GagXCY (Fig. 2, lanes 4). The idea of smaller products arising by proteolytic processing of $p80^{CY}$ was weakened further by pulse-chase labeling experiments. In cells transfected with the parental plasmid, $p80^{CY}$, p74, and $p25^{C}$ were detected after as little as 4 min of labeling, and during long chase periods the relative amounts of all three remained constant (not shown).

The failure to show a precursor-product relationship led to considerations of mRNA splicing as a mechanism for the synthesis of the smaller products. As a first approach to mapping presumptive introns and the portions of the Gag protein which constitute the smaller products, three in-frame deletion mutations were introduced into the *mycy* gene (Fig. 1). These deletions, named R-3J, 3h, and MA1, do not destroy the ability of the Gag protein to direct the production of particles, at least in the absence of the cytochrome sequence (36, 39). The predicted product of the *r3jcy* allele lacks residues from the p10 and CA regions of Gag and is 171 amino acids shorter than $p80^{CY}$. When this mutant was expressed in COS-1 cells, a product of the expected size was released into the medium, along with a product of the size expected for an equally shortened p74 (see two major bands near the top of lanes 6 in Fig. 2). In contrast, the synthesis of p25 was completely unaffected, suggesting that if mRNA splicing occurs, the R-3J deletion must fall in an intron. The second deletion mutation (carried in allele *3hcy*) removed 62 amino acids from the remaining portion of PR at the Gag-cytochrome junction and also failed to affect the synthesis of p25, although the amount of p74 appeared to be increased at the expense of $p80^{CY}$ (lanes 5). A few minor bands of unknown origin were also detected. The third deletion (carried in the *ma1cy* allele) removes 75 residues from the MA sequence near the beginning of $p80^{CY}$. Although this deletion slightly altered the migration of $p80^{CY}$ and p74 (by the expected amount), it appeared to totally abolish the synthesis of p25 (lanes 7). Thus, $p25^{C}$ appears to contain residues encoded by the 5′ end of the *mycy* gene. The failure to detect a shortened p25 product is readily explained by a previous observation that the MA1 deletion removes a major epitope from the MA sequence that is recognized by the anti-RSV antibody used in the experiment (39).

To test the idea that a major exon for $p25^{C}$ is located at the 5′ end of the *mycy* gene and that the R-3J and 3h deletions fall within an intron, two frameshift mutations were created (Fig. 1). In the *mycyxf* allele, the frameshift falls within the sequence deleted by the MA1 mutation. As expected, the synthesis of $p80^{CY}$, p74, and $p25^{C}$ was abolished (Fig. 2, lanes 8). A few minor *gag*-specific bands were detected in the cell lysates, and it is likely that these represent products initiated at AUG codons after the frameshift mutation. The second frameshift mutation, in the *mycynf* allele, falls within the sequence deleted by the R-3J mutation. This mutation was predicted to completely obliterate the synthesis of $p80^{CY}$ and p74 without affecting the synthesis of p25, and this proved to be the case (Fig. 2, lanes 9). What could not be predicted is that the 25-kDa protein (here designated $p25^{Nf}$) would be released with high efficiency into the medium. Centrifugation of the medium revealed that $p25^{Nf}$ was in a particulate form (see below). A product of the size expected

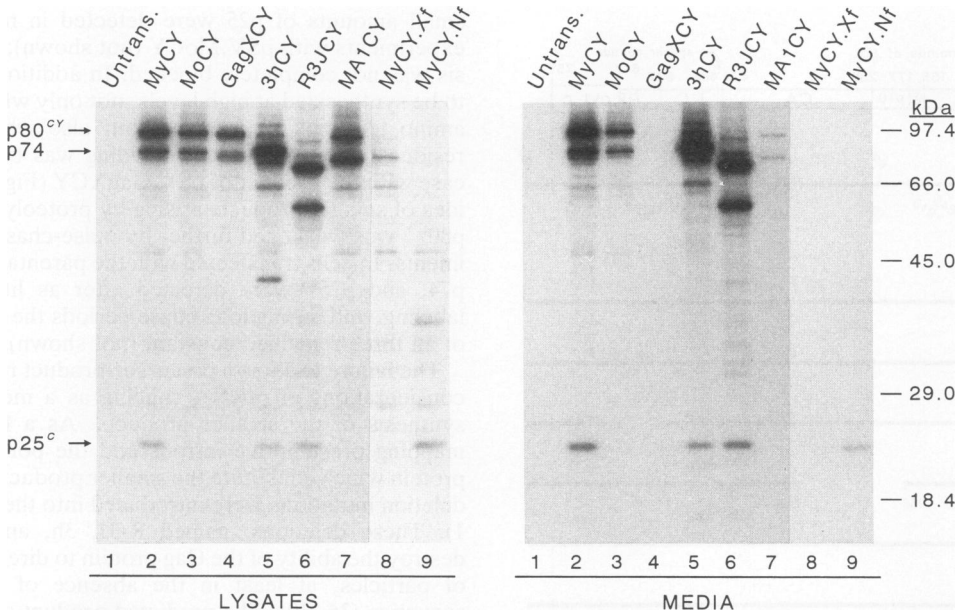

FIG. 2. Effects of mutations on the synthesis of *mcyc* products. Forty-eight hours after transfecting COS-1 cells with no DNA (lanes 1) or with the DNAs indicated, cells were labeled for 2.5 h with [^{35}S]methionine. The culture media were harvested and the cell lysates were prepared as described in the text. Gag-specific proteins immunoprecipitated from the samples by using antibodies to RSV were separated by electrophoresis in SDS–12% polyacrylamide gels and were visualized by fluorography. The positions of the *mycy* gene products (p80^{CY}, p74, and p25^{C}) and the protein size standards are indicated. The asterisk indicates a truncated 32-kDa Gag protein expressed from the MyCY.Nf frameshift mutant (see text). Untrans., untransfected.

for the truncated p80^{CY} (32 kDa) was also observed (Fig. 2, asterisk) but not released.

Because of the unexpected ability of p25^{Nf} to be released into the medium in the absence of any other Gag molecules, subsequent efforts focused on p25 rather than p74. In particular, it was of interest to determine the primary amino acid sequence of p25 and to determine whether this small protein is released into the medium by a mechanism reminiscent of budding. Because the mutational analysis suggested that p25 is a product of mRNA splicing, attempts were made to molecularly clone cDNA copies of such transcripts.

Northern blot analysis, cDNA synthesis, and PCR amplification. On the basis of the molecular weight of p25, the size of its message is predicted to be approximately 1.0 kb. Total RNA from untransfected, pSV.MyCY-transfected, or pSV.MoCY-transfected COS-1 cells was isolated and Northern (RNA) blotting was performed with a ^{32}P-labeled, *gag*-specific riboprobe. Whereas no *gag*-specific RNAs were detected in the untransfected cells, three size classes of RNA were detected in the transfected cell samples (Fig. 3A). The largest species is the size expected for unspliced mRNA. The species slightly smaller than 2.5 kb possibly encode p74, but without further analysis degradation products cannot be ruled out. Finally, in support of the splicing hypothesis, a species of approximately 1.0 kb which presumably encodes p25^{C} was detected. To decrease the background signals, poly(A)$^{+}$ RNA from pSV.MyCY-transfected cells was analyzed with the same antisense riboprobe. The 2.5- and 1.0-kb bands were again detected. Poly(A)-selected RNA was used in the subsequent attempt to clone the mRNA for p25^{C}.

Complementary DNAs were made from poly(A)$^{+}$ RNA isolated from untransfected, pSV.MyCY-transfected, and pSV.MoCY-transfected cells by using an oligo(dT) primer and RT as described in Materials and Methods. PCR was used to amplify all *gag*-specific cDNAs with primers which were designed to anneal to sequences located 5′ of the translational start codon or the polyadenylation signal (Fig. 3B). The antisense primer also contained a *Bss*HII restriction endonuclease site to facilitate cloning of the cDNAs into the expression vector. After 30 rounds of amplification, the cDNAs were separated by agarose gel electrophoresis and visualized by ethidium bromide staining (Fig. 3C). Several bands ranging from 250 to 2.5 kbp were detected in the samples from pSV.MyCY- and pSV.MoCY-transfected cells but not in that from the untransfected cells. The 2.5-kbp band obtained by using pSV.MyCY probably represents the full-length *mycy* cDNA. The lack of a band of the same size in the pSV.MoCY sample may reflect the inherent difficulties of PCR amplifying DNAs larger than 1.0 kbp under the conditions used. Of the remaining bands, only the 900-bp product was predicted to be large enough to encode a 25-kDa protein, and it was used for the subsequent experiments.

cDNA expression and sequence analysis. The 900-kbp PCR product from the pSV.MyCY sample was gel purified, digested with *Sau*I and *Bss*HII, and cloned into the respective sites of pSV.Myr1 to create pSV.25M1 (Fig. 3B). Plasmids from 12 randomly chosen clones were screened by restriction endonuclease analysis, and 6 with cDNA inserts of about 1 kbp were obtained. These clones were used to transfect COS-1 cells; untransfected and pSV.MyCY-transfected cells were included as controls. Three of the clones expressed no Gag-related products; however, two (clones a and b) expressed a single product which migrated at the position of p25^{C} (Fig. 4, compare lanes 2, 3, and 4) while another (clone c) produced one with a size of 23 kDa (lanes 5). Like p25^{Nf} (above), all three products were efficiently released into the growth medium in the absence of any other

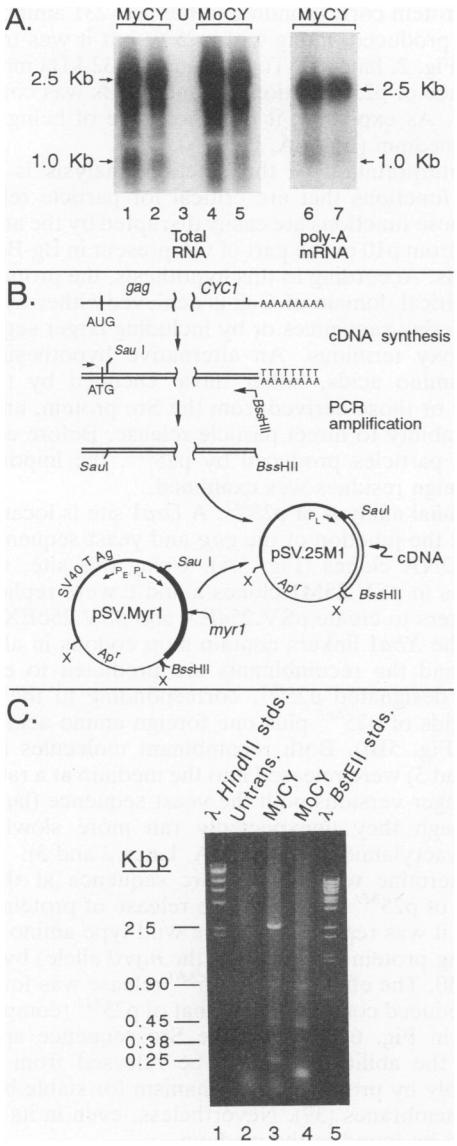

FIG. 3. Purification and cDNA cloning of *mycy*-specific mRNAs. (A) RNA from untransfected (untrans.) cells (lane 3) or cells transfected with the indicated DNAs was purified and subjected to Northern blot hybridization with an antisense *gag*-specific probe. Lanes 1, 3, and 4 contain 10 μg of total RNA; lanes 2 and 5 contain 5 μg of total RNA; lanes 6 and 7 contain 270 ng and 135 ng of poly(A)$^+$ RNA, respectively. (B) Strategy used to cDNA clone *gag*-specific mRNAs from pSV.MyCY- or pSV.MoCY-transfected cells. Poly(A)$^+$ RNA (single line) was converted to cDNA (double lines) with oligo(dT) and AMV RT. The *gag*-specific cDNAs were amplified by PCR with primers at positions indicated by the arrows. The *Sau*I-*Bss*HII cDNA fragment was cloned in place of the corresponding restriction fragment of pSV.Myr1. The double line in pSV.25M1 represents the cDNA. Simian virus 40 (SV40) sequences (medium line) contain the T antigen (T Ag) coding region and the early (P_E) and late (P_L) promoters. The remaining sequences (thin lines) are bacterial plasmid sequences including the ampicillin resistance gene (Ap^r). (C) An ethidium bromide-stained agarose gel of the PCR-amplified products from untransfected cells (lane 2) or cells transfected with pSV.MyCY (lane 3) or pSV.M0CY (lane 4). The sizes of the PCR products were estimated from the migration of the molecular size markers (λ-*Hin*dIII, lane 1; λ-*Bst*EII DNA, lane 5).

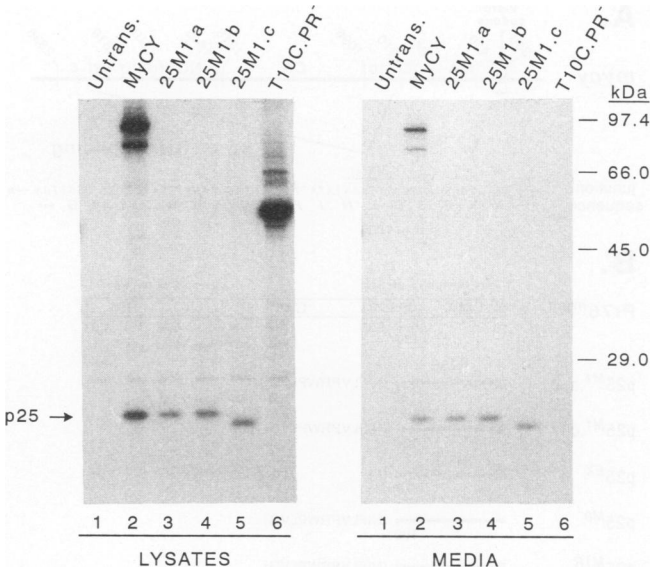

FIG. 4. Expression of the cDNA clones in COS-1 cells. COS-1 cells were transfected with no DNA (lanes 1), pSV.MyCY (lanes 2), three independently isolated clones of pSV.25M1 (lanes 3, 4, and 5), or pSV.T10C.PR$^-$ (lanes 6). Forty-eight hours later, the cells were labeled with [^{35}S]methionine for 2.5 h, and Gag proteins associated with the cells or present in the culture media were analyzed as described in the legend to Fig. 2. The positions of the MyCY proteins are indicated on the left, and the positions of the molecular size markers are indicated on the right. Untrans., untransfected.

gag product. Moreover, they were contained in particulate rather than soluble forms (see below).

As a negative control for budding, a parallel culture was transfected with pSV.T10C.PR$^-$. This plasmid encodes a derivative of the Gag protein that lacks residues 122 to 336 (Fig. 5B) and has an active site mutation in the protease sequence. The T-10C product is defective for particle release even though it includes the small, amino-terminal, membrane-binding domain from the Src protein and appears to interact with the plasma membrane (36). The inability of the T10C.PR$^-$ protein to be released from the cells in spite of its high level of expression (Fig. 4, lanes 6) emphasizes the special property of p25. Thus, it seemed possible that p25 would contain a critical portion(s) of the Gag protein (missing from T-10C) that allows it to direct its own release into the medium, rather than being released by a cell-mediated, membrane blebbing event.

To predict the amino acid sequences of the 25- and 23-kDa proteins, the nucleotide sequences of the cDNA clones were determined. Clones a and b were found to be identical, each containing the first 180 codons of the *gag* gene (nt 380 to 918) fused to the middle of the *CYC1* sequence (Fig. 5A). Moreover, both clones contain a nucleotide substitution at nt 603 (G to A, presumably an artifact of PCR amplification), which would result in a Gly-to-Glu substitution at residue 75 in MA. Translation of these cDNAs is predicted to result in a myristylated RSV Gag protein containing the MA and p2 domains, three amino acids of p10, and 12 foreign residues encoded by the yeast sequence. The clone expressing the 23-kDa protein was found to be identical to the previous clones except that it contains two point mutations. The first (A to G at nt 730) is a silent mutation, and the second (C to A at nt 825) results in an amino acid change of Thr to Ile at

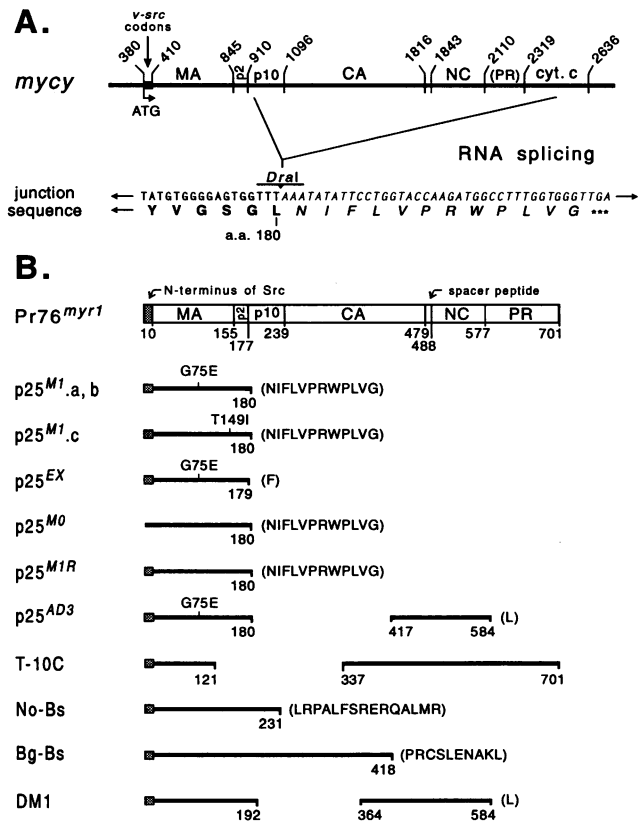

FIG. 5. Sequence analysis of the $p25^{M1}$ cDNAs. (A) The upper line represents the *mycy* gene. The sequence at the splice junction (nt 918 and 2543) in the $p25^{M1}$ cDNA is shown on the next line. A *Dra*I restriction site was fortuitously created at this junction as a result of splicing. The yeast *CYC1* sequence is indicated in italics, and the predicted amino acids (italics) fused to residue 180 of the Gag protein are shown below. cyt. c, iso-1-cytochrome *c*. (B) The upper bar represents the Myr1 precursor, $Pr76^{myr1}$, and the arrangement of its cleavage products. The various deletion mutants of the Gag protein are illustrated below, and any foreign residues introduced as a result of the mutations are indicated in parentheses. Several of the Gag derivatives have internal point mutations (G75E or T149I) which are indicated. The stippled boxes indicate that the first 10 residues of wild-type Gag protein were substituted with the first 10 residues of the Src protein. Those mutants that lack the stippled box contain the wild-type amino terminus of Gag protein.

residue 149 (T149I). The latter mutation presumably accounts for the abnormal migration relative to the other products. It seems unlikely that the three cDNAs described here are artifacts of PCR rather than products from spliced mRNAs because each encodes a polypeptide similar in size to $p25^{C}$ and is efficiently released from cells. However, because the sequence of the original $p25^{C}$ protein (encoded by *mycy*) has not been directly determined, the products of the cDNAs have been given the distinct name $p25^{M1}$ (Fig. 5B).

The deduced sequence of $p25^{M1}$ was interesting in light of previous studies in which it had been found that truncated molecules of a greater length (i.e., including more of the carboxy-terminal sequence) are not released from the cell (35). Two examples of such mutants are Bg-Bs and Es-Bs, which contain the first 418 and 348 amino acids of Gag, respectively. Another can be found in the experiments described above for the *mycynf* allele. In that situation, a 32-kDa protein corresponding to the first 231 amino acids of Gag was produced, along with $p25^{Nf}$, but it was trapped in the cell (Fig. 2, lanes 9). To examine the 32-kDa molecule in the absence of p25, deletion mutant No-Bs was constructed (Fig. 5B). As expected, it was incapable of being released into the medium (Fig. 6A, lanes 1).

One interpretation of the deletion analysis is that p25 contains functions that are critical for particle release, although these functions are easily disrupted by the addition of residues from p10 or the part of CA present in Bg-Bs, Es-Bs, and No-Bs. According to this hypothesis, the proper folding of this critical domain of Gag is achieved either by deleting the interfering sequences or by including larger segments of the carboxy terminus. An alternative hypothesis is that foreign amino acids, either those encoded by the yeast sequence or those derived from the Src protein, endow p25 with the ability to direct particle release. Before characterizing the particles produced by $p25^{M1}$, the importance of these foreign residues was examined.

Mutational analysis of $p25^{M1}$. A *Dra*I site is located fortuitously at the junction of the *gag* and yeast sequences in all of the cDNA clones (Fig. 5A). Using this site, the yeast sequences in pSV.25M1 clones a and c were replaced with *Xba*I linkers to create pSV.25aEX and pSV.25cEX, respectively. The *Xba*I linkers contain stop codons in all reading frames, and the recombinants are predicted to express a product, designated $p25^{EX}$, corresponding to the first 179 amino acids of $p25^{M1}$ plus one foreign amino acid (phenylalanine [Fig. 5B]). Both recombinant molecules (Fig. 6B, lanes 1 and 3) were released into the medium at a rate similar to the longer versions with the yeast sequence (lanes 2 and 4), although they unexpectedly ran more slowly in the SDS-polyacrylamide gel (Fig. 6A, lanes 2 and 3).

To determine whether the Src sequence at the amino terminus of $p25^{M1}$ influences the release of protein into the medium, it was replaced with the wild-type amino terminus of the Gag protein (encoded by the *myr0* allele) by creating pSV.25M0. The efficiency of $p25^{M0}$ release was found to be greatly reduced compared with that of $p25^{M1}$ (compare lanes 2 and 4 in Fig. 6A). Thus, the Src sequence appears to promote the ability of p25 to be released from the cell, presumably by providing a mechanism for stable binding to plasma membranes (39). Nevertheless, even in its absence, p25 could be found in the medium.

To be sure that the amino acid substitutions present in the Gag sequence of $p25^{M1}$ do not contribute to the release of particles, the G75E mutations present in clones a and b were reverted to the wild-type sequence by creating pSV.25M1R (Fig. 5B). The product, $p25^{M1R}$, was released from cells as efficiently as $p25^{M1}$ (compare lanes 2 and 5 in Fig. 6A).

Characterization of p25 particles. Having shown that the release of p25 is mediated by functions associated with the Gag sequence, the nature of the particles produced was examined. Of primary concern was the possibility that p25 might be released from the cells in a non-membrane-enclosed structure by a mechanism that bears little resemblance to budding. If the $p25^{M1}$ in the medium were surrounded by lipid, then it should be resistant to proteases unless detergents are added to solubilize the membrane. To measure sensitivity to trypsin, cells transfected with pSV.25M1 or pSV.Myr1 were labeled with [^{35}S]methionine and the medium from each culture was divided into seven equal aliquots for treatment with either trypsin, Triton X-100, soybean trypsin inhibitor, or a combination thereof. Proteins surviving the treatments were immunoprecipitated

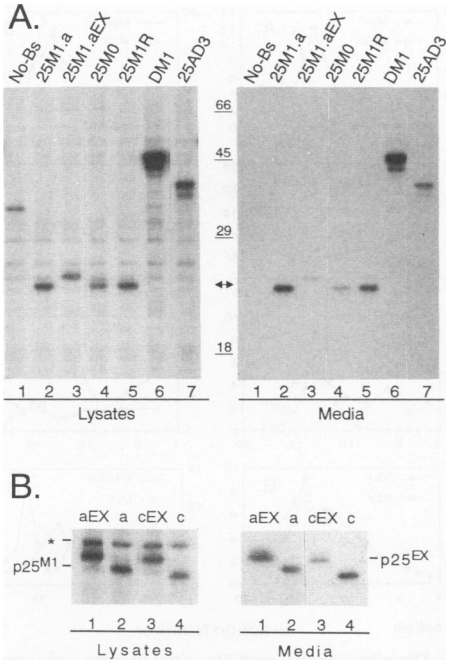

FIG. 6. Expression and release of mutant Gag proteins from transfected cells. (A) Cells were transfected with no DNA (lanes 1) or the indicated DNAs and labeled with [^{35}S]methionine as described above. Gag proteins in the lysate and medium fractions were immunoprecipitated with antibodies against RSV and electrophoresed in an SDS–12% polyacrylamide gel, and the fluorogram was obtained. The positions of the protein size markers and p25^{M1} are indicated. (B) Analysis of proteins expressed by pSV.25M1.aEX and pSV.25M1.cEX (lanes 1 and 3, respectively) which were derived from pSV.25M1 clone a and clone c, respectively (lanes 2 and 4). Only that portion of the resulting fluorogram containing p25^{M1} and p25^{EX} is shown. The asterisk marks the position of a cellular protein that was nonspecifically precipitated.

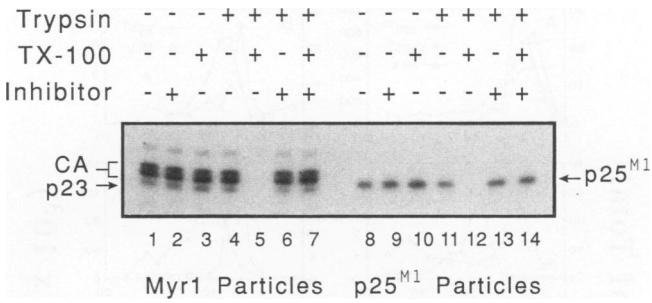

FIG. 7. Trypsin resistance of Myr1 and p25^{M1} particles. COS-1 cells transfected with pSV.Myr1 (lanes 1 to 7) or pSV.25M1 clone a (lanes 8 to 14) were labeled with [^{35}S]methionine for 2.5 h. The medium was removed and divided into seven equal aliquots that were treated with nothing (lanes 1 and 8), soybean trypsin inhibitor (lanes 2 and 9), Triton X-100 (TX-100 [lanes 3 and 10]), trypsin (lanes 4 and 11), trypsin plus Triton X-100 (lanes 5 and 12), trypsin plus inhibitor (lanes 6 and 13), or trypsin plus Triton X-100 and inhibitor (lanes 7 and 14) for 30 min. Trypsin inhibitor was subsequently added to each sample. Any Gag proteins remaining were immunoprecipitated with antibodies to RSV and separated by electrophoresis. Only that portion of the resulting fluorogram corresponding to CA and p25^{M1} is shown. p23 is a processing intermediate composed of MA and p2.

and separated by gel electrophoresis. The resulting fluorogram (Fig. 7) shows that the cleavage products of Pr76^{myr1} (CA and p23) and p25^{M1} were stable in the untreated controls (lanes 1 and 8) or in the presence of inhibitor alone (lanes 2 and 9), Triton X-100 (lanes 3 and 10), trypsin (lanes 4 and 11), trypsin plus inhibitor (lanes 6 and 13), and trypsin plus Triton X-100 and inhibitor (lanes 7 and 14). However, the proteins in both samples were completely susceptible to trypsin digestion in the presence of detergent (lanes 5 and 12), indicating that the p25 molecules were surrounded by a membrane.

The density of the membrane-enclosed, p25 particles was examined by equilibrium centrifugation in sucrose gradients. The density of each of the derivatives of p25 was also measured. Transfected cells were radiolabeled for 8 h with [^{35}S]methionine, the culture supernatant was harvested, and cell debris was removed. Unlabeled Mo-MLV virions were added to each of the culture supernatants to provide an internal control, and the samples were layered onto 10 to 50% sucrose gradients. After centrifugation to equilibrium, the gradients were fractionated into 20 aliquots of equal size. The presence of Mo-MLV particles in each fraction was detected by RT assays, and the Gag-related proteins were collected by immunoprecipitation and separated in an SDS-polyacrylamide gel. The resulting fluorograms were analyzed by scanning densitometry (Fig. 8).

The profile obtained for the Gag-cytochrome fusion protein (p80^{CY}) is identical to that previously reported for the wild-type RSV Gag protein produced in COS-1 cells (39)—the particles were of a uniform but slightly higher density than authentic retrovirions (Fig. 8A). In contrast, p25^{C} was contained in particles of heterogeneous density. Although the majority banded with p80^{CY} (1.1713 g/ml), a prominent shoulder on the profile indicates that some p25^{C} escaped into distinct, low-density particles. In fact, 40% of p25^{C} was found in the top 10 fractions, compared with 5% for p80^{CY}. This phenomenon was repeatedly observed and predicted that p25, in the absence of other Gag species, might be found in low-density forms. Gradient analysis of p25^{M1} molecules (Fig. 8B) revealed that they indeed were contained in particles much lighter than those of MLV (peak densities of 1.1462 and 1.1760 g/ml, respectively). Similar profiles were obtained with particles containing p25^{Nf}, p25^{M0}, p25^{EX}, and p25^{M1R} (Fig. 8, panels C to F, respectively).

The results of the sucrose gradient analysis suggested that the carboxy-terminal half of the RSV Gag protein contains a domain, missing in p25, that is critical for the dense packing of Gag molecules into particles. According to this model, deletion mutants Bg-Bs, Es-Bs, and No-Bs should produce low-density particles, if they produce any particles at all. To test this hypothesis, cells expressing mutant Bg-Bs were labeled with [^{35}S]methionine for an extended period of time (8 h), which allowed low but detectable levels of the truncated protein to accumulate in the medium (data not shown). As predicted, the Bg-Bs molecules (Fig. 8G) were found to be contained in particles of low density (1.14 g/ml) like those of p25.

Because p25 particles are heterogeneous in density, it was of interest to determine whether they are also heterogeneous in size. For this, radiolabeled particles were layered on 10 to 30% sucrose gradients and centrifuged for only 30 min at 80,000 × g. Unlabeled Mo-MLV particles were included in each sample to provide an internal control. The full-length, wild-type RSV Gag protein (Myr0) and the derivative with the Src amino terminus (Myr1) sedimented as a band of uniformly sized particles near the position of the control

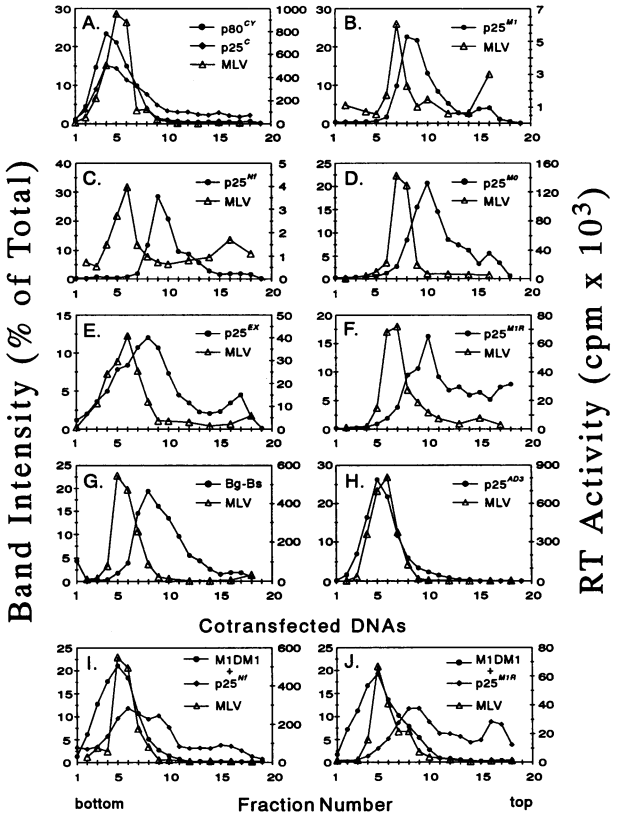

FIG. 8. Measurement of particle density in isopycnic sucrose gradients. COS-1 cells were transfected with pSV.MyCY (A), pSV.25M1 clone a (B), pSV.MyCY.Nf (C), pSV.25M0 (D), pSV.25EX (E), pSV.25M1R (F), pSV.Bg-Bs (G), or pSV.25AD3 (H) or were cotransfected with equal amounts of pSV.DM1 and pSV.MyCY.Nf (I) or pSV.DM1 and pSV.25M1R (J). After 48 h, the cells were labeled with [^{35}S]methionine for 7 to 8 h. The medium was removed, mixed with unlabeled MLV particles, and centrifuged at 70,000 × g for 16 h in 10 to 50% sucrose gradients. Gag proteins in each fraction were immunoprecipitated, separated by electrophoresis in SDS–12% polyacrylamide gels, and visualized by fluorography. Quantitation of the percent of the total specific protein in each fraction was performed by scanning densitometry. Fractions containing MLV were identified with RT assays.

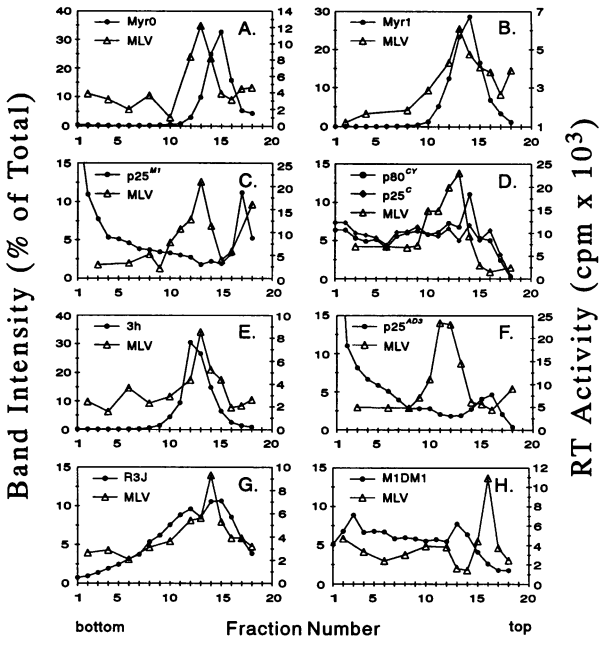

FIG. 9. Distribution of particle size in rate-velocity gradients. COS-1 cells were transfected with pSV.Myr0 (A), pSV.Myr1 (B), pSV.25M1 clone a (C), pSV.MyCY (D), pSV.3h (E), pSV.25AD3 (F), pSV.R-3J (G), or pSV.DM1 (H) and labeled with [^{35}S]methionine as described in the legend to Fig. 8. The medium from each plate was collected, mixed with unlabeled MLV virions, and centrifuged at 70,000 × g for 30 min in 10 to 30% sucrose gradients. Gradient fractions were analyzed for Gag proteins and MLV as described in the legend to Fig. 8.

particles (Fig. 9A and B, respectively). In contrast, the p25^{M1} particles (Fig. 9C) were found throughout the gradient, with the majority located near the bottom, while the Mo-MLV particles were found mostly in the top half. (The heterogeneity of the Mo-MLV particles in these experiments is most likely due to their being pelleted, frozen, and thawed prior to the experiments, manipulations which tend to promote particle aggregation.) Particles containing p80^{CY} from pSV.MyCY-transfected cells were also found throughout the gradient (Fig. 9D), indicating that they are heterogeneous in size. This was unexpected because carboxy-terminal deletion mutant 3h, which contains fewer residues of RSV Gag than p80^{CY}, produced particles of uniform size (Fig. 9E). The size heterogeneity of the MyCY particles may be due to the presence of the *CYC1* gene product, or perhaps p25^{C}, which is also heterogeneous (Fig. 9D).

Derivative of p25 that produces dense particles. If the carboxy-terminal half of the RSV Gag protein contains a discrete domain important for the dense packing of molecules within particles, then it should be possible to add that domain to p25 to endow it with the ability to make particles of high density. That this would be possible was first suggested by RSV Gag mutants that contain large internal deletions that do not abolish budding (36). One such mutant is R-3J, which lacks residues 193 to 363. This deletion has been combined with that of carboxy-terminal deletion mutant 3h to create a double mutant designated DM1 (Fig. 5B), which is released with high efficiency (Fig. 6A, lanes 6) in particles of high density into the medium (36). With the properties of DM1 in mind, codons for residues 417 to 584 were linked to those for p25^{M1} to create p25^{AD3} (Fig. 5B). This recombinant was efficiently released into the medium (Fig. 6A, lanes 7) in particles with a remarkably uniform density, almost identical to Mo-MLV (Fig. 8H). However, the particles were still very large and heterogeneous in size (Fig. 9F), like those of p25^{M1} (Fig. 9C). From these results, it would appear that internal deletions cause disorder in Gag molecules such that particles of very large size are released, even though they are of a uniform density. The heterogeneous distribution of particle sizes for Gag mutants R-3J (Fig. 9G) and DM1 (Fig. 9H) further supports this hypothesis.

Transpackaging of p25 into dense particles. The observation that most p25^{C} molecules band with p80^{CY} in sucrose density gradients (Fig. 8A) suggests that the two are capable of interacting during budding. To test this idea, COS-1 cells were cotransfected with pSV.DM1 and pSV.MyCY.Nf, and the resulting particles were analyzed. The DM1 molecules were contained in particles with a density similar to that of the Mo-MLV control, and much of the p25^{Nf} protein also banded at this position (Fig. 8I). This experiment was

repeated three times, and similar results were obtained each time. Taken together with the low-density profile obtained when $p25^{Nf}$ is expressed by itself (Fig. 8C), this provides good evidence for an interaction with DM1 molecules; however, the bimodal nature of the profile from the cotransfection suggests that many molecules escaped into low-density particles (see Discussion). In contrast, all attempts to rescue $p25^{M1}$ into dense particles failed (not shown). The experiment was also performed with $p25^{M1R}$ because it does not contain the G75E substitution; however, this derivative of p25 also failed to interact with DM1 (Fig. 8J). Experiments with mutant 3h as the rescuing molecule also failed (not shown), suggesting that the inability to copackage $p25^{M1R}$ into dense particles is not due to the rescuing molecule. Further experimentation will be required to determine how the $p25^{Nf}$ product(s) differs from the other derivatives of p25.

DISCUSSION

The results described in this report suggest that $p25^{C}$, the small product of a previously described chimeric RSV *gag* gene (*mycy*), is derived from a spliced transcript rather than by proteolytic processing. On the basis of the nucleotide sequences of the cDNA clones, the resulting 25-kDa protein ($p25^{M1}$) is predicted to contain the first 180 amino acids of the Myr1 derivative of RSV Gag and 12 foreign residues at its carboxy terminus. Although this derivative lacks almost 75% of the Gag protein, it was efficiently released from the cell in membrane-enclosed particles, albeit ones of low density and heterogeneous size. The ability to make particles of high density was regained by placing a fragment containing the NC sequence onto the carboxy terminus of the cloned p25 molecule.

Particles with low density also have been observed with mutant Gag proteins from other retroviruses. In the case of MLV, it has been found that deletion of the NC sequence or small four- to five-amino-acid insertions within the CA sequence result in the release of low-density particles that do not contain plasma membrane marker proteins (14, 15). Alterations in the carboxy-terminal region of the human immunodeficiency virus Gag protein, including removal of its NC domain, also yield particles with abnormal densities and structures (11, 16, 23). What makes the RSV p25 molecule interesting, however, is its very small size. This derivative reveals that the functions needed for the production of low-density particles are contained entirely within the first 180 residues of the RSV Gag protein.

Mechanism of low-density particle production. The release of p25 molecules within low-density particles appears to be mediated by functions retained from the Gag protein rather than by some sort of cell-mediated event (e.g., random membrane shedding). What functions may be required? Clearly, a membrane-binding domain must be involved. In the wild-type Gag protein and the Myr0 form of p25 ($p25^{M0}$), this domain is believed to be located within the MA sequence (37); however, it can be replaced with the membrane-binding domain of the Src oncoprotein (39). This function has been designated assembly domain 1 (AD1 [37]). Of course, to release a protein from the cell within a membrane-enclosed particle requires more than just binding it to the plasma membrane. For example, the Src protein is found on the plasma membrane but is not released into the medium. Moreover, RSV Gag derivatives have been found that are bound to the membrane, yet fail to be released, like p25. One example of this is the Myr1 form of mutant Bg-Bs, which contains all of the sequence included in $p25^{M1}$ and many of the residues from CA (35, 39), the latter of which presumably interfere with the functions contained in the first 180 residues. Another example is mutant T-10C, which lacks residues 123 to 337 (36, 37, and this report). Thus, p25 must contain an additional function, perhaps located within its carboxy-terminal region (i.e., the portion deleted in mutant T-10C). We have tentatively designated this region assembly domain 2 (AD2 [37]).

The ability of p25 to make particles seems certain to require self-association. The sequences that participate in this and the nature of the interaction remain to be determined. Interestingly, cross-linking experiments indicate that the MA molecules of RSV are closely positioned within the mature virion (reviewed in reference 37). Regardless of the mechanism, it is clear that the interactions that occur among p25 molecules are not sufficient to cause the formation of high-density particles.

Particle density and AD3. The increase in particle density observed when residues from the carboxy terminus of Gag are added to p25 (to make $p25^{AD3}$) suggests that a specific region controls particle density. This region has been designated assembly domain 3 (AD3 [37]). A correlation between the presence of AD3 and dense particle formation has also been observed with other deletion mutants of RSV Gag. For example, internal deletion mutant DM1 contains AD3 and is released in dense particles while mutant Bg-Bs lacks AD3 and is released in low-density particles. Moreover, it has been found that the addition of sequences from the NC protein of human immunodeficiency virus or MLV to the carboxy terminus of mutant Bg-Bs also restores dense particle formation, indicating functional similarities among all Gag proteins (2).

What is the physical basis for the difference between low- and high-density particles? It cannot be due to simple differences in the length of the Gag proteins, because deletions of many sizes have no effect on particle density (2, 36, 39). In particular, mutants DM1 and Bg-Bs contain virtually the same number of amino acids (413 and 418, respectively), yet the former is released in dense particles and the latter is released in light particles. Because RNA represents only 1% of the total mass of a wild-type retrovirion, it also seems unlikely that differences in particle density would be due to simple changes in RNA content per se (34), although further experiments will be needed to address this. Nevertheless, it seems reasonable to suggest that differences in particle density reflect the packing density of Gag proteins within the particles (i.e., differences in the protein/lipid ratio).

How might AD3 mediate the close packing of Gag molecules within a particle? This region could be important for establishing direct protein-protein interactions between Gag molecules during particle assembly. Alternatively, since AD3 contains sequences implicated in RNA binding (i.e., the Cys-His motifs and numerous positive charges in the NC domain [1, 7, 8, 12, 21, 24–27]), the dense packing of Gag molecules might arise through interactions with negative charges on RNA. In other words, RNA may indirectly influence particle density by providing a scaffold upon which Gag proteins tightly condense.

If Gag proteins utilize RNA to assemble into dense particles, they do so without apparent sequence specificity. Because the viral RNA packaging signals (ψ) are not present in any of the constructs described here, such a hypothesis would require that cellular RNAs can serve this purpose just as well. This seems reasonable, since it has been estimated that only 50 to 85% of the total RNA encapsidated into

retroviruses is genomic; the rest is made up of smaller, host-encoded species (4, 6, 18, 33). Moreover, RNA-packaging mutant SE21Q1b of RSV has been shown to incorporate cellular RNAs as efficiently as viral RNA (1, 10, 22). Although it is possible that cellular RNAs play no role in particle assembly, an alternative hypothesis is that Gag proteins will utilize cellular RNAs if specific viral RNAs are not present or recognized.

Particle size. Regardless of the mechanism by which AD3 controls particle density, the presence of this domain on Gag proteins is not sufficient for the production of particles of uniform size. Particles produced by mutants R-3J, DM1, and $p25^{AD3}$ have densities similar to those of authentic retroviruses, but they are quite heterogeneous in size. Each of these mutants contains AD3 but lacks most of p10 and CA. In contrast, mutant 3h contains the same carboxy-terminal deletion as DM1 but retains p10-CA and assembles into dense particles of uniform size that cosediment with authentic retroviral particles. These results argue that a function(s) located somewhere in the p10-CA sequence affects the size of the retroviral particle. It will be interesting to determine the morphology of these abnormal particles by electron microscopy.

Transpackaging. The release of low-density particles from cells expressing the *mycynf* allele (with a frameshift within the intron that blocks $p80^{CY}$ expression) suggests that the originally described p25 molecule (encoded by *mycy* and *mycynf*) and the cloned p25 molecule are identical. Unfortunately, the products of the cDNA clones could not be incorporated into dense particles, in spite of many efforts, even though much of the 25-kDa product of the *mycynf* allele could. Likewise, most (but certainly not all) of $p25^{C}$ expressed from the *mycy* allele was found in dense particles with the full-length fusion protein, $p80^{CY}$.

One explanation for the failure to rescue $p25^{M1}$ into dense particles is that additional species of p25 are expressed by the *mycy* allele, and only one was identified by the PCR strategy employed. Support for this hypothesis has been seen in differences in antibody reactivities between $p25^{M1}$ and $p25^{C}$. For example, while $p25^{M1}$ lacks sequences from CA and fails to react with CA-specific anti-peptide antibodies, the same antibodies precipitate a 25-kDa protein released by *mycy*-transfected cells (data not shown). Similar experiments have shown that antibodies directed against iso-1-cytochrome *c* precipitate a 25-kDa protein produced by the *mycy* allele (35); however, the product of the cloned p25 mRNA contains no cytochrome residues. Thus, it may be that the *mycy* and *mycynf* alleles express a 25-kDa protein equivalent to $p25^{M1}$ and at least one other species that contains portions of CA and possibly cytochrome *c*. Perhaps it is this CA-containing protein that is rescued into dense particles in experiments with the *mycynf* allele while the $p25^{M1}$ species is left out (see Fig. 8I). If another p25 species exists, it will be of interest to determine its sequence with respect to regions of the Gag protein important for intermolecular protein interactions. This would require a different cloning strategy (e.g., different primer pairs) because the initial screening of the PCR-amplified cDNA library yielded only one type of p25 cDNA clone.

ACKNOWLEDGMENTS

We thank Joseph Achacoso for technical assistance in several of the plasmid constructions and Alan Rein for providing purified Mo-MLV for use in the sucrose gradient experiments. We appreciate the time given by Rebecca Craven, Michael Verderame, and Sally Weldon for critical reviews of the manuscript. Finally, we thank fellow members of our laboratory, especially Tim Nelle and Robert Bennett, for continued support and many insightful discussions.

Support for this research was provided by NIH grant CA-47482 to J.W.W.

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