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# Myristylation site in Pr65$^{gag}$ is essential for virus particle formation by Moloney murine leukemia virus

**(site-directed mutagenesis/virus assembly/retroviruses)**

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ABSTRACT **It was previously reported that the gag proteins of mammalian type C retroviruses are modified by the addition of myristate to the N-terminal glycine residue. We have performed oligonucleotide-directed mutagenesis to change this glycine codon in the Moloney murine leukemia virus genome to an alanine codon and also to specifically delete the glycine codon. Upon transfection into mammalian cells, these mutant genomes direct the synthesis of gag proteins, but these proteins are not myristylated. The mutants do not form virus particles or any recognizable virus-specific structures visible in thin sections with the electron microscope. Further, the mutant gag proteins appear to remain in the cytosol, whereas the wild type is found principally in particulate fractions of the cell. The results are consistent with the theory that myristate is required for the association of the gag protein with the plasma membrane and that this association is necessary for virus assembly.**

Type C retroviruses assemble at the plasma membrane of virus-producing cells. The *gag* gene product is apparently able to assemble into virus particles in the absence of other virus components (refs. 1–4; A.R. and A.M.S., unpublished results). The nature of the interaction between the gag polyprotein and plasma membranes is not known; the protein that ultimately appears in the virion is not glycosylated and lacks any obvious signal sequence. It is also not known whether interaction with membranes is necessary for virus assembly.

The *gag* gene product of the mammalian type C retroviruses, Pr65$^{gag}$, is modified by the covalent attachment of myristic acid to the N-terminal glycine (5, 6). Although the significance of this highly unusual protein modification for virus replication is unknown, it seems possible that the presence of the myristate group affects the interaction of Pr65$^{gag}$ with cellular membranes.

The exact sequence requirements for protein myristylation in mammalian cells are also unknown; however, the N-terminal glycine is probably required since all known eukaryotic myristylated proteins have N-terminal glycines (7–11) and since glycine has been shown to be necessary for myristylation of pp60$^{src}$ in avian cells (12).

We have employed oligonucleotide-directed mutagenesis to address these questions. Moloney murine leukemia virus (MuLV) was mutagenized so as to change the N-terminal glycine to alanine or to delete the glycine. As described in this report, these mutants encode a Pr65$^{gag}$ that is not myristylated. Further, these mutant gag polyproteins are predominantly soluble cytoplasmic proteins and do not assemble into virus particles.

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## MATERIALS AND METHODS

**Cell Lines.** NIH 3T3 cells (a gift of D. Blair, National Cancer Institute) were maintained in Dulbecco's medium containing 10% (vol/vol) calf serum. Chinese hamster ovary cells (a gift of M. Gottesman, National Cancer Institute) were grown in α medium with 10% (vol/vol) fetal calf serum.

**DNAs.** An infectious clone of Moloney MuLV was obtained from D. Steffen (Worcester Foundation). This clone contained an integrated genome plus flanking sequences, inserted in the *Eco*RI site of Charon 4A. The clone has been subcloned into the *Eco*RI site of the plasmid vector pSV2Neo (13), which was a gift of M. Pearson (National Cancer Institute–Frederick Cancer Research Facility). A 3′ *Akv* subclone, extending from the *Sal* I site in the *pol* gene of clone 623 (14) to an *Eco*RI site in 3′-flanking cellular DNA, inserted in the plasmid vector pBR322, was a gift of D. Lowy (National Cancer Institute). M13mp11 (15) was obtained from Bethesda Research Laboratories and pUC8 (16) was a gift of R. M. Stephens of this laboratory. Recombinant DNA procedures were performed according to standard protocols (17).

**Mutagenesis.** Two-primer oligonucleotide-directed mutagenesis was performed as described by Zoller and Smith (18). The mutagenic oligonucleotides (obtained from Program Resources, Inc., National Cancer Institute–Frederick Cancer Research Facility) were 19 nucleotides long, with 14 nucleotides on the 5′ side of the glycine codon that was the target of mutagenesis. Thus the glycine to alanine substitution was made with the oligonucleotide GTCTGAAAATATGGCCCAG, while the glycine deletion was made with TGTCTGAAAATATGCAGAC. Candidate mutant phage clones were first isolated by plaque hybridization and stepwise melting, using the mutagenic oligonucleotides as probes; the candidates were then analyzed by sequencing the replicative form (19).

**Transfection.** Gene transfer was performed by the calcium phosphate coprecipitation technique. Transfectants were selected by the addition of G-418 (GIBCO) to the cultures 2–3 days after transfection. Pools of G-418-resistant cells were grown and maintained in mass culture in the continuous presence of G-418. It should be noted that in the experiments shown here, the "wild-type controls" are Moloney MuLV while the mutant constructs are Moloney-*Akv* chimeras (see Fig. 1); however, we have subsequently repeated the significant results using wild-type chimeras corresponding exactly to the mutant constructs.

**Virus Assays.** Infectivity assays were performed as described (20, 21). Virion-associated reverse transcriptase activity was assayed as described (22).

Abbreviations: MuLV, murine leukemia virus; M-MCF, Moloney mink cell focus-inducing virus.

**Cell Labeling and Immunoprecipitation.** Labeled cells were lysed as described (6, 23), and immunoprecipitates were then collected using protein A-Sepharose (Pharmacia) and goat anti-p30 serum (24) or the anti-p12 monoclonal F548 (25) (a gift of B. Chesebro, National Institute of Allergy and Infectious Diseases). Washed immunoprecipitates were separated by $NaDodSO_4$/PAGE and visualized by scintillation radioautography.

**Immunoblotting.** Samples were electroblotted onto diazo paper after $NaDodSO_4$/PAGE as described (26). The blots were stained with goat anti-p30 serum (24) followed by $^{125}I$-labeled, affinity-purified rabbit anti-goat IgG and radioautography using an intensifying screen.

**Distribution of $Pr65^{gag}$ in Subcellular Fractions.** All manipulations were performed at 4°C. Monolayers were rinsed with phosphate-buffered saline (36) and scraped into swelling buffer (10 mM KCl/20 mM Tris·HCl, pH 7.8/1 mM EDTA/0.1% 2-mercaptoethanol/1% Trasylol). After 20 min the cells were homogenized with a tight-fitting glass Dounce homogenizer. Nuclei were pelleted at 1,000 × *g* for 5 min. The supernatant was made 0.3 M NaCl and centrifuged at 100,000 × *g* for 30 min. The nuclear pellet was resuspended in swelling buffer; the suspension was made 0.5% Nonidet P-40/0.5% sodium deoxycholate/0.3 M NaCl and was then recentrifuged at 29,000 × *g* for 15 min. The final nuclear and 100,000 × *g* pellets were dissolved by boiling in gel electrophoresis sample application buffer (containing 1% $NaDodSO_4$) and then electrophoresed. Monoclonal antibody F548 was used to collect $Pr65^{gag}$ from the 100,000 × *g* supernatant ("$S_{100}$") and from the supernatant obtained after detergent treatment of the nuclear pellet, and these immunoprecipitates were also electrophoresed. After electroblotting and immune staining with anti-p30 serum and iodine-labeled rabbit anti-goat IgG, the radioactive bands were located, cut from the diazo paper, and quantitated by $\gamma$ scintillation counting. Adjacent areas of the diazo paper immunoblot not containing radioactive bands were cut and counted for background subtraction. In the case of wild-type MuLV, radioactivity from p30 (representing cell-associated virus) was added to $Pr65^{gag}$ radioactivity. Similar results were obtained in several experiments.

**Electron Microscopy.** Cells were washed three times with Tyrodes buffer; then fixed for 2 min with 3% (vol/vol) glutaraldehyde (Sorenson's buffer) before being scraped off the culture flask with a rubber spatula and centrifuged at low speed (1000 × *g*, 5 min) to pellet the cells. The cells were then fixed for an additional 1 hr, washed eight times in Sorenson's buffer, post-fixed in 1% osmium tetroxide (Millonig's buffer) for 3 min at 4°C, and washed twice in Tyrode's buffer. Dehydration, embedding, sectioning, staining, and transmission electron microscopy of the cell pellet in a Phillips EM 201 and 300 were as described (27).

## RESULTS

**Mutagenesis.** $Pr65^{gag}$ of Moloney MuLV begins with the sequence Met-Gly-Gln (28). Normally, the methionine is removed, and the glycine is then myristylated. We employed oligonucleotide-directed mutagenesis to delete the glycine codon or to convert it to an alanine codon. Mutagenesis was performed, and intact viral genomes containing the mutated sequences were reconstructed in the selectable plasmid vector pSV2Neo as illustrated in Fig. 1.

**Biological Properties of Mutants.** The mutant genomes were first tested for their ability to encode infectious MuLV. Intact plasmids were transfected into NIH 3T3 cells, and transfected cells were selected with G-418 at 400 $\mu$g/ml. Culture fluids from pools of G-418-resistant cells contained no infectious MuLV detectable by the $S^+L^-$ focus assay (20) or the standard UV-XC plaque assay (21) (data not shown), which would detect nondefective MuLVs, or by the complementation plaque assay (21), which would detect replication-defective ecotropic MuLVs (Table 1). However, superinfection of the transfected cells with an XC-negative helper virus (Moloney mink cell focus-inducing virus, M-MCF) resulted in the production of complementation plaque forming units, i.e., particles capable of forming XC plaques in the presence of a helper virus (Table 1). Thus the transfected cells contain a rescuable replication-defective MuLV genome (21) with a functional ecotropic *env* gene (30).

**Synthesis of Unmyristylated $Pr65^{gag}$ in Cells Containing Mutant Genomes.** It was of interest to determine whether the mutants directed the synthesis of $Pr65^{gag}$, and whether the mutant $Pr65^{gag}$ molecules, lacking glycine adjacent to the initiator methionine, would be myristylated. The gag proteins encoded by the mutant genomes were analyzed in both NIH 3T3 mouse cells and in Chinese hamster ovary cells; while the results presented here are principally those obtained in hamster cells, essentially similar results have been obtained in mouse cells. [Mouse cells contain an endogenous, myristylated $Pr65^{gag}$. Where necessary, this background band was eliminated by immunoprecipitation of $Pr65^{gag}$ with a monoclonal anti-p12 antibody, F548 (25).] Pools of G-418-resistant transfected cells were tested for the presence of $Pr65^{gag}$ by labeling with [$^{35}S$]methionine, followed by radioimmunoprecipitation and $NaDodSO_4$/PAGE. As shown in Fig. 2, lanes 7 and 8, [$^{35}S$]methionine-labeled $Pr65^{gag}$ was readily detectable in hamster cells transfected with either of the mutants, as well as in cells containing wild-type Moloney MuLV (lane 11) or a mutant with a deletion in *pol* (lane 9). This $Pr65^{gag}$ was not found in control cells (lane 10).

Parallel cultures were also analyzed after metabolic labeling with [$^3H$]myristate. As shown in lanes 4 and 6, $Pr65^{gag}$ synthesized by wild-type MuLV or the *pol* deletion mutant incorporated radioactive label; however, the mutants lacking the glycine residue at the N terminus of $Pr65^{gag}$ (lanes 2 and 3) did not. These data indicate that the mutants in which the glycine was either eliminated or replaced with alanine, synthesize an unmyristylated $Pr65^{gag}$.

**Mutants Synthesizing Unmyristylated $Pr65^{gag}$ Do Not Produce Virus Particles.** It was important to determine whether the unmyristylated gag polyproteins synthesized by the mutant genomes are assembled into virus particles. This was initially tested by assaying for virion-associated reverse transcriptase activity in culture fluid from transfected mouse cells; as shown in Table 2, the results of these tests were negative, while the fluids from mouse cells producing wild-type virus were ≈1,000 times higher than the background in the assay.

These results strongly suggested that mouse cells containing unmyristylated $Pr65^{gag}$ molecules do not synthesize virus particles. However, interpretation of the data was complicated by the fact that wild-type virus, capable of productively infecting the mouse cells, was expressed at a somewhat higher level than the mutant genomes (data not shown). This difference in expression between genes that enter the cell by infection and those that enter the cell by transfection is consistent with the work of Hwang and Gilboa (31).

This difference in expression was eliminated by the use of hamster cells for comparison of wild-type and mutant genomes. Since these cells contain no receptors for ecotropic MuLVs, wild-type genomes transfected into these cells cannot produce virus capable of productively infecting the cells. Thus the wild-type and mutant genomes should be expressed at comparable levels after transfection into these cells.

Expression of the mutant and wild-type genomes was quantitatively compared in hamster cells following pulse-labeling with [$^{35}S$]methionine. As shown in Fig. 3*A*, the two mutant genomes (lanes 1 and 2) and the wild-type genome

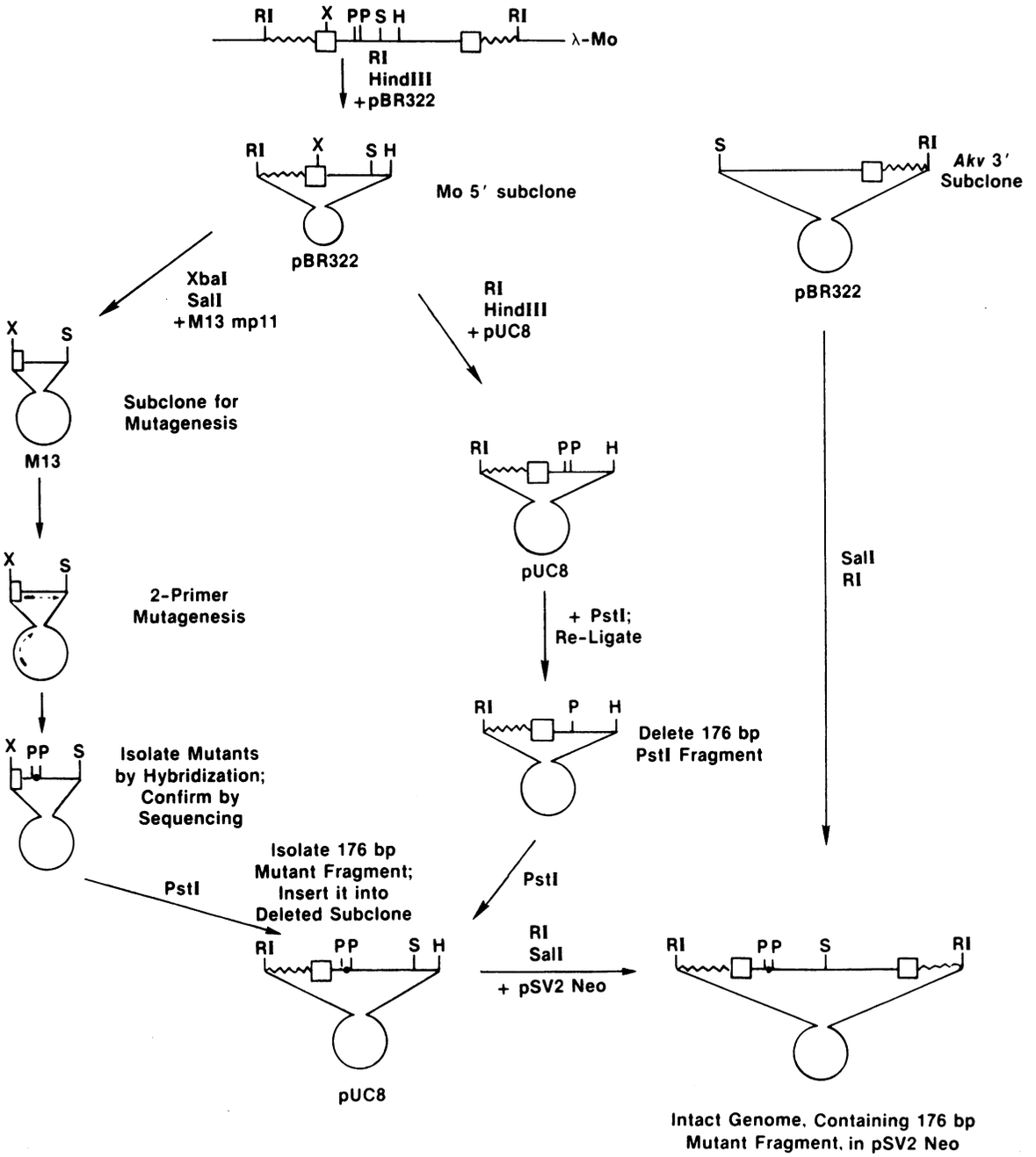


FIG. 1. Scheme for mutagenesis of MuLV and reconstruction of intact viral genomes containing the mutant sequences. A 5′ subclone of Moloney MuLV, from the *Eco*RI site in 5′-flanking sequences to the *Hin*dIII site (nucleotide 4894) (28), was made in pBR322. A smaller subclone, from the *Xba* I site (nucleotide 151) to the *Sal* I site (nucleotide 3705) was made in the M13 vector mp11. Mutants were isolated after two-primer mutagenesis and were analyzed by sequencing. In each case the sequence between the two *Pst* I sites (nucleotides 563 and 739) was determined and was found to be normal except for the desired change. The *Pst* I fragments containing the mutant sequences were then isolated after electrophoresis in low-melting temperature agarose gels. The 5′-*Eco*RI–*Hin*dIII fragment of Moloney MuLV was also inserted in pUC8; the only *Pst* I sites in this subclone are those at nucleotides 563 and 739 in the viral genome. The sequences between these *Pst* I sites were then deleted by digestion with *Pst* I and religation. The *Pst* I fragments containing the mutant sequences were then inserted into the deleted subclone; subclones containing only one *Pst* I insert, in the correct orientation, were identified by restriction mapping. Finally intact viral genomes were reconstructed by combining the mutant fragments, from the 5′-flanking *Eco*RI site to the *Sal* I site (nucleotide 3705), with a 3′ portion of *Akv*, from the corresponding *Sal* I site (*Akv* nucleotide 3720) (29) to the 3′-flanking *Eco*RI site, in the plasmid vector pSV2Neo that had been digested with *Eco*RI.

(lane 3) directed the synthesis of virtually identical levels of $Pr65^{gag}$.

We then examined the transfected hamster cells for virus particle production. As shown in Table 2, no infectious MuLV or particle-associated reverse transcriptase activity could be detected in culture fluids from cells containing the mutant viral genomes, while both activities were present (at relatively low levels) in fluids from cells transfected with the wild-type genome. We estimate that the reverse transcriptase assay could detect 5–10% of the wild-type level of enzyme activity.

The above results suggest that cells containing the mutant genomes do not release virus particles. However, the data do not exclude the possibility that virions lacking active reverse transcriptase are produced. Accordingly, we also tested culture fluids from hamster cells for the presence of virus

Table 1. Presence of replication-defective ecotropic MuLV in NIH 3T3 cells transfected with myristylation-site mutants

| Mutation | Superinfecting virus | CPFU/ml* |
|---|---|---|
| Gly → Ala | None | $<1 \times 10^0$ |
| | M-MCF | $2 \times 10^3$ |
| Gly deletion | None | $<1 \times 10^0$ |
| | M-MCF | $2 \times 10^3$ |

G-418-resistant transfectants were superinfected with M-MCF or were mock-infected. Cell culture supernatants were harvested 4 days later and assayed for complementation plaque-forming units.
*CPFU/ml, complementation plaque forming units/ml of culture supernatant.

particles by immunoblotting virus pellets. The results of these tests are shown in Fig. 3*B*. No p30-related proteins could be detected in pelleted material from cultures with the mutant genomes (lanes 2 and 3), while p30 was easily found in the pelleted material from the culture with the wild-type genome (lane 1). Levels of particle production ≤2% of the wild-type level could have been readily detected in these experiments. These results provide strong evidence that unmyristylated $Pr65^{gag}$ is not assembled into released virus particles.

The cells were also examined in the electron microscope for the presence of virions. No virus particles or recognizable virus-specific structures were seen in 50 cell sections of mouse cells containing the mutant genomes, while 275 particles were observed in the mouse cells with wild-type virus. In the hamster cells, three particles were seen in 50 cells transfected with the wild-type genome, and no virus-specific structures were seen in cells containing the mutant genomes (data not shown). Thus, we find no evidence for assembly of virus-like structures within the cell if $Pr65^{gag}$ molecules are not myristylated.

One trivial explanation for the absence of virus particles in cells containing the mutant genomes would be that unmyristylated $Pr65^{gag}$ is unstable *in vivo*. However, pulse–chase experiments have shown that it is, in fact, much more stable than wild-type $Pr65^{gag}$, with very little loss of radioactivity in a 6-hr chase. In contrast, wild-type $Pr65^{gag}$ is almost entirely processed into p30 during a 2-hr chase (data not shown).

**Unmyristylated $Pr65^{gag}$ Is Not Membrane-Associated.** It

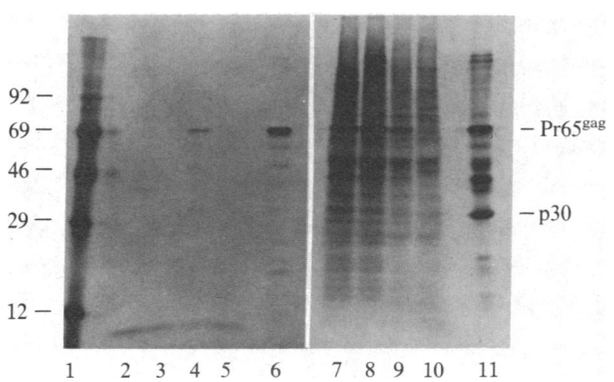


FIG. 2. Synthesis of unmyristylated $Pr65^{gag}$ in hamster cells transfected with myristylation site mutants. Cells were labeled with [$^3$H]myristate (0.5 mCi/ml; 1 Ci = 37 GBq) (lanes 2–6) or [$^{35}$S]methionine (50 Ci/ml) (lanes 7–11) for 1 hr, immunoprecipitated with anti-p30 serum, and analyzed by $NaDodSO_4$/PAGE. Lane 1, molecular size markers (in kDa). G-418-resistant hamster cells were selected following transfection with the following clones in the vector pSV2Neo: lanes 2 and 7, Gly → Ala mutant; lanes 3 and 8, glycine deletion mutant; lanes 4 and 9, Moloney MuLV mutant with 288-base-pair deletion in endonuclease region of *pol* gene (A.R., unpublished results); lanes 5 and 10, pSV2Neo alone. Lanes 6 and 11 contain 1:4 dilutions of extracts of NIH 3T3 cells productively infected with wild-type Moloney MuLV.

Table 2. Lack of virus production by cells containing myristylation site mutants

| Transfected DNA | Cells | RT activity* | Infectivity† |
|---|---|---|---|
| Gly → Ala | Mouse | <0.004 | 0 |
| Gly deletion | Mouse | <0.006 | 0 |
| pSV2Neo | Mouse | <0.004 | 0 |
| MuLV | Mouse | 5 | $1 \times 10^6$ |
| Gly → Ala | Hamster | <0.0015 | 0 |
| Gly deletion | Hamster | <0.0015 | 0 |
| pSV2Neo | Hamster | <0.0015 | 0 |
| MuLV | Hamster | 0.03 | $3 \times 10^3$ |

*RT, reverse transcriptase activity, pmol of [$^3$H]TMP incorporated per ml of culture supernatant.
†Infectivity, focus-inducing units/ml in the $S^+L^-$ focus assay.

seemed possible that the hydrophobic myristate moiety plays a role in the association of wild-type $Pr65^{gag}$ with membranes in the virus-producing cell. We tested this possibility by comparing the distribution of wild-type and mutant $Pr65^{gag}$ molecules in a simple cell fractionation. Mouse cells were lysed and fractionated; the $Pr65^{gag}$ content of individual fractions was then determined by immunoblotting. As shown in Table 3, wild-type and mutant $Pr65^{gag}$s differed dramatically in their distribution: the post-microsomal supernatant contained ≈60–70% of the total $Pr65^{gag}$ in the case of the unmyristylated mutants, but only ≈15% in the case of the wild type.

## DISCUSSION

The gag proteins (Pr65 and p15) of the mammalian type C retroviruses are myristylated at the N-terminal glycine residue (5, 6). We have shown here that when this residue is eliminated, either by deletion or by substitution with alanine, the proteins are not myristylated; further, they neither associate with cellular membranes nor assemble into virions.

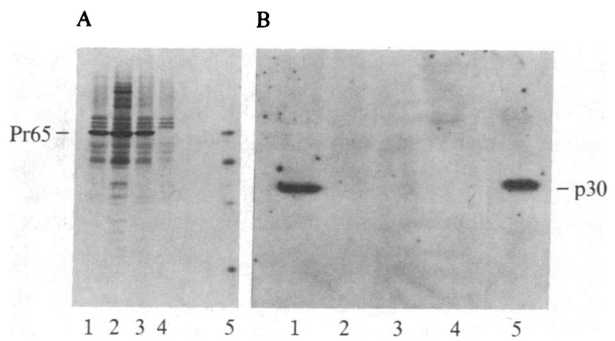


FIG. 3. Rate of synthesis of $Pr65^{gag}$ and lack of detectable virus production in hamster cells transfected with myristylation site mutants. (*A*) G-418-resistant hamster cells were pulse-labeled for 30 min with [$^{35}$S]methionine (50 μCi/ml), and extracts were immunoprecipitated with anti-p30 serum and analyzed by $NaDodSO_4$/PAGE. Cells were transfected with the following clones in the vector pSV2Neo: lane 1, Gly → Ala mutant; lane 2, glycine deletion mutant; lane 3, wild-type Moloney MuLV; lane 4, pSV2Neo alone. Molecular size markers in lane 5 are 69 kDa, 46 kDa, 29 kDa, and 12 kDa. (*B*) Culture fluids were harvested from G-418-resistant hamster cells (lanes 1–4) or from NIH 3T3 cells productively infected with Moloney MuLV (lane 5) and pelleted through 20% (wt/vol) sucrose. The pellets were analyzed by immunoblotting with anti-p30 antiserum. Cells were selected following transfection with the following clones in pSV2Neo: lane 1, wild-type Moloney MuLV; lane 2, Gly → Ala mutant; lane 3, glycine deletion mutant; lane 4, pSV2Neo alone. Lane 5 contains 1/300 of the amount of culture fluid as lanes 1–4.

Table 3. Fraction of mutant and wild-type $Pr65^{gag}$ present in cytosol

| Genotype | % $Pr65^{gag}$ in $S_{100}$ |
|---|---|
| Gly → Ala | 73 |
| Gly deletion | 63 |
| Wild type | 15 |

The amounts of $Pr65^{gag}$ in all four fractions, i.e., final nuclear pellet, detergent extract of initial nuclear pellet, 100,000 × *g* pellet of cytoplasmic fraction, and 100,000 × *g* supernatant of cytoplasmic fraction ($S_{100}$), were determined for each cell line. Table 3 shows the amount in the 100,000 × *g* supernatant as a percentage of the sum of all four fractions.

It seems likely that all of these defects are directly related to each other: we propose that in the wild-type protein, glycine is an essential part of the target sequence recognized by the myristylating enzyme(s), that myristate is required for membrane association of $Pr65^{gag}$, and that membrane association is a necessary prerequisite for virus assembly.

It would not be surprising if an N-terminal glycine residue were required for myristylation in mammalian cells, since all known myristylated eukaryotic proteins have N-terminal glycines (5–11), and since this requirement has already been demonstrated in avian cells for $pp60^{src}$ (12). Nothing else is yet known about the sequence requirements for myristylation in mammalian cells, but in avian cells the N-terminal tetradecapeptide of $pp60^{src}$ has been shown to induce myristic acid addition when fused to heterologous proteins (32). The enzyme(s) responsible for myristylation have not yet been characterized in either cell system.

Extensive studies on mutants of $pp60^{src}$ have shown that the myristate moiety is necessary, but not sufficient, for association with cellular membranes (33). The results reported here indicate that myristate is also essential for membrane association of $Pr65^{gag}$. The fatty acid chain could provide a hydrophobic domain at the N terminus of myristylated proteins, thus facilitating the interaction with the lipid bilayer of the membrane. However, the extreme rarity of myristic acid in lipoproteins raises the possibility that there is a *specific* interaction between the myristylated peptide chain and a cellular component. Further studies will be required to test this idea.

The mechanisms of virus assembly and budding are not yet understood. It seems quite reasonable, however, that localization at a membrane is an essential prerequisite for these events. One effect of membrane association would be to concentrate the $Pr65^{gag}$ molecules by restricting them to the area of the membrane, rather than the volume of the cytoplasm. This concentration effect might be multiplied manyfold if myristylated molecules bind to specific regions of the membrane, as suggested above. In addition, membrane association due to the presence of myristate at the N terminus of $Pr65^{gag}$ would ensure that the $Pr65^{gag}$ molecules are aligned parallel to each other, with their N termini in the membrane and their C termini projecting into the cytoplasm. This configuration is analogous to the arrangement of $pp60^{src}$ molecules in transformed cells (34, 35).

While the present data argue strongly for a critical function of myristate in the assembly of Moloney MuLV, the diversity found among the retroviruses should caution us against overly simplistic models. It is striking that the avian type C viruses, whose assembly processes and mature particles closely resemble those of the mammalian type C viruses in the electron microscope, lack myristic acid. Presumably some other structural feature performs the same function in avian $Pr76^{gag}$ as is performed by myristate in the mammalian gag precursor. Similarly, among the lentiviruses visna virus and equine infectious anemia virus are not myristylated, while the human immunodeficiency virus is (A.M.S., L. E. Henderson, and S. Oroszlan, unpublished data). It should also be noted that some retroviruses, such as mammary tumor virus (type B) and Mason–Pfizer monkey virus (type D), synthesize myristylated gag proteins but assemble within the cell, rather than at the plasma membrane. Thus specific sequences in myristylated proteins must direct these precursors to different sites in the cell.

It seems possible that further mutagenesis experiments, and/or exchange of genetic information between different viruses, will help in answering some of the questions raised here.

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