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A Nucleotide Substitution in the *gag* N Terminus of the Endogenous Ecotropic DBA/2 Virus Prevents Pr65^{gag} Myristylation and Virus Replication

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The endogenous ecotropic provirus *Emv-3* present in DBA/2 mice is poorly expressed in the animal, as well as in cell cultures. Transfection of proviral DNA into NIH 3T3 cells localized the expression defect to the 5′ region of the viral genome, spanning the untranslated region and the N-terminal part of the *gag* gene. Comparison of the nucleotide sequence of the *Emv-3* provirus with the sequence of the highly infectious Akv murine leukemia virus revealed three nucleotide differences within the *gag* coding region. One of these differences was found in codon 3 of the *gag* polyprotein, where a Gln codon is seen in Akv and a Pro codon is seen in *Emv-3*. By site-directed mutagenesis, we showed that the defect of *Emv-3* expression indeed is localized to codon 3 of the *gag* gene. The *gag* polyprotein of mammalian type C retrovirus contains myristic acid covalently linked to the N-terminal glycine. This myristylation is not seen in the *Emv-3*-coded *gag* polyprotein. We showed that the in vitro-mutagenized *Emv-3* genome containing a Gln codon at position 3 of the *gag* gene yields a myristylated *gag* polyprotein. Thus, it seems most likely that the defect of expression of the *Emv-3* provirus is due to the presence of a proline in position 3 of the *gag* polyprotein, preventing the myristylation.

Inbred strains of mice carry in their genome DNA sequences of endogenous ecotropic murine leukemia viruses (MuLVs) (21). The ecotropic MuLVs are produced with variable frequences in different strains, which can be classified as high-virus and low-virus strains (34). The high-virus AKR strain carries the proviral loci *Emv-11* and *Emv-12* and produces high levels of the Akv virus all through the life span of the mice. In the low-virus DBA/2 strain, carrying a single proviral locus, *Emv-3*, virus production was only observed late in the life of the mice. Other mouse strains never express MuLV. These strain differences are also reflected in virus expression after chemical induction (1, 26).

Besides the viral sequence itself, virus expression has been suggested to be under the control of host-coded restriction systems as Fv-1 (47), of *cis*-acting mechanisms residing in the flanking cellular DNA (3), of the chromatin structure (7), and of methylation of the proviral DNA (20). Although the *Emv-3* provirus had a restriction enzyme pattern identical to that of the Akv provirus, results have indicated that the restriction of *Emv-3* virus production originates within the provirus (8). Marker rescue experiments have located the genetic defect to a 1.35-kilobase (kb) 5′ *Sma*I-*Bam*HI nucleotide fragment containing part of the R region, the U5 region, and the untranslated leader region as well as the coding regions for p15^{gag}, pp12^{gag}, and the N terminus of p30^{gag} (10). Sequence analysis showed seven nucleotide differences and a dinucleotide deletion in the *Emv-3 Sma*I-*Bam*HI fragment relative to the Akv virus (10). In the *gag* coding region, three differences were found, of which two would give rise to amino acid changes in the *gag* proteins. One of the changes was localized to codon 3 in p15^{gag}, giving a Gln-to-Pro substitution, whereas the second was located in codon 40 of pp12^{gag}, giving rise to a Leu-to-Pro substitution.

The *gag* gene polyprotein product of mammalian type C retroviruses has been found to be modified by myristylation at the N-terminal glycine (19, 39). It has been suggested that the myristyl group affects the interaction of the *gag* polyproteins with cellular membranes and viral particle formation (35). Copeland et al. (10) reported that the *Emv-3* genome encoded for a nonmyristylated *gag* polyprotein.

In this study, we found evidence that the C nucleotide in position 2 of the proline codon at position 3 of p15^{gag} might be responsible for the defectiveness of *Emv-3* seen in cell culture and in DBA/2 mice. The results also show that the presence of proline in the third amino acid of p15^{gag} prevents the myristylation of the *Emv-3*-coded *gag* polyprotein.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. Strains, phages, and plasmids used are given in Table 1.

Enzymes and reagents. T4 DNA ligase, DNA polymerase I Klenow fragment, DNA polymerase I, exonuclease III, T4 polynucleotide kinase, and restriction enzymes were purchased from Amersham Corp. (Arlington Heights, Ill.), New England BioLabs, Inc. (Beverly, Mass.), and Boehringer Mannheim Biochemicals (Indianapolis, Ind.). The enzymes were used as recommended by the suppliers. [α-^{32}P]dATP (3,000 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, Mass.), TranS^{35}-label (1,027 Ci/mmol) was from ICN Pharmaceuticals, Inc. (Irvine, Calif.), and [^{3}H]dTTP (30 Ci/mmol) and [9,10(*n*)-^{3}H]myristic acid (41 Ci/mmol) were obtained from Amersham. Protein A-Sepharose was obtained from Pharmacia (Uppsala, Sweden), and G418 was from Sigma Chemical Co. (St. Louis, Mo.).

Cell culture media. Newborn calf serum, fetal calf serum, and Dulbecco modified Eagle medium were purchased from Seromed Biochrom Nunc (Roskilde, Denmark). Minimal essential medium without methionine and cysteine was prepared from MEM Select-Amine kit with Earle salts obtained from GIBCO Laboratories (Grand Island, N.Y.). Penicillin-streptomycin (10,000 U:10 mg/ml) was from Biochrom KG.

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TABLE 1. Bacterial strains, bacteriophages, and plasmids

Name	Relevant genotype or DNA inserts	Source or reference
Escherichia coli		
BMH71-18	K-12, Δ(*lac-proAB*) *thi sup*E F′ *lacI*^{q} *lacZM15*, *proA*$^+$*B*$^+$	23
TG1	K-12, Δ(*lac-pro*) *supE thi hsdD5*/F′ *traD36* *proA*$^+$*B*$^+$ *lacI*^{q} *lacZM15*	Amersham
Bacteriophages		
λ-AKR(623)	18-kb *Eco*RI fragment containing a complete Akv proviral genome plus flanking sequences from the murine genome	25
M13mp9		29
Plasmids		
pDBA8.2	8.2-kb *Pst*I fragment of the DBA/2 provirus *Emv-3* (Fig. 1) cloned in pBR322	Nexø
pAKR-59	8.2-kb proviral *Pst*I fragment of AKR(623) cloned in pBR322	24
pSV2*neo*	Simian virus 40 origin and early promoter and the *neo* gene	42

Antiserum. Goat antiserum to purified Rauscher MuLV p30^{gag} was obtained from K. Ulrich, The Fibiger Institute, Copenhagen.

DNA purification. Plasmid DNA and M13 replicative form DNA was purified by the alkali method (4), followed by a CsCl step (28). Restriction enzyme fragments were isolated after electrophoresis in 0.7 or 1.0% agarose gels (Sigma) by using DEAE NA45 paper (Schleicher & Schuell, Inc., Keene, N.H.) (12). Preparation of single-stranded M13 DNA was performed as described by Kramer and Fritz (23). For site-directed mutagenesis, the isolated single-stranded M13 DNA was further purified by fast protein liquid chromatography with a Nucleogen DEAE 4000 column and by the method recommended in the manual from the supplier (Diagen).

Oligonucleotide synthesis. Oligonucleotides were synthesized by the phosphoramidite method on a solid-phase automated DNA synthesizer (Beckmann DNA synthesizer System 1 Plus or Applied Biosystems DNA synthesizer model 381A). The oligonucleotides were purified by fast protein liquid chromatography on a Mono Q HR 5/5 column (Pharmacia) and phosphorylated with T4 polynucleotide kinase.

Oligonucleotide-directed mutagenesis. The 3.1-kb *Pst*I-*Hin*dIII fragment (Fig. 1c) from pDBA8.2 was subcloned into the polylinker site of bacteriophage M13mp9 (29). The fragment contains most of the long terminal repeat (LTR), the 5′ untranslated region, the *gag* gene, and the N-terminal part of the *pol* gene. Site-directed mutagenesis was performed with the recombinant single-stranded M13 DNA by using an oligonucleotide-directed in vitro mutagenesis system (Amersham).

DNA sequencing. Nucleotide sequences were determined by the dideoxynucleotide method (37). Plasmid DNA sequencing was performed as described by Hattori and Sakaki (18). Polyacrylamide gels (0.2 mm, 8%) for the electrophoresis were prepared as described by Garoff and Ansorge (16).

Transfection. DNA transfections were done by a modification of the calcium phosphate method (17). Before transfections, CsCl-prepared plasmid DNA was digested with *Pst*I and ligated to generate concatemers of viral genomes flanked by intact LTRs. Mouse fibroblast NIH 3T3 cells were grown as monolayers in Dulbecco modified Eagle medium containing 10% newborn calf serum and 1% penicillin-streptomycin solution and incubated at 37°C under 5.7% CO_2 and 90% relative humidity. The calcium phosphate-DNA coprecipitates were allowed to stand for 25 min at room temperature before being pipetted onto the semiconfluent cell cultures. Recipient cells were exposed to 15% glycerol–10% newborn calf serum–1% penicillin-streptomycin for 3 min at room temperature 4 to 5 h after DNA treatment. All transfections were done in duplicate.

RT assay. The reverse transcriptase (RT) assay was a modification of the one described by Gallagher and Gallo (14). Medium which had been in contact with the cells for 24 h was cleared for cell debris by centrifugation at 500 × *g* for 10 min in a Heraeus centrifuge. The retroviral particles from 1 ml of cleared supernatant were sedimented at 10,000 × *g* for 60 min in an Eppendorf centrifuge. The pellets were suspended in a volume of 50 μl containing 50 mM Tris hydrochloride (pH 8.0), 0.2 mM $MnCl_2$, 60 mM NaCl, 0.05% Nonidet P-40, 5 μg of oligo(dT) per ml, 10 μg of poly(rA) per ml, 20 mM dithiothreitol, 0.625 μCi [^{3}H]dTTP and 4 μM dTTP. After 60 min of incubation at 37°C, the extent of DNA synthesis was determined by spotting the reaction mixture on DEAE paper. The papers were washed three times in 1 M NaH_2PO_4 and once in 96% ethanol, and the amount of radioactivity incorporated was counted.

Cell labeling and immunoprecipitation. Semiconfluent monolayers of NIH 3T3 cells were cotransfected with 1.2 μg of pSV2*neo* DNA and 6 μg of *Pst*I-digested and ligated DNA from pDBA8.2, pAKR-59, or pE(a3-e40). Transfectants were selected with G418 (750 μg/ml), and the subcultures were analyzed after 6 weeks without further isolation of individual G418-resistant clones. Nearly confluent monolayer cultures in T-25 flasks were labeled for 1 h with either [^{3}H]myristic acid (0.3 mCi/ml) or [^{35}S]methionine-cysteine (60 μCi/ml) (38, 39). At the end of the labeling period, the cells were rinsed with phosphate-buffered saline and lysed. Lysates were clarified by centrifugation at 15,000 × *g* for 1 h, and volumes containing 10^4 cpm of ^{3}H or 10^6 cpm of ^{35}S were immunoprecipitated with goat anti-Rauscher-MuLV p30^{gag} serum, using protein A-Sepharose (40). Washed immunoprecipitates were suspended in sodium dodecyl sulfate (SDS) electrophoresis sample buffer, boiled for 2 min, and subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE). Gels were impregnated with Amplify (Amerham), and the radioactive bands were visualized by fluorography with preflashed Hyperfilm-MP (Amersham). Protein concentrations in the lysates were measured with the bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, Ill.).

Radioactivity in the immunoprecipitated *gag* polyprotein bands was quantified by scanning the fluorograms in a Shimadzu TLC scanner (model CS-930).

RESULTS

Mutagenesis. The endogenous ecotropic provirus *Emv-3* is carried by the inbred DBA/2 mouse strain. The restriction enzyme map of molecularly cloned *Emv-3* DNA was found to be identical to that of the highly infectious Akv virus (9). However, the *Emv-3* provirus is poorly expressed in mice

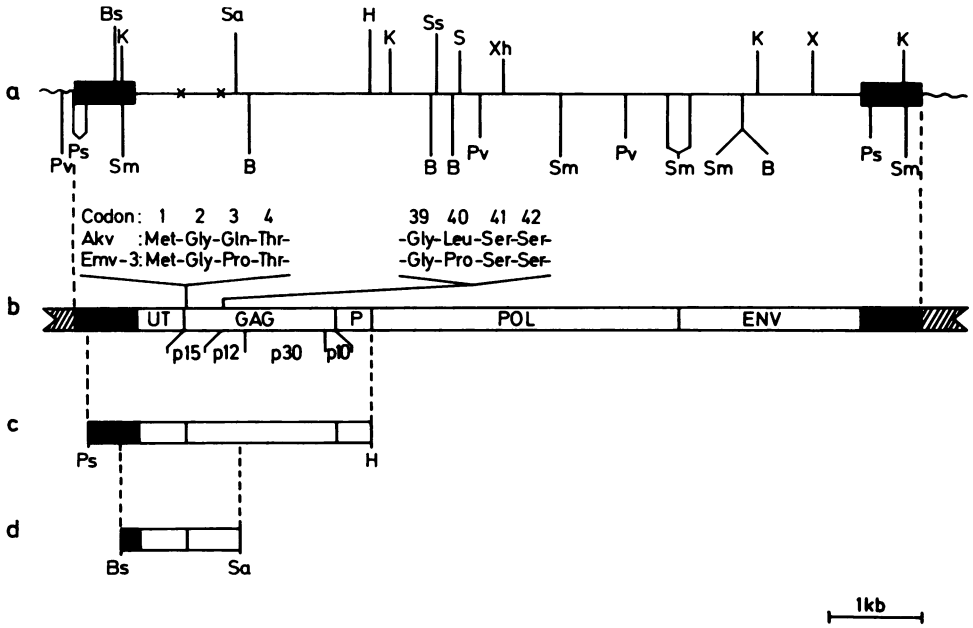

FIG. 1. (a) Restriction enzyme map of *Emv-3* provirus. B, *Bam*HI; H, *Hin*dIII; Pv, *Pvu*II; Sm, *Sma*I; Ps, *Pst*I; K, *Kpn*I; X, *Xba*I; S, *Sal*I; Xh, *Xho*; Bs, *Bss*HII; Sa, *Sac*II. (b) Genome of an ecotropic MuLV provirus. Filled boxes, LTR sequences; hatched boxes, flanking host DNA; UT, the 5′ untranslated sequences between the LTR and the *gag* gene; GAG, group-specific antigens; P, protease gene; POL, RT gene; ENV, envelope gene. The relevant amino acid sequences of Akv and *Emv-3* are indicated above the provirus. The positions of the *gag* proteins are given below the provirus. (c and d) Strategy of in vitro mutagenesis. The 3.1-kb 5′ *Pst*I-*Hin*dIII fragment from pDBA8.2 was subcloned into M13mp9. Oligonucleotide-directed mutagenesis was used to introduce the desired nucleotide substitution in codon 3 of $p15^{gag}$ or codon 40 of $pp12^{gag}$ or in both. After mutagenesis, the modified 1.25-kb *Bss*HII-*Sac*II fragments were subcloned into plasmids pDBA8.2 and pAKR-59, respectively.

(30) as well as in cell cultures (8). Cotransfection and marker rescue experiments localized the region responsible for the poor expression to a 1.35-kb *Sma*I-*Bam*HI fragment at the 5′ end of the *Emv-3* genome (Fig. 1). Comparison of the *Emv-3* DNA sequence with the corresponding sequence of the Akv virus revealed seven nucleotide differences and one dinucleotide deletion in this region (10). Four nucleotide differences and the deletion are localized to the 5′ untranslated region. Three differences are localized within the coding region of the *gag* gene. Of these, one occurs in the third nucleotide position of a Val codon in $p15^{gag}$ and does not result in an amino acid change. The remaining two differences in the N-terminal part of the *gag* coding region (Fig. 1) give rise to amino acid substitutions in the *Emv-3 gag* proteins as compared with the Akv *gag* proteins. The A-to-C transversion in codon 3 of $p15^{gag}$ results in a glutamine-to-proline substitution, while the T-to-C transition in codon 40 of $pp12^{gag}$ leads to a leucine-to-proline substitution. To test whether any of these amino acid changes might be the cause of the restricted expression of the *Emv-3* provirus, we carried out oligonucleotide-directed mutagenesis to convert the *Emv-3* codons to those of the Akv virus.

TABLE 2. Plasmids used for transfection studies

Plasmid[a]	Vector	5′ *Bss*HII-*Sac*II insert[b]	Codon 3 of $p15^{gag}$[c]	Codon 40 of $pp12^{gag}$[c]
pE(Emv)	pDBA8.2	Emv-3	e	e
pE(a3-e40)	pDBA8.2	Iv mut	a	e
pE(e3-a40)	pDBA8.2	Iv mut	e	a
pE(a3-a40)	pDBA8.2	Iv mut	a	a
pE(Akv)	pDBA8.2	Akv	a	a
pA(Emv)	pAKR-59	Emv-3	e	e
pA(a3-e40)	pAKR-59	Iv mut	a	e
pA(e3-a40)	pAKR-59	Iv mut	e	a
pA(a3-a40)	pAKR-59	Iv mut	a	a
pA(Akv)	pAKR-59	Akv	a	a

[a] For plasmid construction, the 1.25-kb 5′ *Bss*HII-*Sac*II DNA fragments were excised either from the in vitro mutagenized replicative form of M13 clones or from nonmutated pDBA8.2 and pAKR-59. The fragments were reinserted into either pDBA8.2 (pE series) or pAKR-59 (pA series).
[b] Iv mut, In vitro mutagenized.
[c] e, Codons from *Emv-3*; a, codons from Akv.

In vitro mutagenesis was performed on the 3.1-kb *Pst*I-*Hin*dIII DNA fragment (Fig. 1c) from the plasmid pDBA8.2 subcloned into bacteriophage M13mp9. Oligodeoxynucleotides of 40 and 44 nucleotides with sequences corresponding to nucleotides 622 to 661 and 1118 to 1161 in Fig. 2 in Copeland et al. (10) were used to introduce the required single-nucleotide replacements. Three different revertants were constructed, one with a C-to-A replacement in codon 3 of $p15^{gag}$, another with a C-to-T replacement in codon 40 of $pp12^{gag}$, and the third having both nucleotide replacements. The sequences of the constructed mutants were verified by DNA sequencing of the single-stranded M13 DNA (37).

After the in vitro mutagenesis, the 1.25-kb *Bss*HII-*Sac*II fragments (Fig. 1d) were excised from the replicative form DNA of the M13 clones and used to substitute the corresponding fragment in the plasmid pDBA8.2 (Table 2). To test whether the mutated fragments had similar effects on Akv and *Emv-3* expression, we also inserted the fragments into the plasmid pAKR-59 (Table 2). As controls, the nonmutated *Bss*HII-*Sac*II fragments of pDBA8.2 and pAKR-59 were cross-cloned into pAKR-59 and pDBA8.2.

TABLE 3. Infectivity of different viral genomic DNAs assayed by RT

DNA[a]	RT activity in culture fluid (10^4 cpm/ml)[b]		
	7 days	15 days	20 days
Blank	<0.02	<0.02	<0.02
Carrier DNA[c]	<0.02	<0.02	<0.02
pDBA8.2	<0.02	<0.02	<0.02
pAKR59	3.5	3.0	5.5
pE(Emv)	<0.02	<0.02	<0.02
pA(Emv)	<0.02	<0.02	<0.02
pE(a3-e40)	3.1	3.5	5.5
pA(a3-e40)	3.4	2.7	4.6
pE(e3-a40)	<0.02	<0.02	<0.02
pA(e3-a40)	<0.02	<0.02	<0.02
pE(a3-a40)	1.4	3.1	5.4
pA(a3-a40)	3.5	3.6	5.5
pE(Akv)	3.2	3.1	6.3
pA(Akv)	2.0	3.3	5.3
λ-AKR(623)	4.5	3.0	5.2

[a] The DNAs were transfected into NIH 3T3 cells. To generate viral genomes flanked by intact LTR sequences prior to transfection, the DNA of the plasmids indicated was digested with *Pst*I and the DNA fragments were ligated. In λ-AKR(623), the viral genome is flanked by two complete LTR sequences.

[b] As a measure of the production of viral particles, the RT activity in the culture medium was determined at the day indicated.

[c] Salmon sperm DNA was included as carrier DNA and as a negative control.

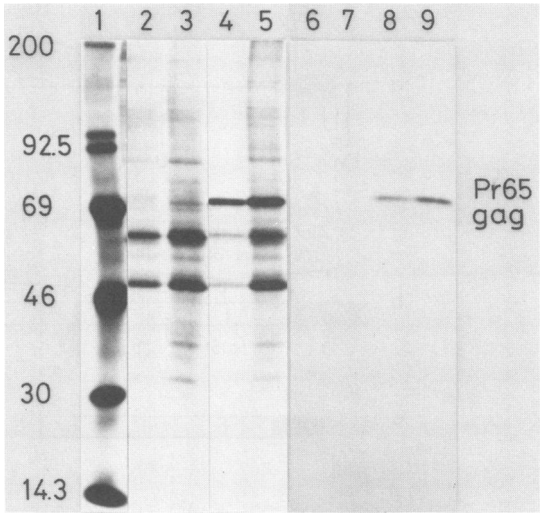

FIG. 2. SDS-PAGE analysis of *gag* polyproteins encoded by the *Emv-3*, E(a3-e40), and Akv genomes. NIH 3T3 fibroblast cells were cotransfected with DNA from pSV2*neo* and pDBA8.2, pE(a3-e40), or pAKR-59 or with pSV2*neo* alone. To generate intact proviral DNA sequences, we treated the plasmid DNAs as described in Table 3, footnote *a*. Pools of transfected G418-resistant cells were labeled for 1 h with [^{35}S]methionine-cysteine (lanes 2 through 5) or with [^{3}H]myristic acid (lanes 6 through 9). Cells were lysed and immunoprecipitated with antiserum to the p30^{gag} component of the *gag* polyprotein, followed by SDS-PAGE and fluorography. The films were exposed for 28 days (^{35}S) and 63 days (^{3}H). Lane 1, Radioactive size markers: myosin, 200,000; phosphorylase *b*, 92,500; bovine serum albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; lysozyme, 14,300. Lanes 2 and 6, pSV2*neo* control; lanes 3 and 7, pDBA8.2; lanes 4 and 8, pE(a3-e40); lanes 5 and 9, pAKR-59.

The structure of the plasmid reconstructions and the presence of the nucleotide replacements were verified by endonuclease digestion and sequencing analysis.

Infectivity of mutants. To determine the effect of these nucleotide replacements on virus expression, we transfected mouse fibroblast NIH 3T3 cell cultures with DNA from each of the mutated plasmids in parallel with DNA from pDBA8.2 and pAKR-59. In pDBA8.2 and pAKR-59 and in the derived plasmids, the LTR sequences are present in a permuted form. Prior to transfection, the plasmids were digested with *Pst*I and concatemers were formed by ligation to generate viral genomes flanked by complete LTR regions. The release of viral particles was tested 7, 15, and 20 days after transfection by assays for virion-associated RT activity in the culture fluid from the transfected cells. The infectious genomic clone λ-AKR(623), containing a complete nonpermuted proviral Akv genome, was used as a positive transfection control. Salmon sperm DNA was included as a carrier and as a negative control. The results of the transfection experiments are given in Table 3.

As described earlier, the *Emv-3* DNA was poorly infectious in NIH 3T3 cells (8). In cells transfected with pAKR-59 DNA, an increase in RT activity was observed 7 days after transfection. The activity measured was similar to that observed for λ-AKR(623), indicating that the manipulations of the plasmid DNA did not greatly influence the infectivity of the DNA. Cell cultures transfected with DNA from the control plasmid constructions pE(Emv) and pA(Emv) and pE(Akv) and pA(Akv) gave RT values identical to those for pDBA8.2 and pAKR-59, respectively (Table 3). DNA from the two mutant plasmids pE(a3-e40) and pA(a3-e40) yielded transfected cell cultures with RT values as high as those for pAKR-59 and λ-AKR(623). In contrast, transfection with DNA from pE(e3-a40) and pA(e3-a40) did not result in the production of RT-containing particles.

Together, these results suggest that the proline in position 3 of p15^{gag} is involved in the defectiveness of *Emv-3* expression, whereas the proline in position 40 in pp12^{gag} has no obvious deleterious effect.

Myristylation of *gag* polyproteins. The N-terminal amino acid sequence of the *gag* polyprotein of Akv has been identified as Met-Gly-Gln-Thr-Val- (13). The methionine is subsequently removed, followed by myristylation of the N-terminal glycine (19, 39). Copeland et al. (10) have shown a lack of myristylation of the *Emv-3*-coded *gag* polyprotein with the N-terminal sequence Met-Gly-Pro-Thr-Val-. Therefore, it was of interest to determine whether a change of Pro to Gln in codon 3 of p15^{gag} of *Emv-3* would lead to the production of a myristylated *gag* polyprotein. NIH 3T3 fibroblast cells were cotransfected with pSV2*neo* DNA and *Pst*I-digested and ligated DNA from pDBA8.2, pAKR-59, or pE(a3-e40). After 4 weeks, the culture fluids from G418-resistant cells were assayed for production of RT containing viral particles to confirm a successful transfection.

After another 2 weeks, subcultures were tested for the presence of *gag* polyproteins and myristylated *gag* polyproteins by labeling with [^{35}S]methionine-cysteine (60 μCi/ml) or [^{3}H]myristic acid (300 μCi/ml) for 1 h. The cells were lysed, and immunoprecipitated material was analyzed by SDS-PAGE and fluorography (Fig. 2). In cell cultures in which no virus was produced, only those cells which received the proviral gene during transfection (or descendants from such cells) produced the *gag* polyprotein.

A [^{35}S]methionine-cysteine-labeled *gag* polyprotein was detectable at the same level (Table 4) in NIH 3T3 cells transfected with DNA from either pE(a3-e49) or pAKR-59 (Fig. 2, lanes 3 and 4). The *gag* polyprotein was present in

TABLE 4. Quantitation of incorporation of myristic acid into the *gag* precursor[a]

Plasmid	[^{3}H] myristic acid	[^{35}S] methionine-cysteine
pSV2*neo*	<1	NP[b]
pDBA8.2	<1	11
pE(a3-e40)	82	99
pAKR-59	100	100

[a] The plasmid indicated was transfected into NIH 3T3 cells and the *gag* polyprotein was analyzed by SDS-PAGE (Fig. 3). The X-ray film was scanned. The scanning data were normalized to the data of pAKR-59, which was set to 100.

[b] NP, No peak detected.

smaller amounts in cell cultures transfected with DNA from pDBA8.2 (Fig. 2, lane 3) (Table 4). In parallel cultures labeled with [^{3}H]myristic acid (Fig. 2, lanes 6, 7, 8, and 9), the *gag* polyprotein of cells transfected with DNA from pE(a3-e40) and pAKR-59 had incorporated radioactive label (lanes 8 and 9), whereas no radioactivity was observed at the position of the *gag* polyprotein in cells transfected with pDBA8.2 DNA (lane 7). Control cell cultures did not show the presence of any radioactivity at the location of the *gag* polyprotein after either [^{35}S]methionine-cysteine or [^{3}H]myristic acid labeling (Fig. 2, lanes 2 and 6).

An approximate quantitation of the synthesized *gag* polyproteins was obtained by scanning the fluorograms, which were all exposed within the region of proportionality of the films. The incorporation of ^{3}H and ^{35}S into the *gag* polyproteins was estimated (Table 4).

These results confirm that the *gag* polyproteins with a proline in position 3 of p15^{gag} are nonmyristylated and that a substitution by in vitro mutagenesis of this amino acid with glutamine restores the myristylation of the *Emv-3*-derived *gag* polyprotein.

DISCUSSION

The endogenous ecotropic provirus *Emv-3* carried in the genome of the DBA/2 mouse strain is poorly expressed in the mice (30). Similarly, the cloned *Emv-3* proviral DNA is poorly expressed when transfected into NIH 3T3 fibroblasts (8). The defect causing the poor expression was localized to the 1.35-kb *Sma*I-*Bam*HI DNA fragment at the 5′ end of the *Emv-3* genome (Fig. 1). The sequence of 1,400 base pairs covering the R, the U5, and the 5′ untranslated region as well as part of the *gag* gene has recently been determined (10). When this nucleotide sequence was compared with the corresponding sequence of the highly infectious Akv MuLV, a number of nucleotide differences were noted (10). Four nucleotide differences and a dinucleotide deletion were seen in the 5′ untranslated region and three nucleotide differences were found in the N-terminal-coding region of the *gag* gene. Only two of these changes, in codon 3 of p15^{gag} and in codon 40 of pp12^{gag}, would cause an amino acid substitution in the *gag* gene product of the *Emv-3* provirus compared with the Akv *gag* polyprotein.

To verify which of these nucleotide changes was responsible for the differences in virus production, we performed site-directed mutagenesis to change the two *gag* codons of the *Emv-3*-derived plasmid pDBA8.2 to those of the Akv virus. Transfection experiments showed that only plasmid constructs with a changed codon 3 of p15^{gag} restored virus expression. In these constructs, the proline codon of *Emv-3* was replaced the glutamine codon of Akv virus.

The amino acid variation of codon 40 of pp12^{gag}, in which a Leu codon is present in Akv and a Pro codon is present in *Emv-3*, does not seem to influence the viral expression [Table 3, pE(a3-e40), pE(a3-a40)]. It might also be inferred from our results that the other nucleotide differences which have been shown to exist between *Emv-3* and Akv do not have any major influence on virus production.

The *gag* polyprotein of MuLV has been shown to be myristylated (41). However, the *gag* polyprotein coded by *Emv-3* provirus is not myristylated (10). During assembly of the type C retroviruses, the *gag* polyprotein is located at the plasma membrane of the host cell (11). Although the function of the myristylation is not understood, attachment of the hydrophobic myristyl group to the N-terminal glycine may promote interaction of the *gag* polyprotein with the cellular membranes and thereby promote the formation of the viral particles.

To test whether the proline in the third amino acid position of p15^{gag} of *Emv-3* might interfere with the process of myristylation, we did labeling experiments with [^{35}S]methionine-cysteine or with [^{3}H]myristic acid with NIH 3T3 cells transfected with either *Emv-3* DNA (pDBA8.2) or with DNA from plasmid pE(a3-a40). Only the proviral genomes coding for glutamine in position 3 of p15^{gag} yielded a myristylated *gag* polyprotein (Fig. 2; Table 4).

Towler et al. (44, 45) have analyzed in vitro the removal of the initiator methionine and the myristylation of the N-terminal glycine using synthetic octapeptides as substrates for yeast *N*-myristyl transferase (NMT) purified to various degrees. It was demonstrated that the first six amino acids were important for the interaction with NMT (44). Furthermore, NMT showed an absolute requirement for an N-terminal glycine (45). Besides the presence of glycine at the second position, there is no obvious sequence homology between the amino acid termini of N-myristylated proteins (32, 43). In contrast, not all proteins with mature N-terminal glycine residues are myristylated (46).

In vivo, the first 10 amino acids of Rous sarcoma virus transforming protein p60^{src} were found to be required for efficient myristylation (33). It has been shown that mutagenesis or deletion of the glycine residue blocks myristylation and membrane association of the *gag* polyprotein of type C and type D retrovirus (35, 36) and of p60^{src} (22).

The process of myristylation occurs cotranslationally before peptide synthesis is completed (41, 48). It requires an aminopeptidase for the removal of the initiator methionine and NMT for the coupling of myristic acid to the glycine (15, 44).

Our results do not permit us to decide whether the absence of myristylation is caused by a deficiency in the removal of the methionine residue or by a change in the recognition site of the NMT. However, in studies with the octapeptide Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg, which is normally myristylated, it was shown that the replacement of Asn with Pro gave a peptide which was neither myristylated nor bound to NMT (44). This suggests that a Pro in the position next to the Gly to be myristylated, as found in the *gag* precursor of *Emv-3*, interferes with the affinity of the NMT, most likely owing to an incorrect presentation of the N-terminal Gly. Supposing that the initiator Met residue of the *Emv-3 gag* precursor is removed, this offers an explanation of the defective phenotype of the *Emv-3* provirus.

Although most of the acetylated proteins in the mammalian cells are membrane associated (27, 31, 49), not all known myristylated proteins are membrane bound (2, 5, 6, 32). For mammalian retroviruses, *gag* polyprotein myristylation has

been shown to be required for membrane association and virus particle formation (35, 36), suggesting that the myristylation is essential for transport to and association with other viral proteins at special locations on the cell membrane.

We mapped the genetic defect of the *Emv-3* locus of DBA/2 virus to codon 3 of the precursor of the *gag* proteins. Substitution of the C in the second position of this codon with the A detected in the replication-competent Akv made it possible for the sequence to produce replication-competent retrovirus upon transfection into NIH 3T3 fibroblasts. In virus genomes, a nucleotide sequence often harbors information for more than one function. The region of the mapped mutation carries information for the *gag* gene. Our results do not permit us to exclude the possibility that the defect of virus expression of the *Emv-3* provirus is due to an altered structure of the *Emv-3* viral RNA caused by the presence of the C nucleotide located in position 2 of codon 3 of the *gag* gene. However, we presented evidence that the defective pattern of myristylation of the *gag* polyprotein in *Emv-3* was restored when the C was replaced with an A.

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