JOURNAL OF VIROLOGY, Oct. 1989, p. 4331–4343
0022-538X/89/104331-13$02.00/0

Vol. 63, No. 10

Creation and Expression of Myristylated Forms of Rous Sarcoma Virus Gag Protein in Mammalian Cells

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Received 31 March 1989/Accepted 18 June 1989

Rous sarcoma virus (RSV), a member of the avian sarcoma and leukosis family of retroviruses, has long been known to be capable of infecting and transforming mammalian cells; however, such transformed cells do not release virus particles. The RSV *gag* product ($Pr76^{gag}$) produced in these cells is not released into the culture medium or proteolytically processed to release mature products. Thus, the behavior of $Pr76^{gag}$ in mammalian cells is much like that of mammalian retroviral Gag proteins which have been altered so as to block the addition of myristic acid at residue 2 (Gly). Because the RSV *gag* product does not possess a myristic acid addition site, we hypothesized that the creation of one by oligonucleotide-directed mutagenesis might permit particles to be released from mammalian cells. Two myristylated forms of Pr76 were created. In $Pr76^{myr1}$, the first 10 amino acids have been exchanged for those of $p60^{v\text{-}src}$, which are known to be sufficient for myristylation. In $Pr76^{myr2}$, the Glu at the second residue has been substituted with Gly. The alleles encoding the modified and wild-type forms of Pr76 have been expressed at high levels in mammalian (CV-1) cells by using an SV40-based vector. Surprisingly, we have found that expression of high levels of the unmodified (wild-type) product, $Pr76^{myr0}$, results in low levels of particle formation and precursor processing. This indicates that myristic acid is not the sole determinant for targeting. However, the addition of myristic acid to $Pr76^{myr1}$ or $Pr76^{myr2}$ resulted in a fivefold enhancement in Gag function. In all aspects examined, the behavior of myristylated Pr76 was identical to that of the authentic product produced in avian cells. We also show that processing is mediated by the *gag*-encoded protease and that removal of the amino terminus to create $Pr76^{gagX}$ results in an inability to form particles or be processed. This suggests that proper targeting is prerequisite for activation of the RSV protease in mammalian cells.

It has long been established that certain strains of Rous sarcoma virus (RSV), an avian retrovirus, will infect and transform mammalian cell cultures (reviewed in reference 50). Such transformed cells carry an integrated proviral DNA, but they do not release infectious or noninfectious virus particles. This failure to produce particles is not due to alteration of the proviral DNA sequences, because infectious virions can be obtained if the transformed cells are injected into chickens, cocultivated with chicken cells, or fused to uninfected avian cells (2, 40). The blocks to RSV replication that exist in mammalian cells are poorly understood but are of great interest because of the potential for shedding light on the normal process of RSV assembly (in avian cells) and for revealing differences between avian and mammalian retroviruses. We are interested in relieving the blocks to RSV replication that exist in the mammalian cell and focus here on the RSV *gag* gene and its product, $Pr76^{gag}$.

The *gag* gene (which encodes five RSV structural proteins) is one of three genes common to all replication-competent retroviruses, the others being *pol* (which codes for the reverse transcriptase and related functions) and *env* (which codes for the envelope glycoproteins). RSV is unique in that it also carries an oncogene, *src*, alongside these three structural genes. A great deal of genetic evidence, obtained through the characterization of spontaneous mutations, has suggested that *gag* is the only viral gene needed for budding and particle formation (reviewed in reference 4). That is, noninfectious particles can be released from the cells in the absence of reverse transcriptase, envelope glycoproteins, tumor-inducing protein, or genomic RNA. It is only when *gag* is mutated that the ability to form particles is lost.

$Pr76^{gag}$ is a polyprotein precursor which is synthesized on cytoplasmic ribosomes from an unspliced proviral transcript that is identical to the viral genome (4). It is subsequently targeted to the plasma membrane (the site of virus assembly) by a mechanism that is not understood. Like all type C retroviruses, RSV does not preassemble core structures in the cytoplasm, but rather these arise concurrently with the envelopment or budding process. The five internal virion proteins that arise through proteolytic processing of $Pr76^{gag}$ are designated as follows (20), according to their order in the precursor: NH_2-p19 (the matrix or membrane-associated protein [MA]), p10 (of unknown function), p27 (the capsid protein [CA]), p12 (the nucleocapsid protein [NC]), and p15-COOH (the protease [PR]). As for other retroviruses, the processing of $Pr76^{gag}$ is poorly understood, but it is believed to occur after the arrival of the precursor at the plasma membrane (reviewed in reference 18). However, processing itself does not appear to be a prerequisite to the budding process, since RSV mutants have been found that synthesize truncated forms of $Pr76^{gag}$ which are not cleaved but are released from the cells in the form of particles (48). It is not clear what minimal portion of the *gag* product can still promote budding.

In RSV-transformed mammalian cells, the levels of $Pr76^{gag}$ expressed are low, and detection generally requires the sensitive method of immunoblotting rather than metabolic labeling followed by immunoprecipitation. In those cases in which analysis has been successful, mature cleavage products have been detected neither in the cells nor in the cell culture medium (6, 44). The nonfunctional precursors

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apparently are conformationally correct, since they seem to be cleaved properly if the cells are injected with the protease, p15 (47). These observations have led to the interesting hypothesis that the block to RSV replication in mammalian cells may be due to a failure to initiate particle formation as a result of improper targeting of $Pr76^{gag}$ to the plasma membrane (44).

It is not at all clear how retroviruses target their *gag* products to the plasma membrane, though it is widely believed that the MA protein plays a critical role. For example, cross-linking experiments with RSV have shown that p19 is associated with the viral membrane (28, 29). Among mammalian retroviruses, almost all encode Gag proteins having a 14-carbon fatty acid, myristate, at the amino terminus, and this hydrophobic moiety may play a role in membrane interactions during targeting (11, 36). The addition of myristic acid appears to occur cotranslationally (24, 51) and results in an amide bond between the acyl group and the α-amino group of glycine after removal of the initiation methionine (reviewed in references 35, 38, and 42). Elimination of the myristic acid addition site on the Gag protein of Mason-Pfizer monkey virus (M-PMV) by means of site-specific mutagenesis abrogates M-PMV particle release and Gag precursor processing (33); similar results have been found for murine leukemia virus (32). Conversely, the creation of a myristic acid addition site on the Gag protein of an endogenous murine leukemia virus, *Emv-3*, has enabled virus expression (14).

The observations reported for the nonmyristylated mammalian Gag proteins were striking to us since those mutant precursors seem to behave like the wild-type RSV Gag precursor in RSV-transformed mammalian cells. Since the RSV Gag protein does not have glycine at position 2 and is not myristylated but is instead acetylated (25, 36), we hypothesized that the failure of $Pr76^{gag}$ to be targeted, processed, and released by budding from mammalian cells might be due to a requirement for myristic acid addition. The primary goal of the experiments described below was to determine whether the block to $Pr76^{gag}$ function that exists in mammalian cells could be alleviated by the creation of an amino-terminal myristic acid addition site. The results demonstrate that is so. Furthermore, we have found that low, but easily detected, levels of particle formation occur when the wild-type (unmodified) Pr76 is expressed at high levels in mammalian cells.

MATERIALS AND METHODS

DNAs, viruses, and cells. The wild-type RSV *gag* gene was obtained from pATV-8 (16), a molecular clone containing an infectious, sequenced copy of the RSV Prague C genome (37). Plasmid pJD100 carries an infectious but unsequenced copy of the Prague A strain of RSV (41). The RSV protease mutant, D37I (in which the aspartic acid at residue 37 is changed to isoleucine), was kindly provided by Volker M. Vogt, Cornell University, Ithaca, N.Y., and was contained in pSR.*gag*, a plasmid bearing the Schmidt-Ruppin A *gag* gene. D37I has been shown to lack protease activity when expressed in avian cells (V. M. Vogt, personal communication) or *Escherichia coli* cells (17). RSV was grown in turkey cell cultures which were prepared from fertile eggs (Hudson Farms, Muskogee, Okla.) and propagated in supplemented F10 medium (primary growth medium) by previously published methods (12). The turkey cell cultures were found to contain no sequences capable of recombining with RSV *gag* sequences and produced no RSV-specific antigens. Prague A (JD100) and Prague C (ATV-8) viruses were obtained by transfecting secondary turkey cell cultures with pJD100 or pATV-8 DNA, respectively. Recombinant plasmids were propagated in *E. coli* DH-1 (8), using solid or liquid Luria broth medium containing 25 μg of ampicillin per ml. Recombinant M13 bacteriophages were propagated in Luria broth medium without ampicillin. The simian virus 40 (SV40) vector used for the expression of wild-type and mutant RSV *gag* genes in mammalian cells was derived from a previously described construction (52, 53), as explained below. African green monkey kidney cells (CV-1) used for the transfection of SV40-*gag* DNAs were propagated in Dulbecco modified Eagle medium supplemented with 3% fetal bovine serum and 7% adult bovine serum (Hyclone Laboratories, Inc., Logan, Utah).

Oligonucleotide-directed mutagenesis. The coding sequence for the *gag* gene lies between nucleotides (nt) 380 and 2482 in the RSV genome (37). The *Sac*I-*Hin*dIII fragment containing this region (nt 255 to 2740, respectively) was cloned into the polylinker region of M13mp19. The resulting clone was named MGAG. Mutagenesis of MGAG was accomplished by the method of Kunkel et al. (19). Briefly, MGAG phage was sequentially propagated three times in CJ236, a *dut ung* strain of *E. coli*, in order to replace thymine with saturating amounts of uracil. Single-stranded DNA was then isolated for use as the template for mutagenesis. Mutagenic oligonucleotides were synthesized by using an Applied Biosystems DNA synthesizer, gel purified, phosphorylated with T4 polynucleotide kinase, hybridized with the uracil-containing template, made double-stranded by using T4 DNA polymerase, and sealed by using T4 DNA ligase. The products of these reactions were transfected into a Dut^+ Ung^+ strain and plated to allow selection and segregation of the mutations. The resulting plaques were picked and the phages were grown to obtain replicative-form DNA and single-stranded DNA. Clones containing the desired mutations were identified by means of the dideoxy method of DNA sequencing of single-stranded DNA (34).

To substitute the first 10 codons of *gag* for those of RSV *src*, a 57-mer was used (5′-CCCGGTGGATCAAGCATGG GATCCAGCAAAAGCAAGCCTAAGGACGCGTGTAAAA CC-3′) which was designed to maximize complementarity (Fig. 1). As this change is rather complex, presumptive clones were initially identified by the presence of a newly introduced *Mlu*I site (ACGCGT) contained in the 57-mer. The resulting allele, confirmed by DNA sequencing, is designated myr_1. To change the second codon of *gag* so that it codes for Gly, a single point mutation (A to G) was required (Fig. 1). This was accomplished by using a 24-mer (5′-CAAGCATGGGAGCCGTCATAAAGG-3′), and the resulting allele is designated myr_2. Fragments containing the *gag* mutations were excised from the replicative-form DNAs by digestion with *Sac*I and *Bgl*II (nt 1630) for transfer to the mammalian expression vector.

Construction of the SV40-*gag* expression vectors. The wild-type (myr_0) and mutant (myr_1 and myr_2) *gag* genes were transferred to an SV40-based vector called $p\Delta SV.Myr_x$ to allow high levels of transient expression in mammalian cells. In this vector, transcription is driven from the SV40 late promoter. The parent of $p\Delta SV.Myr_x$ is pΔSV.GAGX, which expresses a truncated Gag protein whose amino terminus is altered presumably because of the presence of an upstream, out-of-frame initiation codon in the SV40 sequence (see Results). A description of these two vectors follows.

(i) pΔSV.GAGX. Plasmid pΔSV.GAGX contains DNA fragments from three sources: the RSV genome, the SV40

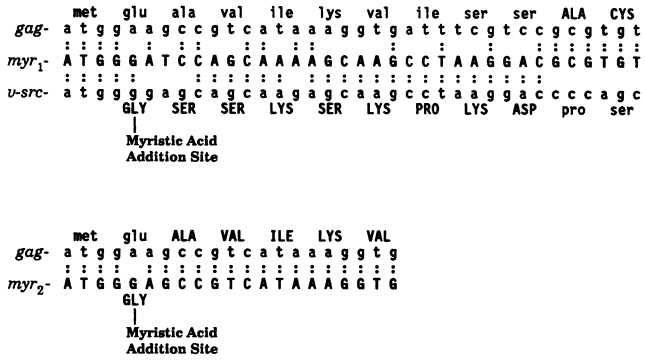

FIG. 1. Creation of myristic acid addition sites at the amino terminus of Pr76. (Top) The 5′ nucleotide sequences for RSV *gag* and v-*src* aligned with the relevant sequence of the mutagenic oligonucleotide used to create myr_1. The complete oligonucleotide (Materials and Methods) was designed such that the first 10 amino acids of $Pr76^{gag}$ would be exchanged for those of $p60^{v\text{-}src}$ while the maximum amount of base pairing with *gag* was maintained. (Bottom) The single nucleotide change in the second codon of *gag* to change Glu and Gly in $Pr76^{myr2}$. The predicted amino-terminal sequences of $Pr76^{myr1}$ and $Pr76^{myr2}$ are represented with uppercase amino acid names, and the sites for addition of myristic acid are indicated. Colons indicate positions having identical nucleotides.

genome, and the bacterial plasmid pAT153. The RSV fragment, *Sac*I to *Hin*dIII (see above), contains the *gag* gene and was modified by inserting an *Xba*I linker (5′-CTCTAGAG-3′) into the *Hpa*I site (nt 2731) by means of blunt-end ligation. The *Sac*I end was made blunt by using the Klenow fragment of *E. coli* DNA polymerase I, and a *Cla*I linker (5′-CATCGATG-3′) was attached by using T4 DNA ligase. The *Hin*dIII end was not modified. The SV40 fragment was obtained from *dl*2005, an SV40 mutant lacking approximately 230 base pairs of the T-antigen intron (39). This viable mutant produces fully functional T antigen. The fragment used in our study extends from the *Bam*HI site (wild-type SV40 nt 2533) to the *Hpa*II site (nt 346) and includes the early region, replication origin, and late promoter; the portion of the SV40 genome which codes for capsid proteins is missing. The *Hpa*II end was made blunt by using the Klenow fragment of DNA polymerase I, and the *Cla*I linker was attached with T4 DNA ligase. The *Bam*HI end was modified with a polylinker, resulting in the following sequence of sites: *Bam*HI-*Xba*I-*Bam*HI-*Cla*I. The portion of pAT153 used lacks the 6-base-pair region between the *Cla*I and *Hin*dIII sites, and the *Eco*RI site has been removed by using *Eco*RI, Klenow fragment, and T4 DNA ligase. Several subcloning steps were required to assemble pΔSV.GAGX (22), but the final product is linked as follows. The destroyed *Hpa*II end near the SV40 late promoter is joined to the destroyed *Sac*I end of the RSV fragment by means of the *Cla*I linker. The 3′ end of the RSV fragment is joined to pAT153 via their intact *Hin*dIII sites. The intact *Cla*I end of the pAT153 sequence is joined to the SV40 fragment via the *Cla*I site of the polylinker *Bam*HI-*Xba*I-*Bam*HI-*Cla*I.

(ii) pΔSV.Myr_x. Because the RSV *Sac*I site was destroyed during the construction of pΔSV.GAGX, a new *Sac*I site was inserted adjacent to the SV40 late promoter to permit the transfer of myr_0, myr_1, and myr_2 into the expression vector. For this purpose, pΔSV.Myr_x was created by digesting pΔSV.GAGX with *Asp*718I (a *Kpn*I isoschizomer), making the ends blunt by using Klenow fragment, and then adding a *Sac*I linker (5′-CGAGCTCG-3′), using T4 DNA ligase. This manipulation did not destroy the *Asp*718I (or *Kpn*I) site. Digestion of pΔSV.Myr_x with *Sac*I and *Bgl*II removed the 5′ end of the *gag* gene as well as the upstream, out-of-frame SV40 initiation codon, but it had no effect on the SV40 late promoter. Replacement with the *Sac*I-*Bgl*II fragments from MGAG.myr_0, MGAG.myr_1, or MGAG.myr_2 created pΔSV.Myr_0, pΔSV.Myr_1, and pΔSV.Myr_2, respectively. The restriction map of pΔSV.Myr_1, which was used for most of the experiments, is illustrated in Fig. 2.

Transfection of mammalian cells. Prior to transfection, the SV40-*gag* DNAs were digested with *Xba*I to remove the bacterial plasmid sequence (Fig. 2) and then ligated at low DNA concentrations to connect the 3′ end of the *gag* gene with the late SV40 polyadenylation signal. CV-1 cells were transfected by using a variation of the DEAE-dextran and chloroquine method previously described (53). Briefly, 60-mm plates containing 90 to 95% confluent monolayers were washed twice with phosphate-buffered saline and twice with Tris-buffered saline (TBS) (12) immediately before 500 μl of the DNA mixture (TBS containing 1 to 2 μg of ligated DNA and 0.5 mg of DEAE-dextran) was added. After incubation at 37°C in a CO_2 incubator for 45 to 60 min, the DNA was removed from the monolayers and replaced with regular CV-1 growth medium containing 100 μM chloroquine for 4 h. The latter step enhances the delivery of the transfected DNA to the nucleus (21), and we have found it to be essential for high levels of *gag* expression. After chloroquine treatment, the monolayers were returned to normal CV-1 growth medium.

Transfection of avian cells. Turkey cells were transfected in 60-mm plates (80 to 90% confluent), using the DEAE-dextran method described above, except that only 100 μg of DEAE-dextran was used per 500 μl of DNA in TBS, and the cells were incubated in serum-free medium, instead of medium with chloroquine, for 4 h before being returned to normal growth medium. Cells transfected with infectious RSV DNA by this procedure exhibit complete morphological transformation after 3 to 4 days.

Metabolic labeling of transfected cells. CV-1 cells were labeled with radioisotopes 48 h after transfection, and transfected turkey cells were labeled after the monolayers had become completely transformed. For labeling with L-[^{35}S]methionine (>1,000 Ci/mmol; ICN Biomedicals), the cells were washed once with phosphate-buffered saline, and then 800 μl of methionine-free, serum-free medium containing 50 μCi of [^{35}S]methionine was added. After 30 min of labeling, cold methionine was added to a final concentration of 1/10 the amount found in normal Dulbecco modified Eagle medium, and the labeling was continued for 2 h more.

Transfected CV-1 cells were labeled with [9,10(*n*)-^{3}H] myristic acid (47.5 Ci/mmol; Amersham International) by the general method of Rhee and Hunter (33). [^{3}H]myristic acid was dried under a gentle stream of nitrogen to evaporate the toluene solvent and dissolved in dimethyl sulfoxide at a concentration of 30 μCi/μl. The isotope was then added to complete CV-1 growth medium to give a final concentration of 1 mCi/ml. Each 60-mm plate was labeled with 400 μl (0.4 mCi) of this medium for 1 h at 37°C.

Treatment of particles with trypsin. The culture fluid was collected from pΔSV.Myr_1-transfected CV-1 cells and pJD100-transfected turkey cells after radiolabeling for 2.5 h with [^{35}S]methionine in serum-free medium as described above. Any loose cells present in the medium were removed by centrifugation at 15,000 × *g* for 5 min, and the supernatant was immediately divided into six equal portions and processed as follows. Portion 1 received nothing further, portion 2 received soybean trypsin inhibitor (500 μg/ml, final

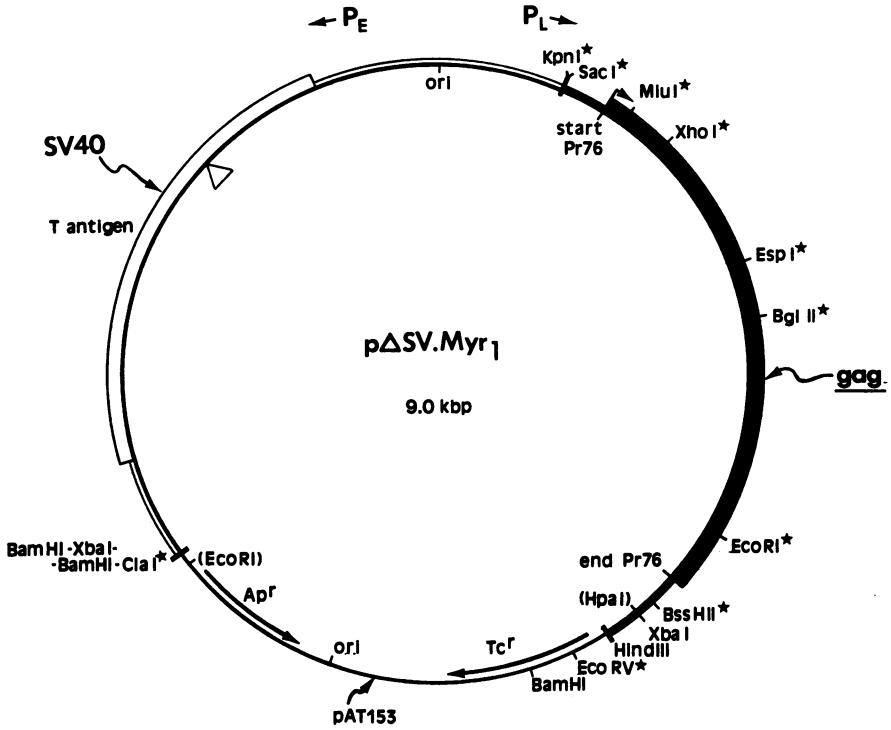

FIG. 2. Vector used for expression of $Pr76^{myr1}$. DNA sequences derived from the RSV genome are represented by the solid line from *Sac*I to *Hin*dIII. The beginning and end of the *gag* coding sequence are indicated within this region. The *Mlu*I site near the 5′ end of *gag* was created as a result of the mutagenesis. SV40 sequences containing the T-antigen gene, the origin of replication (ori), and the late promoter are indicated by the open double line extending from *Cla*I to *Kpn*I. This arrangement places the *gag* coding sequence downstream from the late promoter (P_L). T antigen is expressed from the SV40 early promoter (P_E). Sequences from the bacterial plasmid pAT153, necessary for the propagation of the plasmid in *E. coli*, are represented by the thin line from *Hin*dIII to *Cla*I. Sites in parentheses (*Eco*RI and *Hpa*I) were destroyed during the construction. Unique sites are indicated (★). The position of a noninterfering deletion within the intron of the T-antigen gene is indicated (△). Substitution of the appropriate *gag* alleles allows the expression of $Pr76^{myr0}$ and $Pr76^{myr2}$. kbp, Kilobase pairs.

concentration), portion 3 received Triton X-100 to 1%, portion 4 received 200 μg of trypsin per ml, portion 5 received Triton X-100 plus trypsin, and portion 6 received soybean trypsin inhibitor plus trypsin. The activity of the trypsin was 11,500 U/mg, and a sixfold excess of soybean trypsin inhibitor activity was used. All of the samples were incubated for 30 min at room temperature, and then trypsin inhibitor was added to the tubes that had previously received none. The treated samples were mixed with 5× lysis buffer B (125 mM Tris hydrochloride [pH 8.0], 0.75 M NaCl, 0.5% sodium dodecyl sulfate [SDS], 5% Triton X-100, 5% deoxycholate) for immunoprecipitation.

Fractionation of cell cultures. After the labeling, the medium from each plate (800 μl) was removed and mixed with 200 μl of 5× lysis buffer B containing 5× protease inhibitors (500 μg of phenylmethylsulfonyl fluoride per ml, 5 μg of pepstatin per ml, 5 μg of leupeptin per ml). Monolayers were lysed by using 500 μl of 1× lysis buffer A (25 mM Tris hydrochloride [pH 8.0], 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate) containing 1× concentrations of protease inhibitors. The plates were washed again with 500 μl of lysis buffer A, and nuclei were removed from the 1 ml of lysate by centrifugation at 15,000 × *g* for 1 min. The supernatant was transferred to a clean tube and mixed with 10 μl of 10% SDS.

Immunoprecipitation of Gag proteins. Samples (500 μl) were incubated with an excess of antiserum at 4°C for 12 to 16 h. For most of the experiments, rabbit anti-p27 serum was used. This antiserum (generously provided by Eric Hunter, University of Alabama at Birmingham) recognizes primarily the RSV capsid protein and processing intermediates that contain p27, but it also has low reactivity with other RSV *gag* products. To better collect other *gag* products, goat antiserum against whole RSV (Microbiological Associates, Inc.) was used, followed by a 2-h incubation with rabbit serum against goat immunoglobulin G (Cooper Biomedical, Inc., West Chester, Pa.). All antigen-antibody complexes were collected with fixed *Staphylococcus aureus* by using standard procedures (10). The complexes were washed twice with 1× lysis buffer B and once with 20 mM Tris hydrochloride (pH 8.0) and then disassociated in 20 μl of sample buffer (60 mM Tris hydrochloride [pH 6.8], 10% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.001% bromophenol blue) by being heated at 90°C for 1 to 2 min. Immediately prior to electrophoresis, the free *S. aureus* cells were removed by centrifugation.

SDS-polyacrylamide gel electrophoresis. Immunoprecipitated proteins were electrophoresed in 1.5-mm-thick SDS-polyacrylamide gels by standard methods (10). Resolving gels and stacking gels were prepared by using a 29:1 ratio of acrylamide monomer and cross-linker (*N*,*N*′-methylenebisacrylamide). The resolving portion of the gels contained acrylamide (7, 10, or 15% as noted), 0.1% SDS, and 400 mM Tris hydrochloride (pH 8.8). The stacking gels contained 3% acrylamide, 0.1% SDS, and 60 mM Tris hydrochloride (pH 6.8). After electrophoresis, the separated proteins were fixed and stained with Coomassie blue R250 (0.003% Coomassie blue in 10% acetic acid–50% ethanol). Subsequently, the gels were destained in a solution of 5% methanol–7% acetic acid.

The radioactive bands were detected by fluorography, using Fluoro-Hance (Research Products International, Inc.) and X-OMAT AR5 film (Eastman Kodak Co., Rochester, N.Y.) at −70°C. Typically, exposures of 1 to 16 h were required for the detection of [^{35}S]methionine-labeled proteins, while [^{3}H]myristic acid-labeled proteins required exposures of 1 to 2 weeks.

RESULTS

Oligonucleotide design and mutagenesis. The acyl group of myristylated proteins is always added to an amino-terminal glycine which becomes exposed after the removal of the initiator methionine. This absolute requirement for glycine at residue 2 might explain why Pr76^{gag}, which has glutamic acid at this position, is not myristylated. To test this idea, myr_2 was constructed (Fig. 1) by changing the second codon of RSV *gag* from GAA (Glu) to GGA (Gly). On the other hand, since the importance of residues adjacent to Gly-2 for the recognition by *N*-myristyl transferase is not fully understood (35), it was not certain that this simple substitution would be sufficient for myristylation. Therefore, myr_1 was made (Fig. 1), in which the first 10 codons of *gag* are changed so as to encode the first 10 amino acids of p60$^{v\text{-}src}$. Pr76^{myr1} would be expected to be myristylated since it has been shown that the first 14 amino acids of p60$^{v\text{-}src}$ are sufficient for myristylation, even when attached to a heterologous protein (26). The oligonucleotide used to create myr_1 was designed to maximize homology with the first 10 codons of *gag* by making use of the redundancy of the genetic code. This oligonucleotide also introduced new restriction endonuclease sites, one of which, *Mlu*I, was used for the identification of presumptive mutants. Because this new *Mlu*I site is unique, it provides access to the 5′ coding sequence for future alterations.

The template for mutagenesis was single-stranded DNA from MGAG, an M13mp19 recombinant bearing the *gag* gene on a *Sac*I-*Hin*dIII fragment from the RSV genome. After mutagenesis (see Materials and Methods), approximately 75% of the resulting clones were found to possess the desired mutations, as analyzed by restriction mapping and DNA sequencing. To guard against the effects of errant mutations at other sites, two steps were taken. First, only a segment of the mutant *gag* gene was transferred to the expression vector so that mutations outside that segment would be left behind, and second, multiple clones from each mutagenesis were tested in mammalian cells to make sure that they exhibited identical phenotypes.

Transfer of the mutant *gag* genes to a mammalian expression vector. Though it would be interesting to study the effects of Pr76 myristylation in RSV-transformed mammalian cells, several considerations made it unlikely that infectious particles could be obtained from transfected avian cells by using RSV genomes that contain myr_1 or myr_2. The most serious problem, in the case of myr_1, is that the mutation eliminates an essential splice donor site required for the synthesis of *env* mRNA (37). Also, since there are no avian sarcoma or leukosis viruses known to encode myristylated *gag* products, it might well be the case that Pr76^{myr1} or Pr76^{myr2} would be deleterious to RSV replication in avian cells. Finally, since RSV-transformed mammalian cells synthesize only small amounts of Pr76^{gag}, a vector with reliably high expression levels was preferred. For these reasons, we developed an SV40-based, transient expression vector similar to that used previously for the expression of the RSV *env* gene (9, 30, 52, 53).

The 5′ portion of each of the *gag* alleles was transferred from mutant MGAG replicative-form DNA into the SV40 vector, using *Sac*I and *Bgl*II. As an example, the restriction endonuclease map for the resulting construction made by using myr_1, pΔSV.Myr$_1$, has been illustrated (Fig. 2). In this plasmid, the myr_1 allele is placed downstream from the SV40 late promoter such that there are no initiation codons upstream from that of *gag* (at nt 380). Also present are the SV40 early region (which codes for T antigen) and origin of replication, both of which are needed for DNA replication in monkey cells. Though the early region is not needed if the vector is used in COS-1 cells (which constitutively express T antigen [7]), including it allows *gag* expression in the less metabolically active CV-1 cell line. This ability becomes important when labeling with [^{3}H]myristic acid, which can be converted by the cell into radioactive amino acids upon extended labeling periods (see below). Sequences from the bacterial plasmid pAT153 separate the 3′ end of *gag* from the polyadenylation signal of the SV40 late region and were removed prior to transfection by digestion with *Xba*I. Ligation at low DNA concentrations allows circularization of the SV40-*gag* fragment.

As mentioned above, this vector has two important advantages over other approaches for the expression of *gag* in mammalian cells. First, by placing the *gag* alleles under the control of the SV40 late promoter, very high levels of expression can be achieved. This was important because we wished not only to examine effects of myristylation but also the effects of high concentrations of wild-type *gag* products on particle formation in mammalian cells. That is, the block to processing and particle formation in RSV-transformed mammalian cells might be due to low (below-threshold) levels of Pr76^{gag} and might be overcome simply by producing a lot of protein (17, 44). The second advantage of the vector is that it enables rapid analysis of mutants, since *gag* products are analyzed only 48 h after transfection.

Have myristylation sites been created? To determine whether myr_1 or myr_2 encodes myristylated products, duplicate plates of CV-1 cells were transfected with either no DNA or pΔSV.Myr$_0$ (wild-type), pΔSV.Myr$_1$, or pΔSV.-Myr$_2$ DNA. After 48 h, one plate of each pair was labeled with [^{35}S]methionine (to determine the relative levels of *gag* expression) or with [^{3}H]myristic acid for 1 h. After labeling, the medium was discarded, the cells were lysed, and nuclei were removed by centrifugation. The *gag* products were collected from the lysates by immunoprecipitation with an anti-p27 antibody, separated by SDS-polyacrylamide electrophoresis in a 10% gel, and visualized by fluorography.

It is clear from the [^{35}S]methionine results (Fig. 3A) that each of the SV40-*gag* DNAs expressed a full-length product (Pr76), whereas untransfected cells showed only nonspecific background bands. The differences in intensity between Pr76^{myr0}, Pr76^{myr1}, and the two clones of Pr76^{myr2} reflect differences in the amounts of DNA used in this particular experiment. Results from many other experiments have shown that the DNAs that encode these proteins have equal expression potential. Labeling with [^{3}H]myristic acid demonstrates that both Pr76^{myr1} and Pr76^{myr2} indeed are myristylated (Fig. 3B). A comparison of the relative band intensities obtained with [^{35}S]methionine and [^{3}H]myristic acid suggests that the myristic acid addition site on Pr76^{myr2} is less frequently used than that of Pr76^{myr1}. Also apparent are bands that probably represent proteolytic processing intermediates (Fig. 3, arrowheads). Those detected with the [^{3}H]myristic acid label presumably represent intermediates that contain the amino-terminal portion of Pr76. (The broad

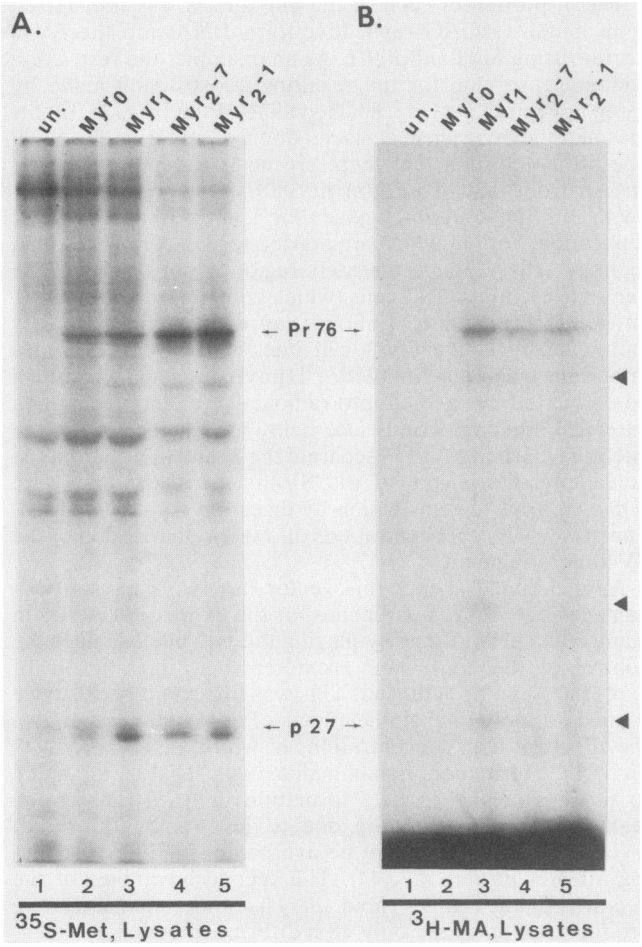

FIG. 3. Labeling of transfected cells with [^{3}H]myristic acid. Untransfected CV-1 cells (lanes 1) and cells transfected with pΔSV.Myr$_0$ (lanes 2), pΔSV.Myr$_1$ (lanes 3), and two clones of pΔSV.Myr$_2$ (lanes 4 and 5) were labeled for 1 h with [^{35}S]methionine (A) or with [^{3}H]myristic acid (B). Gag proteins in the cell lysates were immunoprecipitated with anti-p27, electrophoresed in a 10% SDS-polyacrylamide gel, and detected by fluorography. The positions of Pr76 and p27 in transfected cells are indicated. The positions of myristylated processing intermediates are shown at the right (◀).

band present at the bottom of all the lanes is due to the binding of unincorporated but hydrophobic [^{3}H]myristic acid to *S. aureus*.)

When using [^{3}H]myristic acid, lengthy labeling periods must be avoided; otherwise the labeled molecules may be metabolized by the cells, and the tritium may be incorporated into nonmyristylated proteins. It is evident that the conditions used here avoided that problem because of the lack of labeling of the wild type, Pr76^{myr0}. We have obtained identical results with periods of up to 2 h; however, trace amounts of ^{3}H-labeled proteins that are known to be nonmyristylated can be seen after 2.5 h (data not shown).

Does myristylation enable the release and processing of *gag* products? To further characterize the abilities of the various forms of Pr76 to be processed and released by budding, transfected CV-1 cells were labeled for 2.5 h with [^{35}S]methionine, and the culture media were analyzed along with the cell lysates, using antibodies against p27 (Fig. 4B). Turkey cells infected with RSV were also labeled to obtain authentic *gag* products for comparison and to show the antibody specificity (Fig. 4A). Preliminary experiments (results not shown) revealed half-lives on the order of 30 min for all of the forms of Pr76. The continued presence of labeled precursors in the cell lysates at the end of our standard 2.5-h labeling conditions suggests that steady-state conditions were approximated.

For the RSV control experiment, two molecularly cloned RSV strains were used, JD100 (Prague A) and ATV-8 (Prague C). Both gave identical profiles of radiolabeled proteins on the fluorogram (Fig. 4A). (The uppermost band seen even in the uninfected control is fibronectin, which binds to *S. aureus* cells during the immunoprecipitation.) In the lysates, the most conspicuous bands are Pr76^{gag}, two processing intermediates of approximately 60 and 47 kilodaltons, and a characteristic doublet of bands running at the expected position of p27. We always observe these five bands with these two widely used infectious clones. The "p27- doublet" represents mature products, since it is also seen in the medium samples, whereas the three larger polypeptides are not. The lack of significant amounts of processing intermediates in the medium has been confirmed in pulse-chase experiments (data not shown) and is indicative of the efficiency of cleavage during the budding process.

Positive identification of the proteins in the p27 doublet awaits further experimentation, but on the basis of the apparent difference in molecular mass between the two proteins (approximately 1 kilodalton), it is plausible that the lower band represents p27 proper and the upper band represents p27 with a 9-amino-acid extension corresponding to the peptide located between p27 and p12 (37). That is, the doublet may arise because of incomplete processing of Pr76. This interpretation is confounded by the presence of a myristylated cleavage product from Pr76^{myr1} and Pr76^{myr2} in this region of the gel (Fig. 3). Thus, it is possible that one of the bands represents a cleavage product containing p19 and p10 that was copurified with p27 during preparation of the antiserum. We could not, of course, specifically label this amino-terminal product from the authentic Pr76 in turkey cells (or Pr76^{myr0} in CV-1 cells), since the wild-type product does not contain a site for myristylation.

With the authentic RSV protein profile in mind, the products produced in CV-1 cells were examined after transfection with two clones of pΔSV.Myr$_0$ and two clones of pΔSV.Myr$_1$. Three striking observations were obtained from the experiment. First and perhaps most important, it can be seen that products of the wild-type protein (Pr76^{myr0}) were released with low efficiency into the CV-1 culture medium (Fig. 4B, lanes 3 and 4). This indicates that Pr76 can function in mammalian cells, at least if expressed at high levels. Second, placement of the p60$^{v\text{-}src}$ myristic acid addition site onto Pr76 resulted in a great enhancement (fivefold) in its ability to be released from the cell (Fig. 4B, lanes 5 and 6). An identical result was obtained with the point mutation (Glu to Gly) that creates Pr76^{myr2} (Fig. 5). In conjunction with the first observation, this suggests that myristic acid is an important component but not the sole determinant for targeting of *gag* products to the plasma membrane. The third striking result was that processing of Pr76^{myr0} and Pr76^{myr1} in mammalian cells occurred in a manner very similar, if not identical, to that of authentic RSV. The five p27-related bands described above for RSV were also seen in the mammalian cell lysates. Furthermore, the processing efficiency is quite high, since almost no intermediates were detected in the medium (Fig. 4B).

Since the ability of Pr76^{gag} to function in mammalian cells may be dependent (in part) on the levels of expression, it was

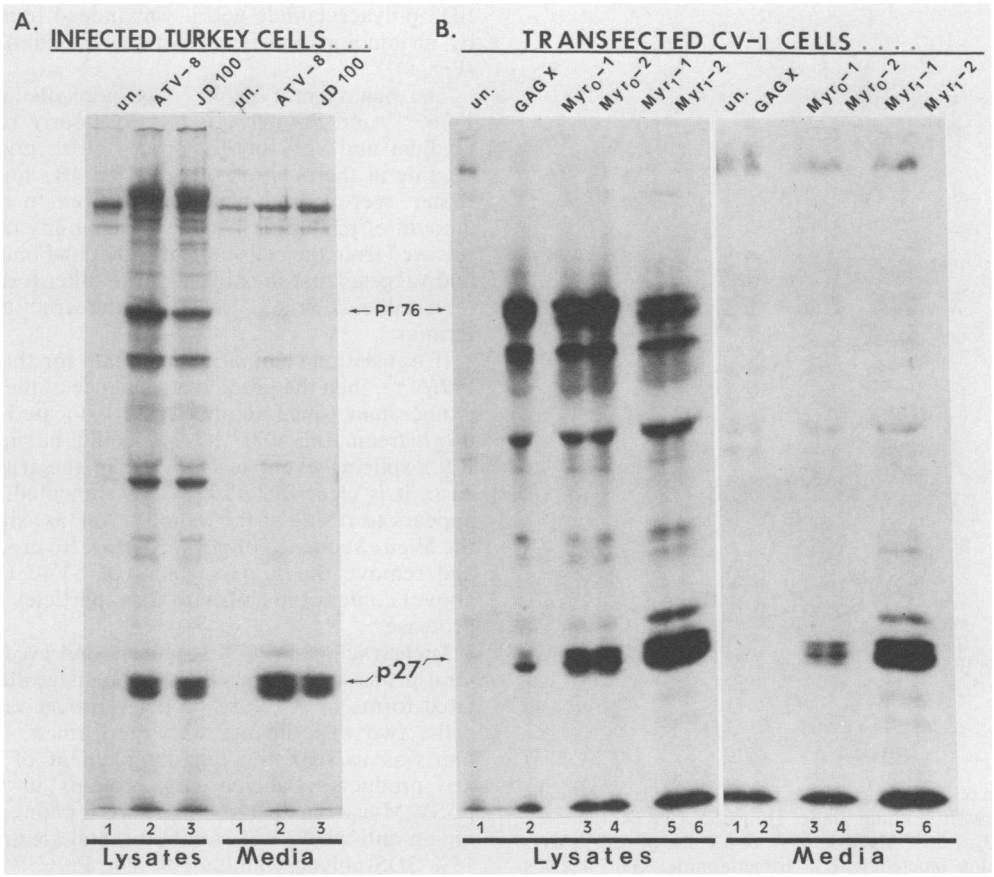

FIG. 4. Comparison of Gag proteins expressed in avian and mammalian cells. (A) Uninfected turkey cells (lanes 1) and cells transformed with RSV strain ATV-8 (Prague C, lanes 2) or JD100 (Prague A, lanes 3) were labeled with [^{35}S]methionine. Gag proteins were immunoprecipitated from the cell lysates and culture media with anti-p27, electrophoresed in a 10% SDS-polyacrylamide gel, and detected by fluorography. (B) Cell lysates and media from untransfected CV-1 cells (lanes 1) and cells transfected with pΔSV.GAGX (lanes 2), two clones of pΔSV.Myr$_0$ (lanes 3 and 4), or two clones of pΔSV.Myr$_1$ (lanes 5 and 6) were analyzed for Gag proteins in the same manner used for turkey cells. The positions of Pr76 and p27 are indicated. The samples from the turkey cells were not run on the same gel as those from the mammalian cells in this particular experiment.

of interest to estimate how much protein is produced by the SV40 vector. For this purpose, the efficiency of transfection was measured by an indirect immunofluorescence assay with anti-p27. Typical for the CV-1 cell line and the DEAE-dextran and chloroquine method of transfection (21), it was found that approximately 30% of the cells expressed Gag antigens (data not shown). Taking this efficiency into account, we calculated that the amount of *gag* products released into the medium during a 2.5-h labeling period (at 48 h posttransfection) is approximately equal (on a per cell basis) to that released from RSV-infected turkey cells during the same period. Consistent with this estimate, we have noticed that the amount of Myr$_1$ protein released in 2.5 h from monolayers on 60-mm plates generally can be detected by Coomassie blue staining after immunoprecipitation and electrophoresis (data not shown).

Are the *gag* products released by budding? To test the hypothesis that Pr76^{myr1} cleavage products are released by an actual budding process, their containment within a viral membrane was assessed by means of their susceptibility to trypsin. Transfected CV-1 cells and JD100-infected turkey cells were radiolabeled, the medium was collected and divided into six portions for the various treatments, and the surviving *gag* products were immunoprecipitated and separated by electrophoresis. The resulting fluorogram (Fig. 6) shows that the released products of Pr76^{myr1} and authentic Pr76^{gag} were completely stable during incubations in the presence of nothing (lanes 1), trypsin inhibitor alone (lanes 2), Triton X-100 alone (lanes 3), trypsin (lanes 4), and trypsin plus inhibitor (lanes 6). For both samples, the *gag* products became susceptible to the protease only when the membrane-dissolving agent and trypsin were present together (lanes 5).

Further evidence that Pr76^{myr1} products are released within a viral envelope was obtained by sedimentation analysis. It was found that conditions suitable for pelleting authentic RSV (45 min at 70,000 × *g*) also quantitatively pelleted the Myr$_1$ particles out of the CV-1 medium (data not shown). Preliminary experiments also indicate that Myr$_1$ particles have a density in sucrose gradients that is similar to that of RSV run in parallel gradients, and the proteins present in the gradient-purified Myr$_1$ particles include the mature p27 doublet, which can be detected after electrophoresis by Coomassie blue staining (data not shown).

Is the amino terminus required for particle formation? The results described above for Pr76^{myr0} show that myristylation of Pr76 is not an absolute requirement for targeting, budding, and processing in the mammalian cell. Moreover, from the

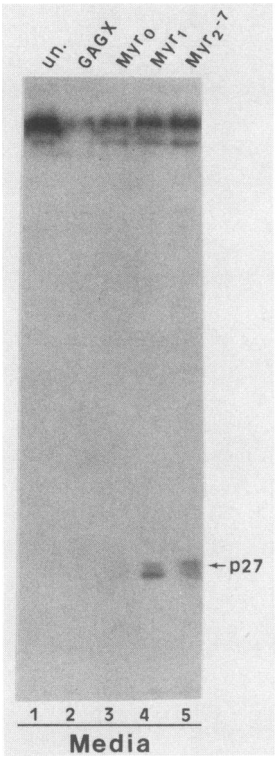

FIG. 5. Release of Pr76^{myr2} products from CV-1 cells. CV-1 cells were transfected with nothing (lane 1), pΔSV.GAGX (lane 2), pΔSV.Myr$_0$ (lane 3), pΔSV.Myr$_1$ (lane 4), or a clone of pΔSV.Myr$_2$ (lane 5). After being labeled with [^{35}S]methionine, Gag proteins present in the media were immunoprecipitated by using anti-p27, electrophoresed in a 10% SDS-polyacrylamide gel, and detected by fluorography. The position of p27 is indicated. Bands near the top of the figure correspond to fibronectin.

results for Pr76^{myr1} and Pr76^{myr2}, it would appear that the precise sequence of the first 10 amino acids of Pr76 is nonessential for these events. These observations led us to consider the possibility that the various forms of Pr76 might be released by a nonspecific, cell-blebbing mechanism perhaps driven by high expression levels. The entrapment and release of cytoplasmic proteins by such a nonspecific event would be ruled out by the identification of an altered but cell-bound form of Pr76. To explore this possibility, we characterized the product of an SV40-*gag* vector called pΔSV.GAGX which expresses a product whose amino terminus is truncated.

In essence, pΔSV.GAGX differs from pΔSV.Myr$_0$ (and also from pΔSV.Myr$_1$ and pΔSV.Myr$_2$) only by the presence of an additional 52 base pairs from the SV40 late region. More specifically, the SV40 sequences from *Kpn*I to *Hpa*II are present, with a *Cla*I linker inserted at the junction of SV40 and RSV sequences (Fig. 7). This fragment contains the initiation codon for the SV40 agnoprotein which we previously have found to be used efficiently in late-region replacement vectors (30). This codon is out of reading frame with the *gag* initiation codon (nt 380), and any translation initiated at the agno-ATG would not be expected to terminate until the *gag* ATG had been passed. If translation was to resume at the next internal methionine codon (nt 464), then Pr76^{gagX} would lack the first 28 amino acids of Pr76 (Fig. 7). When Pr76^{gagX} was compared with Pr76^{myr0}, Pr76^{myr1}, and authentic Pr76^{gag} (from JD100) on a 7% SDS-polyacrylamide gel, it was indeed found to be shorter by an amount consistent with a 28-amino-acid truncation (Fig. 8).

The behavior of Pr76^{gagX} is quite distinct from that of Pr76^{myr0} and Pr76^{myr1}. It is very poorly released into the medium and very poorly processed to give products that migrate at the position of p27 (Fig. 4B, lanes 2). Indeed, a rather overexposed film was required to reveal the small amount of p27 in the lysate. The inability of Pr76^{gagX} to be released from the cells indicates that blebbing does not occur and suggests that the ability of the other forms of Pr76 to be released (e.g., Pr76^{myr1}) is due to the structure of their amino termini.

If translation reinitiation accounts for the smaller size of Pr76^{gagX}, then the predicted sequence at the amino terminus is uncertain, since another ATG is located only 11 codons downstream (nt 497). It also could be imagined that an RNA-splicing event is involved in the truncation. In any case, it is clear that Pr76^{gagX} is truncated. The truncation appears to reside at the amino terminus, since exchange of the 5′ *gag* sequence of pΔSV.GAGX (to create *myr*$_1$ or *myr*$_2$ and remove the 52 base pairs of SV40 DNA mentioned above) confers the ability to form particles and activate the protease.

Is cleavage of Pr76^{myr1} accurate and accomplished by the viral protease? To corroborate the suggestion that myristylated forms of Pr76 are properly processed in mammalian cells, two experiments were performed. The purpose of one was to seek the full complement of Pr76^{myr1} cleavage products. Labeled *gag* proteins in the medium of pΔSV.Myr$_1$-transfected cells were immunoprecipitated, using an anti-whole RSV antibody, and electrophoresed on a 15% SDS-polyacrylamide gel. The Pr76^{myr1} cleavage products were found to comigrate with the authentic RSV products obtained from turkey cells infected with either JD100 (Fig. 9) or ATV-8 (data not shown). The pattern observed with JD100, ATV-8, and Myr$_1$ is similar to previously reported profiles (6, 46), though it should be noted that variations exist between these and other RSV strains (1, 6, 45, 46). (As before, the uppermost band in Fig. 9 corresponds to fibronectin.)

The other experiment which was designed to test the hypothesis that processing in the mammalian system is

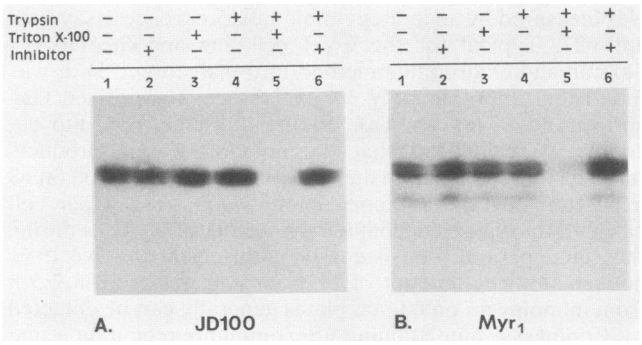

FIG. 6. Trypsin resistance of Pr76^{myr1} particles and RSV virions. JD100-infected turkey cells (A) and pΔSV.Myr$_1$-transfected CV-1 cells (B) were labeled with [^{35}S]methionine. The medium was harvested, divided into six equal portions, and treated with nothing (lanes 1), trypsin inhibitor (lanes 2), Triton X-100 (lanes 3), trypsin (lanes 4), trypsin plus Triton X-100 (lanes 5), or trypsin plus inhibitor (lanes 6). Subsequently, trypsin inhibitor was added to each of the samples, and the surviving Gag proteins were collected, using anti-p27. The immunoprecipitates were analyzed by electrophoresis in a 10% SDS-polyacrylamide gel, followed by fluorography.

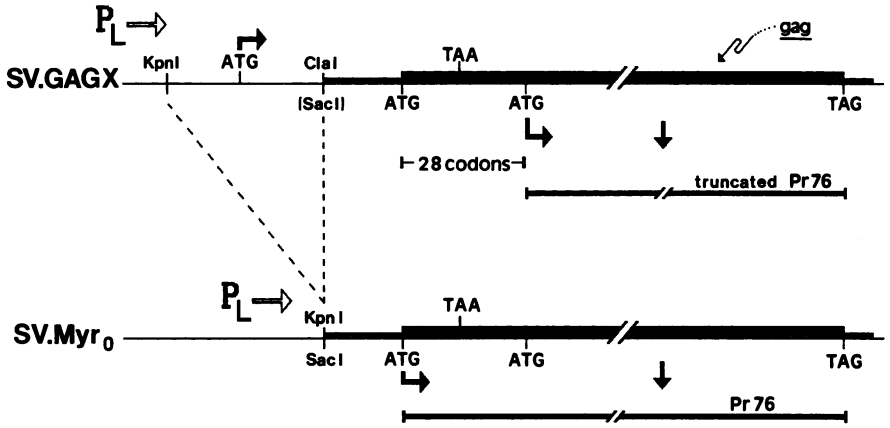

FIG. 7. Comparison of pΔSV.GAGX and pΔSV.Myr$_0$. RSV sequences are indicated by the shaded bars, within which the *gag* coding sequence is indicated by the wider bars. SV40 sequences are indicated by the thin lines. Transcription occurs from the SV40 late promoter (P_L). The *Sac*I site of *gag* (in parentheses) and the *Hpa*II site of SV40 were destroyed, and the ends were joined to a *Cla*I linker during the construction of pΔSV.GAGX. In this construction, an upstream ATG from the SV40 agnoprotein gene lies out of frame with the *gag* initiation codon. Ribosomes initiating at the upstream site bypass the normal *gag* initiation codon and terminate as a result of the indicated TAA. The next available initiation codon lies within the *gag* reading frame. Reinitiation at this internal site would result in the synthesis of a Gag precursor that is truncated by 28 amino acids. Another possible internal site for reinitiation (not indicated) is located 11 codons further downstream. The *Kpn*I to *Cla*I region of pΔSV.GAGX was removed during the construction of pΔSV.Myr$_0$ (and also pΔSV.Myr$_1$ and pΔSV.Myr$_2$). This eliminates the upstream ATG and allows proper initiation and synthesis of full-length Pr76.

accurate and virally encoded involved the transfer of an inactive RSV protease into Pr76^{myr1}. In this protease mutant, the codon for aspartic acid at residue 37 of the active site has been changed to encode isoleucine, and it is referred to here as D37I. The *Bgl*II-*Bss*HII fragment bearing this mutation was engineered into pΔSV.Myr$_1$ to replace the wild-type RSV Prague C fragment (Fig. 2) and create pΔSV.D37I. Because the original mutation was contained in the *gag* gene of the Schmidt-Ruppin strain of RSV, control plasmids were constructed which likewise possess 5′ sequences from Prague C (*Sac*I to *Bgl*II) and 3′ sequences from wild-type RSV Schmidt-Ruppin A (*Bgl*II to *Bss*HII). These control plasmids were designated pΔSV.D37.

Two clones of pΔSV.D37 and two clones of pΔSV.D37I were transfected into CV-1 cells. Other plates received no DNA, pΔSV.GAGX, or pΔSV.Myr$_1$. As expected, the exchange of Prague C residues for those from wild-type Schmidt-Ruppin A had no effect on targeting, budding, and processing (Fig. 10A and B, lanes 3, 4, and 5). That is, Pr76^{D37}, like Pr76^{myr1}, was processed to mature products (as indicated by the p27 doublet) and formed particles that were released into the medium. Also, Pr76^{gagX} again failed to form particles or mature products (Fig. 10, lanes 2), and untransfected cells resulted in the appearance of only nonspecific bands (Fig. 10, lanes 1).

The results with the protease mutant were considerably different (Fig. 10A and B, lanes 6 and 7). The major product was a precursor of 76 kilodaltons, but also apparent was a small amount of a protein of higher molecular mass immediately above Pr76^{D37I} (arrowhead). We interpret the latter protein to be a product of ribosomal frameshifting (13) between the *gag* coding sequence and the truncated *pol* sequence that remains in our vectors, but confirmation of this awaits further experimentation. More relevant here, however, is that Pr76^{D37I} did not give rise to the p27 doublet; it was completely absent in the cell lysate (Fig. 10A) and the culture medium (Fig. 10B). Smaller cleavage products also have not been detected by using antibodies against whole RSV and higher-percentage gels (data not shown). This demonstrates that the viral protease is indeed responsible for

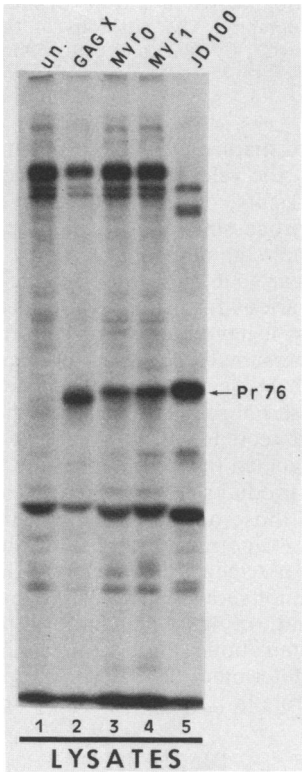

FIG. 8. Comparison of Pr76^{gagX} with other Gag precursors. CV-1 cells transfected with nothing (lane 1), pΔSV.GAGX (lane 2), pΔSV.Myr$_0$ (lane 3), or pΔSV.Myr$_1$ (lane 4) and JD100-infected turkey cells (lane 5) were pulse-labeled with [^{35}S]methionine. Gag precursors were collected from cell lysates by immunoprecipitation with anti-p27, electrophoresed in a low-concentration (7%) SDS-polyacrylamide gel, and detected by fluorography. Pr76^{gagX} appears to be about 3,000 daltons smaller than the other forms of Pr76.

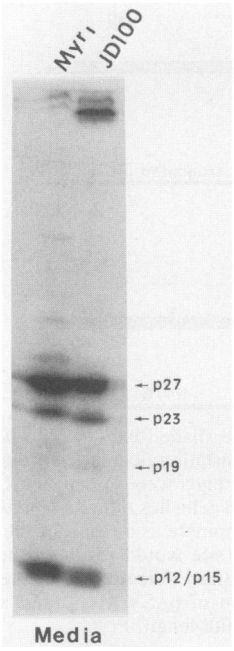

FIG. 9. Identification of other cleavage products of $Pr76^{myr1}$. CV-1 cells transfected with pΔSV.Myr_1 and JD100-infected turkey cells were labeled with [^{35}S]methionine. The Gag proteins released into the medium were immunoprecipitated with goat anti-RSV and analyzed by electrophoresis in a 15% SDS-polyacrylamide gel followed by fluorography. The positions of the authentic RSV cleavage products (p27, CA; p23 and p19, MA; p12, NC and p15, PR) are indicated.

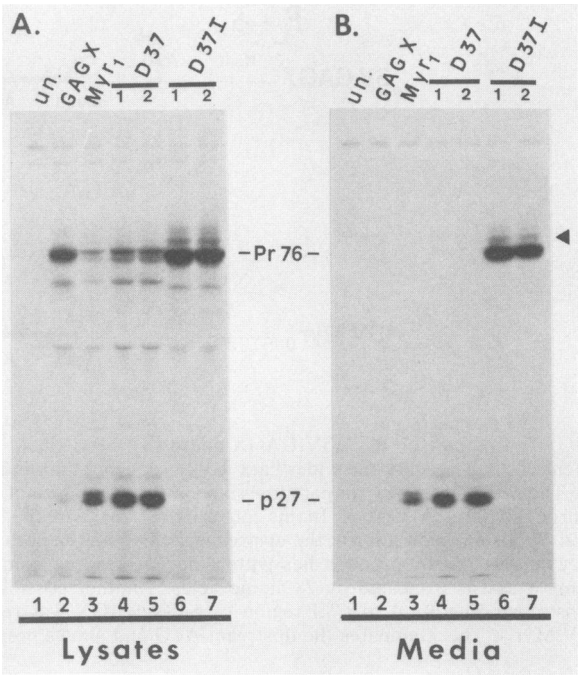

FIG. 10. Effects of a protease mutation on $Pr76^{myr1}$ processing. pΔSV.D37 encodes a functionally wild-type but hybrid form of Pr76. The amino-terminal portion is from RSV Prague C while the carboxy-terminal portion is from RSV Schmidt-Ruppin A. pΔSV.D37I is identical except for a mutation that changes aspartic acid to isoleucine at residue 37 in the active site of the RSV protease. CV-1 cells were transfected with nothing (lanes 1), pΔSV.GAGX (lanes 2), pΔSV.Myr_1 (lanes 3), two clones of pΔSV.D37 (lanes 4 and 5), and two clones of pΔSV.D37I (lanes 6 and 7). Cells were labeled with [^{35}S]methionine, and the Gag proteins were immunoprecipitated with anti-p27 from cell lysates (A) and from media (B). Proteins were analyzed by electrophoresis in a 10% gel and fluorography. The position of presumptive Pr76 fusion proteins arising by ribosomal frameshifting into the adjacent *pol* sequences is indicated (◀).

the production of mature cleavage products in mammalian cells. Moreover, the release of unprocessed $Pr76^{D37I}$ confirms previous results, obtained with this mutant in avian cells, that show processing not to be prerequisite for budding (V. Vogt, personal communication).

While it is clear that the viral protease is essential for processing the various forms of Pr76 to mature products in mammalian cells, it nevertheless appears that another protease activity, presumably cellular in origin, could be involved. This can be seen by the presence of *gag*-specific products of a size intermediate between those of Pr76 and p27 which are detected with all forms of Pr76, including $Pr76^{D37I}$, when labeled for extended periods (Fig. 4 and 10). These cleavage products seem to be the same processing intermediates as those observed with JD100 and ATV-8 in avian cells (Fig. 4), not only because of their size but also because they are not seen after shorter labeling periods (Fig. 3 and 8 and data not shown). It is also interesting that these bands correspond, at least in size, to the myristylated intermediates noted above (Fig. 3). Further investigation will be required to determine whether this additional protease activity plays a role in particle formation and processing.

DISCUSSION

The Gag protein plays a central role in the assembly of infectious retroviruses, and our experiments directly confirm the findings of others that it is the only viral protein required for particle formation (4). In order for Gag to make particles, three poorly understood but distinct molecular events must occur. Specifically, the Gag protein must (i) be synthesized and folded, (ii) be targeted to the plasma membrane, and (iii) form intermolecular connections to cause the membrane evagination that results in budding. There are, of course, many other steps required for the assembly of infectious virions (for example, packaging of reverse transcriptase, viral RNA, and glycoproteins), but these three steps are prerequisite to and independent of all other steps in assembly.

Myristylation and particle formation. The primary purpose of the experiments described here was to determine whether particle formation by the RSV *gag* product, Pr76, could be promoted in mammalian cells by the creation of a myristic acid addition site at its amino terminus. We have shown that this is indeed the case. $Pr76^{myr1}$ and $Pr76^{myr2}$ appear to be remarkably proficient in their ability to induce budding and virus-specific processing. Furthermore, we have been unable to find any aspect of their behavior that differs from that of the authentic Pr76 produced in avian cells. Because the amino termini of $Pr76^{myr1}$ and $Pr76^{myr2}$ are so different (Fig. 1), the increased activity (over that of the nonmodified $Pr76^{myr0}$) seems almost certain to be due to the presence of myristate. Whether the enhancing effects of myristic acid addition can be mimicked by other amino-terminal alterations will require further experimentation.

It should be mentioned that a personal communication cited within another work (3) suggested that high-level

expression of Pr76 in mammalian cells, using an SV40 vector, did not enable particle formation. That preliminary information was based on results for pΔSV.GAGX, which at the time was thought to produce the wild-type RSV *gag* product. The experiments presented here, however, show that $Pr76^{gagX}$ is truncated at its amino terminus. Experiments are in progress to elucidate the precise structure of this protein.

The creation of a myristic acid addition site by changing the second residue from glutamic acid to glycine ($Pr76^{myr2}$) could not have been predicted with surety, since the importance of adjacent amino acids is poorly understood. In fact, a very recent compilation of known addition sites (42) predicts that $Pr76^{myr2}$ would not be a good substrate for myristylation since it possesses a large, positively charged residue (lysine) at the fifth position after the initiator codon. Our experiments show that the site on $Pr76^{myr2}$ is quite adequate for the addition of myristic acid, though somewhat less efficient than that on $Pr76^{myr1}$.

That the less highly myristylated $Pr76^{myr2}$ forms particles as readily as $Pr76^{myr1}$ leads to the interesting hypothesis that nonmyristylated precursors can find their way into particles by interacting with myristylated partners in the molecular population. Previously reported cross-linking data are consistent with, but of course do not prove, this suggestion of cooperative interactions among $Pr76^{gag}$ molecules (27). The alternate hypothesis would be that only the myristylated proteins within the population are capable of budding from the cell. One way to distinguish between these two possibilities would be to perform complementation experiments in which attempts are made to rescue nonmyristylated, cell-bound mutant proteins such as $Pr76^{gagX}$. We have recently obtained data (to be presented elsewhere) that show that $Pr76^{gagX}$ can be rescued (i.e., released in particles) from the mammalian cell when coexpressed with properly targeted Pr76 molecules. Further investigation of the minimum number of myristylated molecules needed for particle formation is warranted.

We were surprised to find that the unmodified Gag protein, $Pr76^{myr0}$, is capable of promoting 20% of the level of particle formation of the myristylated forms. This result was unexpected for two reasons. One reason is that previous studies of RSV-transformed mammalian cells failed to show any processing or release of $Pr76^{gag}$ (6, 44). Because of this observation, it has been widely held that a fundamental difference exists between the avian and mammalian cell with regard to Gag function. Our results suggest that the basic cellular machinery exploited by retroviruses for particle formation is the same from one cell species to another. The reason that others have not observed particle formation in RSV-transformed mammalian cells may be in part due to the very low levels of $Pr76^{gag}$ protein made. As has been suggested previously (17, 44), there may be a critical threshold amount of Pr76 below which particle formation is unable to proceed. The low-level production of particles obtained when $Pr76^{myr0}$ is expressed at high levels supports this suggestion to some degree. But since the addition of myristic acid leads to a fivefold increase in particle formation, levels of *gag* expression cannot account entirely for the previously observed lack of particle formation in RSV-transformed mammalian cells. Nevertheless, the idea of threshold levels deserves further investigation. It would be enlightening, for example, to modulate the expression of $Pr76^{myr0}$ in a single cell type and determine how the rates of budding correlate with the amounts of protein made.

The other reason that particle formation with $Pr76^{myr0}$ was unexpected is that mammalian retroviruses that normally synthesize myristylated Gag proteins fail to make particles when acylation is disrupted. This has been demonstrated for murine leukemia virus, a C-type retrovirus (32), and M-PMV, a D-type retrovirus (33). It may be that the alterations that were placed into the Gag proteins of those viruses happened to more thoroughly disrupt the function of the MA domain. It would be of interest to determine whether other substitutions within the MA domains of MuLV and M-PMV can lead to an intermediate ability for particle formation.

Role of the amino terminus. How are Gag proteins targeted to the plasma membrane? What forces drive the budding process? What are the roles of myristic acid and the amino terminus in these functions? Gag proteins are too poorly understood for these questions to be answered. However, there are good reasons to expect that retroviruses utilize existing cellular machinery, and not simple diffusion, for dispatching their Gag proteins to the site of assembly. For example, nonmyristylated forms of M-PMV assemble particles that appear to be trapped deep within the cell rather than throughout the cytoplasm (33). Furthermore, there is evidence that suggests that the Gag proteins of murine retroviruses may interact with cytoskeletal elements (5).

Our results with $Pr76^{myr0}$ demonstrate that myristic acid is not the primary determinant for enabling Gag proteins to interact with the machinery of the mammalian cell. This idea is strongly supported by the fact that many nonviral myristylated proteins are targeted to compartments other than the plasma membrane; some are not even membrane associated (42). Furthermore, the Gag proteins of some mammalian retroviruses (e.g., equine infectious anemia virus and visna virus) are not myristylated at all (42), while those of certain avian retroviruses unrelated to RSV (e.g., reticuloendotheliosis virus) are myristylated (43). Nevertheless, our experiments with the truncated protein, $Pr76^{gagX}$, do seem to suggest that the amino terminus of Pr76 carries a function essential for targeting or particle formation. Since myristylation seems only to enhance Pr76 function, it may be that there is nothing special about myristic acid, but rather the important feature at the amino terminus simply might be hydrophobicity, for example. This idea could be tested by progressively altering the amino terminus of $Pr76^{myr0}$ such that it became increasingly more hydrophobic but not myristylated.

We are unaware of any data that unequivocally demonstrate a direct role for the amino terminus of Gag in the targeting process. An alternate role would be to stabilize the Gag protein at the plasma membrane or initiate the budding process once the protein arrives there. It might even be that the amino terminus serves only indirectly by enabling the proper folding of an important functional domain elsewhere on the molecule. That is, myristylation of Pr76 might enhance particle formation by satisfying a conformational requirement that permits better interaction of the polypeptide with the machinery of the mammalian cell. For example, the addition of myristic acid might permit a minor adjustment in the structure of a domain to allow it to fit more perfectly into a mammalian cell "chaperon protein" situated along the pathway to the plasma membrane.

Ideas about the conformation of retrovirus MA proteins require careful consideration. An amino terminus possessing a very hydrophobic, 14-carbon fatty acid would not be expected to remain exposed to the aqueous environment but rather to be buried within the Gag protein, within another protein, or within a membrane. The latter possibility can be eliminated, at least immediately after protein synthesis,

since Gag proteins are made on free ribosomes (4) and are most likely myristylated cotranslationally (24, 51). Distinguishing between the former two possibilities is not straightforward.

Activation of the protease and processing. Consistent with the findings of others (15, 48), we have shown that processing of Pr76 is not prerequisite to particle formation, since inactivation of the viral protease did not abolish budding. In contrast, targeting and particle formation appear to be prerequisites to processing. By cleaving the Gag proteins late in assembly (concurrent with or after budding), the need for each of the mature cleavage products to be targeted separately to the plasma membrane is eliminated. As a consequence of this strategy, retroviral proteases must be tightly regulated to prevent premature cleavage. The protease of RSV (and other members of the avian sarcoma and leukosis virus family) might be expected to be even more tightly controlled than those of other retroviruses, since it is encoded as part of the *gag* gene and produced in equimolar amounts relative to its substrate. All other retroviruses encode their protease in a downstream reading frame and produce amounts that are on the order of 20-fold less (reviewed in reference 18).

Though it is not understood why RSV must produce such large proportions of protease, it is clear that the proteolytic activity is tightly and properly controlled in mammalian cells. Inspection of Fig. 4 reveals the clear correlation of processing with particle formation. Virtually no protease activity is seen with the cell-bound $Pr76^{gagX}$; low activity is seen with the poorly released $Pr76^{myr0}$; and the highest protease activity is seen with $Pr76^{myr1}$, which also most readily forms particles. The failure of $Pr76^{gagX}$ (which carries a wild-type protease sequence) to be cleaved suggests that even high levels of expression in a eucaryotic cell are insufficient to activate the RSV protease if it is not properly targeted to the plasma membrane. Consistent with this hypothesis, we have found that when $Pr76^{gagX}$ is rescued (see above), its protease is activated (to be presented elsewhere). Thus, it may be that membrane targeting is required to allow formation of protease (p15) dimers which constitute the active form of the RSV protease (23, 49). That we observe small amounts of Gag intermediates in the absence of viral protease activity leaves open the possibility for the involvement of a cellular protease in initiating the processing pathway.

Whatever the means by which RSV regulates its protease, once it is activated it is exceedingly efficient in producing the mature cleavage products. In our mammalian expression system, as in RSV-infected avian cells, almost no intact precursors or processing intermediates could be detected in the medium even during steady-state labeling conditions. The efficiency and speed of the protease are highlighted by the 30-min half time of particle formation. Is processing accurate in our mammalian system? It certainly seems likely to be. Each of the $Pr76^{myr1}$ cleavage products comigrated with an authentic RSV cleavage product, and the kinetics of cleavage appear to be the same between the two. However accurate, it is clear that processing is mediated by the viral protease, since mature cleavage products were no longer produced when the active site of the protease was disrupted.

We are presently investigating whether the *gag* products produced in our mammalian system are biologically active. Specifically, we have exchanged the myr_2 allele for the wild-type *gag* gene in pJD100 and are testing the ability of the recombinant RSV genome to cause the release of particles, from mammalian cells, which are infectious for avian cells. These experiments are not straightforward, and many potential problems can be imagined. In particular, it may be that a multitude of blocks to RSV replication in mammalian cells exist, each sufficient to prevent the production of particles that are infectious for avian cells. For example, it has been reported that RNA splicing in RSV-transformed mammalian cells is anomalous and results in reduced levels of genomic (35S) RNA (31). Furthermore, since wild-type RSV does not encode a myristylated Gag protein, the synthesis of such a protein as a result of the myr_2-*gag* exchange might interfere with replication in avian cells.

In any case, aside from myristylation, we have not yet found any differences in the behavior of $Pr76^{myr1}$ and $Pr76^{myr2}$ relative to that of authentic Pr76 produced in avian cells. The ease and efficiency of our mammalian system should allow rapid analysis of the effects of *gag* mutations on particle formation and processing, without the rather difficult task of obtaining cell clones that harbor integrated copies of the mutant genomes.

ACKNOWLEDGMENTS

We thank Eric Hunter and Volker Vogt for open and stimulating discussions and for providing the materials noted above. We especially thank the fellow members of our laboratory, Robert Weldon and Christine Erdie, for careful review of the manuscript and supportive interactions.

This work was supported by Public Health Service grant CA-47482 from the National Institutes of Health. Support for J.A.A. was provided by Louisiana State University Medical Center and is gratefully acknowledged.

LITERATURE CITED

1. **Bhown, A. S., J. C. Bennett, and E. Hunter.** 1980. Alignment of the peptides derived from acid-catalyzed cleavage of an aspartylprolyl bond in the major internal structural polypeptide of avian retroviruses. J. Biol. Chem. **255**:6962–6965.
2. **Copeland, N. G., A. D. Zelenetz, and G. M. Cooper.** 1979. Transformation of NIH/3T3 mouse cells by DNA of Rous sarcoma virus. Cell **17**:993–1002.
3. **Dickson, C., R. Eisenman, and H. Fan.** 1985. Protein biosynthesis and assembly, p. 135–145. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, vol. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
4. **Dickson, C., R. Eisenman, H. Fan, E. Hunter, and N. Teich.** 1984. Protein biosynthesis and assembly, p. 513–648. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Edbauer, C. A., and R. B. Naso.** 1984. Cytoskeleton-associated $Pr65^{gag}$ and assembly of retrovirus temperature-sensitive mutants in chronically infected cells. Virology **134**:389–397.
6. **Eisenman, R. N., V. M. Vogt, and H. Diggelmann.** 1974. Synthesis of avian RNA tumor virus structural proteins. Cold Spring Harbor Symp. Quant. Biol. **39**:1067–1075.
7. **Gluzman, Y.** 1981. SV40 transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
8. **Hanahan, D.** 1985. Techniques for transformation of *E. coli*, p. 109–135. *In* D. M. Glover (ed.), DNA cloning, vol. 1. IRL Press Ltd., Oxford.
9. **Hardwick, J. M., K. S. Shaw, J. W. Wills, and E. Hunter.** 1986. Amino-terminal deletion mutants of the Rous sarcoma virus glycoprotein do not block signal peptide cleavage but can block intracellular transport. J. Cell Biol. **103**:829–838.
10. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Henderson, L. E., H. C. Krutzsch, and S. Oroszlan.** 1983. Myristyl amino-terminal acylation of murine retrovirus proteins: an unusual post-translational protein modification. Proc. Natl. Acad. Sci. USA **80**:339–343.
12. **Hunter, E.** 1979. Biological techniques for avian sarcoma vi-

ruses. Methods Enzymol. **58**:379–393.
13. **Jacks, T., H. D. Madhani, F. R. Masiarz, and H. E. Varmus.** 1988. Signal for ribosomal frameshifting in the Rous sarcoma virus *gag-pol* region. Cell **55**:447–458.
14. **Jørgensen, E. C., N. O. Kjeldgaard, F. S. Pedersen, and P. Jørgensen.** 1988. A nucleotide substitution in the *gag* N terminus of the endogenous ecotropic DBA/2 virus prevents $Pr65^{gag}$ myristylation and virus replication. J. Virol. **62**:3217–3223.
15. **Katoh, L., Y. Yoshinaka, A. Rein, M. Shibuya, T. Odaka, and S. Oroszlan.** 1985. Murine leukemia virus maturation: protease region required for conversion from "immature" to "mature" core form and for virus infectivity. Virology **145**:280–292.
16. **Katz, R. A., C. A. Omer, J. H. Weis, S. A. Mitsialis, A. J. Faras, and R. V. Guntaka.** 1982. Restriction endonuclease and nucleotide sequence analyses of molecularly cloned unintegrated avian tumor virus DNA: structure of large terminal repeats in circle junctions. J. Virol. **42**:346–351.
17. **Kotler, M., R. A. Katz, and A. M. Skalka.** 1988. Activity of avian retroviral protease expressed in *Escherichia coli*. J. Virol. **62**:2696–2700.
18. **Krausslich, H.-G., and E. Wimmer.** 1988. Viral proteinases. Annu. Rev. Biochem. **57**:701–754.
19. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
20. **Leis, J., D. Baltimore, J. M. Bishop, J. Coffin, E. Fleissner, S. P. Goff, S. Oroszlan, H. Robinson, A. M. Skalka, H. M. Temin, and V. Vogt.** 1988. Standardized and simplified nomenclature for proteins common to all retroviruses. J. Virol. **62**:1808–1809.
21. **Luthman, J., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. **11**:1295–1308.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Miller, M., M. Jaskolski, J. K. M. Rao, J. Leis, and A. Wlodawer.** 1989. Crystal structure of a retroviral protease proves relationship to aspartic protease family. Nature (London) **337**:576–579.
24. **Olson, E. N., and G. Spizz.** 1986. Fatty acylation of cellular proteins: temporal and subcellular differences between palmitate and myristate acylation. J. Biol. Chem. **261**:2458–2466.
25. **Palmiter, R. D., J. Gagnon, V. M. Vogt, S. Ripley, and R. N. Eisenman.** 1978. The NH_2-terminal sequence of the avian oncovirus *gag* precursor polyprotein ($Pr76^{gag}$). Virology **91**:423–433.
26. **Pellman, D., E. A. Garber, F. R. Cross, and H. Hanafusa.** 1985. An N-terminal peptide from $p60^{src}$ can direct myristylation and plasma membrane localization when fused to heterologous proteins. Nature (London) **314**:374–377.
27. **Pepinsky, R. B., D. Cappiello, C. Wilkowski, and V. M. Vogt.** 1980. Chemical crosslinking of proteins in avian sarcoma and leukemia viruses. Virology **102**:205–210.
28. **Pepinsky, R. B., and V. M. Vogt.** 1979. Identification of retrovirus matrix proteins by lipid-protein cross-linking. J. Mol. Biol. **131**:819–837.
29. **Pepinsky, R. B., and V. M. Vogt.** 1984. Fine-structure analyses of lipid-protein and protein-protein interactions of *gag* protein p19 of the avian sarcoma and leukemia viruses by cyanogen bromide mapping. J. Virol. **52**:145–153.
30. **Perez, L., J. W. Wills, and E. Hunter.** 1987. Expression of the Rous sarcoma virus *env* gene from a simian virus 40 late-region replacement vector: effects of upstream initiation codons. J. Virol. **61**:1276–1281.
31. **Quintrell, N., S. H. Hughes, H. E. Varmus, and J. M. Bishop.** 1980. Structure of viral DNA and RNA in mammalian cells infected with avian sarcoma virus. J. Mol. Biol. **143**:363–393.
32. **Rein, A., M. R. McClure, N. R. Rice, R. B. Luftig, and A. M. Schultz.** 1986. Myristylation site in $Pr65^{gag}$ is essential for virus particle formation by Moloney murine leukemia virus. Proc. Natl. Acad. Sci. USA **83**:7246–7250.
33. **Rhee, S. S., and E. Hunter.** 1987. Myristylation is required for intracellular transport but not for assembly of D-type retrovirus capsids. J. Virol. **61**:1045–1053.
34. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
35. **Schultz, A. M., L. E. Henderson, and S. Oroszlan.** 1988. Fatty acylation of proteins. Annu. Rev. Cell Biol. **4**:611–647.
36. **Schultz, A. M., and S. Oroszlan.** 1983. In vivo modification of retroviral *gag* gene-encoded polyproteins by myristic acid. J. Virol. **46**:355–361.
37. **Schwartz, D. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. Cell **32**:853–869.
38. **Sefton, B. M., and J. E. Buss.** 1987. The covalent modification of eukaryotic proteins with lipid. J. Cell Biol. **104**:1449–1453.
39. **Sleigh, M. J., W. C. Topp, R. Hanich, and J. F. Sambrook.** 1978. Mutants of SV40 with an altered small t protein are reduced in their ability to transform cells. Cell **14**:79–88.
40. **Steimer, K. S., and D. Boettiger.** 1977. Complementation rescue of Rous sarcoma virus from transformed mammalian cells by polyethylene glycol-mediated cell fusion. J. Virol. **23**:133–141.
41. **Stoltzfus, C. M., L.-J. Chang, T. P. Cripe, and L. P. Turek.** 1987. Efficient transformation by Prague A Rous sarcoma virus plasmid DNA requires the presence of *cis*-acting regions within the *gag* gene. J. Virol. **61**:3401–3409.
42. **Towler, D. A., J. I. Gordon, S. P. Adams, and L. Glaser.** 1988. The biology and enzymology of eukaryotic protein acylation. Annu. Rev. Biochem. **57**:69–99.
43. **Tsai, W.-P., T. D. Copeland, and S. Oroszlan.** 1985. Purification and chemical and immunological characterization of avian reticuloendotheliosis virus *gag*-gene-encoded structural proteins. Virology **140**:289–312.
44. **Vogt, V. M., D. A. Bruckenstein, and A. P. Bell.** 1982. Avian sarcoma virus *gag* precursor polypeptide is not processed in mammalian cells. J. Virol. **44**:725–730.
45. **Vogt, V. M., R. H. Eisenman, and H. Diggelmann.** 1975. Generation of avian myeloblastosis virus structural proteins by proteolytic cleavage of a precursor polypeptide. J. Mol. Biol. **96**:471–493.
46. **Vogt, V. M., R. B. Pepinsky, and L. E. Southard.** 1985. Primary structure of p19 species of avian sarcoma and leukemia viruses. J. Virol. **56**:31–39.
47. **Von der Helm, K., J. Kempeni, W. Wille, and K. Willecke.** 1980. Rous sarcoma virus precursor protein Pr76 is processed in avian sarcoma virus-transformed mammalian cells after fusion-injection of viral protein p15. Virology **106**:310–316.
48. **Voynow, S. L., and J. M. Coffin.** 1985. Truncated *gag*-related proteins are produced by large deletion mutants of Rous sarcoma virus and form virus particles. J. Virol. **55**:79–85.
49. **Weber, I. T., M. Miller, M. Jaskolski, J. Leis, A. M. Skalka, and A. Wlodawer.** 1989. Molecular modeling of the HIV-1 protease and its substrate binding site. Science **243**:928–931.
50. **Weiss, R.** 1984. Experimental biology and assay of RNA tumor viruses, p. 209–260. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
51. **Wilcox, C., J.-S. Hu, and E. N. Olson.** 1987. Acylation of proteins with myristic acid occurs cotranslationally. Science **238**:1275–1278.
52. **Wills, J. W., J. M. Hardwick, K. Shaw, and E. Hunter.** 1983. Alterations in the transport and processing of Rous sarcoma virus envelope glycoproteins mutated in the signal and anchor regions. J. Cell. Biochem. **23**:81–94.
53. **Wills, J. W., R. V. Srinivas, and E. Hunter.** 1984. Mutations of the Rous sarcoma virus *env* gene that affect the transport and subcellular location of the glycoprotein product. J. Cell Biol. **99**:2011–2023.