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Poorly Expressed Endogenous Ecotropic Provirus of DBA/2 Mice Encodes a Mutant Pr65^{gag} Protein That Is Not Myristylated

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DBA/2 mice carry a single endogenous ecotropic murine leukemia provirus designated *Emv-3*. Although this provirus appears to be nondefective by genomic restriction enzyme mapping, weanling mice do not produce virus and only about one-third of adult mice ever express virus. 5-Iododeoxyuridine and 5-azacytidine, two potent inducers of ecotropic virus expression, are relatively ineffective at inducing *Emv-3* expression. However, the chemical carcinogen 7,12-dimethylbenz(*a*)anthracene can induce ecotropic virus expression in approximately 95% of treated DBA/2 mice. Previous experiments involving DNA transfection and marker rescue analysis of molecularly cloned *Emv-3* DNA suggested that *Emv-3* carries a small defect(s) in the *gag* gene, not detectable by restriction enzyme mapping, that inhibits virus expression in vivo and in vitro. Using a combination of approaches, including DNA sequencing, peptide mapping, and metabolic labeling of cells with [^{3}H]myristate, we have demonstrated that the defect in *Emv-3* most likely results from a single nucleotide substitution within the gene for p15^{gag} that inhibits myristylation of the Pr65^{gag} N terminus. Myristylation of Pr65^{gag} is thought to be required for this protein to associate with the plasma membrane and is essential for virus particle formation. These results provide a conceptual framework for understanding how *Emv-3* expression is regulated during development and after chemical induction.

Most inbred mouse strains carry ecotropic murine leukemia virus (MuLV) DNA sequences that were presumably acquired by germ line infection and are now transmitted in a normal Mendelian fashion (for a review, see reference 6). These endogenous retroviruses have been studied extensively because of both their ability to infect and replicate in mouse cells and their causal role in the induction of lymphomas.

The expression of endogenous ecotropic MuLVs varies considerably among inbred strains: some strains express ecotropic virus early in life, and other strains express virus only late in life or never at all (6). Differences in expression among strains are also seen in vitro and after chemical induction (1, 2, 19, 31). The variability in MuLV expression is the consequence of both *trans*- and *cis*-acting regulatory factors, and the elucidation of these factors has provided an important model system for the study of eucaryotic gene expression. The major *trans*-acting locus affecting ecotropic virus expression is the autosomal, dominant *Fv-1* locus (40, 41, 45). A number of *cis*-acting factors have been postulated to regulate virus replication, including proviral DNA methylation (10, 18, 22, 26, 38, 54), cellular control sequences linked to the viral integration site (3), chromatin structure (11), and mutations within the proviral DNA sequences themselves (12, 24, 47).

To determine which of these control mechanisms regulate ecotropic virus expression, we have begun to molecularly clone and characterize some of these proviral loci. One provirus under study is *Emv-3* (endogenous ecotropic proviral locus 3). The integration of this provirus is causally associated with the dilute (*d*) coat color mutation and has been identified in all strains of mice carrying the mutant *d* allele (27). Although this provirus appears nondefective by genomic restriction mapping, it seldom produces infectious virus in vivo. For example, DBA/2 mice that carry *Emv-3* as their sole endogenous ecotropic provirus do not express detectable ecotropic virus until about 5 months of age, and then only about one-third of adult mice will ever express virus. Furthermore, 5-iododeoxyuridine and 5-azacytidine, two potent inducers of ecotropic virus expression, are relatively ineffective in inducing *Emv-3* expression in vitro (unpublished results). However, repeated percutaneous application of 7,12-dimethylbenz(*a*)anthracene (DMBA) on weanling DBA/2 mice can induce ecotropic virus expression in approximately 95% of treated animals (36). Increased concentrations of the viral antigen p30^{gag} can be detected in the blood as early as 1 week after DMBA treatment has started.

Ecotropic viruses isolated from both normal and DMBA-treated DBA/2 mice can be classified into three major groups, E_a, E_b, and E_c, that differ in protein structure and pathogenicity (35, 37, 57). Two of the groups, E_a and E_b, are composed of N-tropic viruses and are closely related to the prototypic endogenous ecotropic viruses of AKR mice. Both groups of viruses have similar envelope (*env*) gene-encoded proteins, but differ in the fine structure of the viral core (*gag*) gene products.

E_a viruses have been isolated by cocultivating DBA/2 spleen cells from animals exhibiting little or no overt viremia with SC-1 cells, a cell line permissive for replication of most ecotropic viruses. E_a viruses have an ecotropic *env* gene but carry xenotropic sequences within *gag*. E_a viruses replicate poorly in cell lines derived from strains such as C3H and do not produce detectable viremia when injected into C3H mice. E_a viruses are nonpathogenic. Substitution of xenotropic sequences within *gag* thus appears to generate viruses that are less virulent than the prototypic ecotropic virus and which grow poorly in some cells.

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E_b viruses are regularly isolated from viremic adult animals and from young animals treated with DMBA. E_b viruses are closely related to the prototypic endogenous ecotropic viruses of AKR mice. E_b viruses induce moderate levels of viremia in susceptible hosts and are pathogenic after long latency periods. The increased in vivo growth advantage of E_b viruses relative to E_a viruses probably accounts for their frequent isolation from viremic adult mice.

E_c viruses have been isolated only infrequently from DBA/2 mice. E_c viruses are NB-tropic and differ extensively from ecotropic viruses in both *gag* and *env*. The origin of these isolates is unclear. E_c viruses induce the highest levels of viremia in susceptible hosts and are pathogenic, with short latency periods.

DNA transfection and marker rescue experiments with molecularly cloned *Emv-3* DNA have suggested that *Emv-3* expression is not regulated by linkage to *cis*-acting cellular DNA sequences or by DNA methylation (12). Instead, *Emv-3* appears to carry a small mutation(s) within *gag* that is responsible for the lack of replication of this virus in vivo and in vitro. The nature of the replication defect in *Emv-3* has now been identified by a combination of approaches, including DNA sequencing, peptide mapping, and in vitro metabolic labeling studies.

MATERIALS AND METHODS

DNA sequencing. Restriction enzymes were from New England BioLabs or Boehringer Mannheim. T4 DNA ligase and Bal31 exonuclease were from New England BioLabs. The Klenow fragment of *Escherichia coli* polymerase was from Boehringer Mannheim. [^{32}P]dATP was from Amersham (2,000 Ci/mmol). Plasmid p.*Emv-3* (12) was prepared by standard procedures (32). The M13 DNA sequencing strategy was performed as described previously (42, 46). Single-stranded M13 DNA was prepared as described previously (34). Acrylamide gels for sequencing were prepared by the method of Garoff and Ansorge (16).

Peptide mapping of viral proteins. Viral proteins in cells and virions were labeled by published procedures (37, 58). Preparation of cell extracts, immunoprecipitation, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and digestion of gel pieces containing electrophoretically purified proteins with diphenylcarbamylchloride-treated trypsin were performed as described previously (37). [^{3}H]lysine-labeled p15^{gag} from virions was purified by two-dimensional PAGE rather than immunoprecipitation, followed by ordinary SDS-PAGE. Chromatographic separation of peptides on thin-layer plates followed the procedure of Krantz et al. (29), except that the solvent used in the second dimension was modified (1-butanol–pyridine–acetic acid–water, 13:8:2:8). Kodak XAR films were exposed at −60°C with a fluorophor (En^3 Hance; New England Nuclear Corp.).

DNA transfection, cell labeling, immunoprecipitation, and SDS-PAGE. NIH 3T3 cells were cotransfected with 6 μg of p.*Emv-3* DNA and 1 μg of pSV2neo DNA (53) by calcium phosphate precipitation as described previously (9). Transfectants were selected in G-418 (400 μg/ml; Schering) and grown and analyzed in mass culture, without isolation of individual G-418-resistant clones.

Confluent monolayer cultures in T75 flasks were labeled for 1 h with either [^{3}H]myristic acid (1 mCi/ml) or [^{35}S] methionine (25 μCi/ml) as described previously (48, 49). Cell extracts were immunoprecipitated with monospecific antiserum to p30^{gag} of MuLV and protein A-sepharose, and the washed immunoprecipitates were separated by SDS-PAGE as described previously (51). Radioactive proteins were detected by fluorography (30).

RESULTS AND DISCUSSION

DNA transfection and marker rescue assays suggest that *Emv-3* is defective in the *gag* region. Although *Emv-3* DNA is poorly infectious in DNA transfection assays [>10^5-fold less infectious than AKR(623) DNA], it can nonetheless produce infectious virus (12). However, virus appears only 3 to 4 weeks and several subcultures after transfection and most likely represents the rare spontaneous production of a nondefective revertant virus via mutation or via recombination with NIH 3T3 endogenous proviral sequences. The recovered viruses have been designated N(*Emv-3*) viruses.

The location of the replication defect(s) in *Emv-3* was determined by marker rescue. Briefly, several subgenomic fragments of wild-type infectious AKR(623) DNA were isolated after digestion with *Kpn*I. *Kpn*I produces three internal viral DNA fragments of 4.0, 2.8, and 1.4 kilobases (kb) (Fig. 1). These fragments were mixed with *Emv-3* DNA, and the specific infectivity of the mixtures was assayed by transfection. The specific infectivity of *Emv-3* DNA was significantly increased by the addition of the 2.8-kb fragment but not by the 4.0- or 1.4-kb fragment (12). The viruses generated by marker rescue assay have been designated N[*Emv-3* + AKR(623)] viruses.

Subsequent transfection experiments (data not shown) further localized the defective region to a 1,346-nucleotide *Sma*I-*Bam*HI fragment located at the extreme 5′ end of *Emv-3* (Fig. 1). This fragment includes part of R and all of U5 from the 5′ long terminal repeat as well as the *gag* N terminus. The *gag* gene is translated as a polyprotein of 65 kilodaltons, designated Pr65^{gag}. This polyprotein is cleaved within the virion to yield the viral core proteins p15^{gag}, pp12^{gag}, p30^{gag}, and p10^{gag} (listed in the order they are encoded). The *Sma*I-*Bam*HI fragment contained p15^{gag}, pp12^{gag}, and the N terminus of p30^{gag} (Fig. 2).

Nucleotide sequence of the region defective in *Emv-3*. To more precisely localize the defect(s) in *Emv-3*, we sequenced the 1,346-nucleotide *Sma*I-*Bam*HI fragment and compared it with the corresponding sequence of AKR(623) (Fig. 2). Very few sequence differences between *Emv-3* and AKR(623) were observed in this region; *Emv-3* and AKR(623) differed by only seven nucleotide substitutions and a single TG dinucleotide deletion. Limited sequence variation between another ecotropic provirus, *Emv-1*, and AKR(623) has also been reported (24), consistent with the hypothesis that endogenous ecotropic proviruses were recently acquired by mice and that few cycles of reverse transcription separate these viral genomes. *Emv-1* encodes a replication-competent virus and, like *Emv-3*, encodes T's at positions 129 and 333.

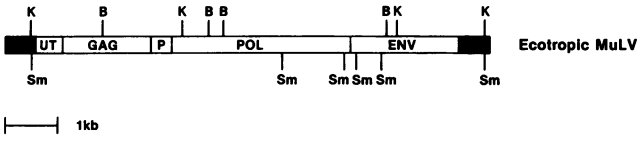

FIG. 1. Restriction enzyme map of AKR(623). The provirus is drawn 5′ to 3′ with respect to viral RNA. Solid blocks represent the viral long terminal repeat sequences. The approximate boundary for the 5′ untranslated sequences (UT), group-specific antigens (GAG), protease (P), polymerase (POL), and envelope (ENV) regions are indicated. Cleavage sites are denoted as follows: Sm, *Sma*I; B, *Bam*HI; K,*Kpn*I.

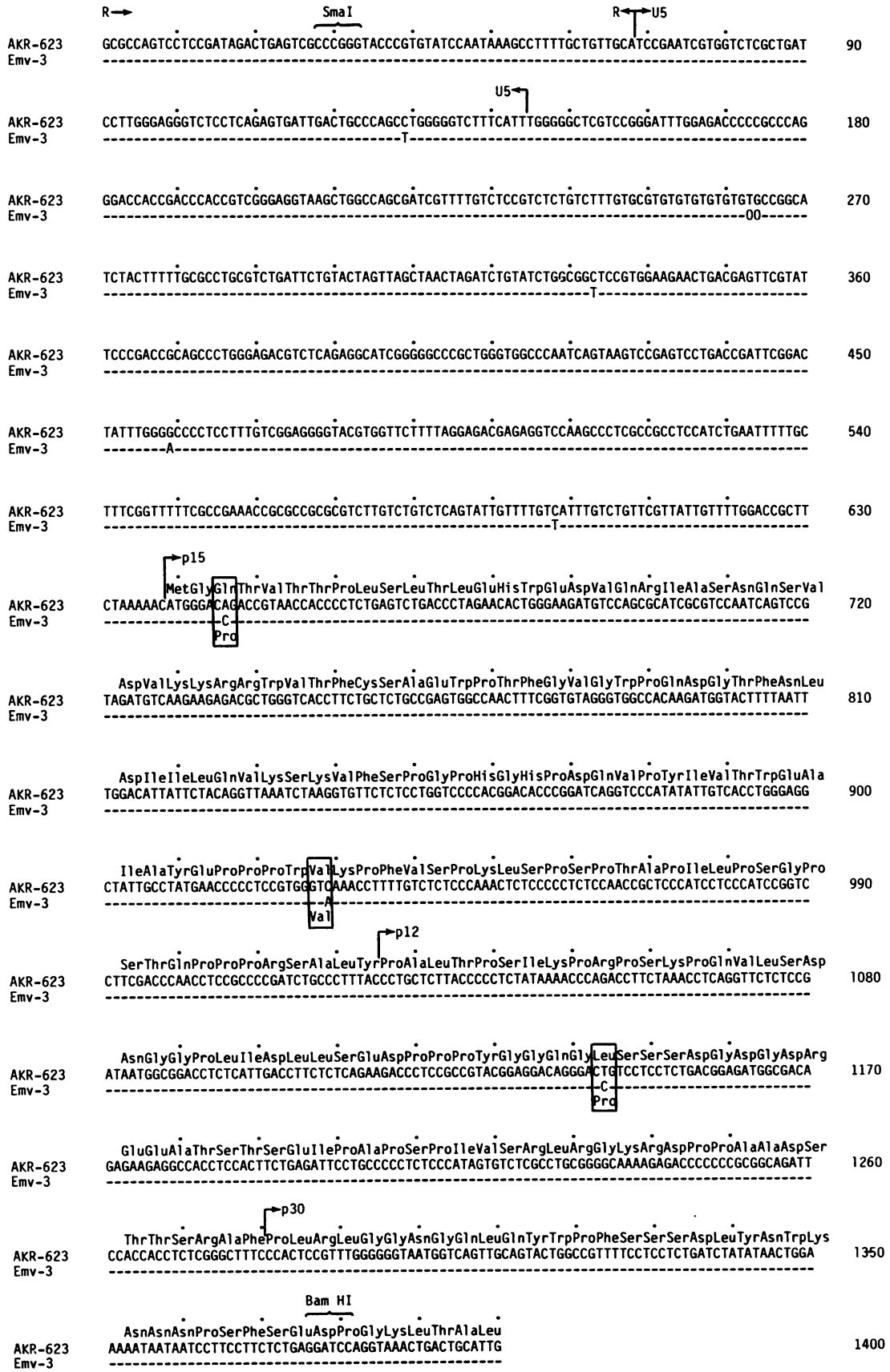

FIG. 2. DNA sequence of the region defective in *Emv-3*. The sequence of the 1,346-nucleotide *Sma*I-*Bam*HI fragment from the 5′ end of p.*Emv-3* (Fig. 1) was determined as described in the text. This sequence is compared with that of the corresponding region in AKR(623) (15, 21), with homologous regions shown with a dotted underline. Seven nucleotide substitutions and one TG deletion (OO) were identified.

Emv-1 also appears to be missing one TG dinucleotide pair relative to AKR(623) (28). The combined data suggest that these changes are not responsible for the poor replication of *Emv-3* and that the defect in *Emv-3* is in one or more of five nucleotide substitutions. Two of these nucleotide substitutions (positions 459 and 598) were, like the changes described above, in the 5′ untranslated region and unlikely to affect *Emv-3* expression. The other three nucleotide substitutions occurred within the *gag* coding region. The C to A transversion at nucleotide 929 was silent. An A to C transversion in p15^{gag} resulted in a Gln to Pro substitution, whereas a T to C transition in pp12^{gag} led to a Leu to Pro substitution. Either or both of these amino acid changes could render *Emv-3* defective.

Tryptic peptide analysis of viral *gag* proteins. We performed tryptic peptide analyses of the *gag* proteins encoded by viruses isolated in transfection and marker rescue assays of *Emv-3* DNA to further localize the defective region. The peptide maps of [^{3}H]leucine-labeled *gag* proteins from three independent N[*Emv-3* + AKR(623)] isolates were virtually identical. However, the Pr65^{gag} of all viruses contained a minor peptide not present in any of the N(*Emv-3*) viruses (compare Fig. 3A and D, and data not shown). The same spot was also present in the peptide map of AKR(623) (Fig. 3E). It was not present in maps of two E_b viruses, 1413-1^k and 1362-1^k, isolated previously from DBA/2 mice (37) (Fig. 3B, and data not shown). We have not yet localized the source of this peptide, but it was not present in the maps of p15^{gag} or p30^{gag} (data not shown).

Analyses of maps of the same proteins labeled with [^{3}H]arginine revealed another common difference between the *gag* precursors of these two classes of viruses. The maps from three independent N(*Emv-3*) isolates all contained a spot close to the origin not found in the N[*Emv-3* + AKR(623)] viruses (compare Fig. 3C and F, and data not shown). The peptide was not found in the map of AKR(623) (Fig. 3J). This peptide mapped to pp12^{gag} of N(*Emv-3*) (compare Fig. 3G and I). These data are consistent with the hypothesis that at least part of the *gag* region of N[*Emv-3* + AKR(623)] viruses, including pp12^{gag}, is derived from AKR(623) and that the difference between N[*Emv-3* + AKR(623)] and N(*Emv-3*) viruses arises because AKR(623) encodes a Leu instead of a Pro in pp12^{gag} (Fig. 2). The presence of Leu in pp12^{gag} may introduce a site that can be cleaved by the chymotryptic activity, which is difficult to suppress during trypsin digestion. Importantly, these data suggest that the N(*Emv-3*) viruses retain the proline-encoding pp12^{gag} and yet are replication competent. If so, the defect in *Emv-3* most likely results from the single nucleotide substitution in the gene for p15^{gag}.

Tryptic peptide analysis of the p15^{gag} proteins from three N[*Emv-3*] viruses was consistent with this prediction. For

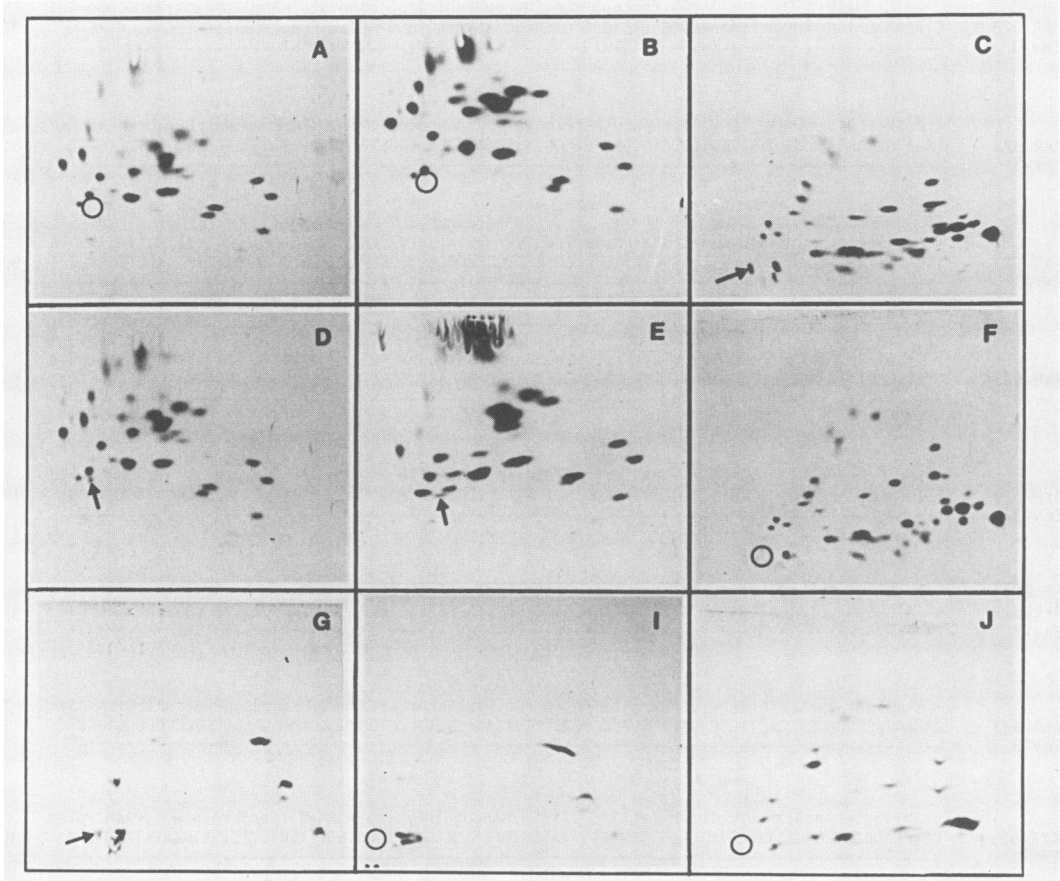

FIG. 3. Peptide maps of *gag* proteins from N[*Emv-3* + AKR(623)] viruses. The origin of the viruses and the peptide mapping methods are described in the text. A, [^{3}H]leucine-labeled N(Emv-3)5 Pr65^{gag}; B, [^{3}H]leucine-labeled 1413-1^k Pr65^{gag}; C, [^{3}H]arginine-labeled N(*Emv-3*)2 Pr65^{gag}; D, [^{3}H]leucine-labeled N[*Emv-3* + AKR(623)]45 Pr65^{gag}; E, [^{3}H]leucine-labeled AKR(623) Pr65^{gag}; F, [^{3}H]arginine-labeled N[*Emv-3* + AKR(623)]45 Pr65^{gag}; G, [^{3}H]arginine-labeled N(*Emv-3*)4 pp12^{gag}; I, [^{3}H]arginine-labeled N[*Emv-3* + AKR(623)] pp12^{gag}; J, [^{3}H]arginine-labeled AKR(623) Pr65^{gag}. Peptides that were identified in only some viral isolates are indicated with an arrow. The absence of a peptide is indicated with a circle.

example, analysis of [^{3}H]leucine-labeled Pr65^{gag} from one virus, N(*Emv-3*)4, revealed a peptide not present in the maps of the other two N(*Emv-3*) viruses, N(*Emv-3*)2 and N(*Emv-3*)5 (compare Fig. 4A, B, and C). The unique peptide in N(*Emv-3*)4 was located in p15^{gag} (Fig. 4D and E). A second difference among the *gag* precursors of these viruses was identified when [^{3}H]lysine was used as a label. The map of N(*Emv-3*)2 contained a spot not present in the maps of N(*Emv-3*)4 or N(*Emv-3*)5 viruses (compare Fig. 4G, H, and I). This peptide may result from a mobility shift in a peptide whose intensity was reduced in the map of N(*Emv-3*)2 (Fig. 4G). Again, this peptide was located in p15^{gag} (compare Fig. 4J, K, and L). Thus, each of the three N(*Emv-3*) viruses analyzed carried a p15^{gag} with a unique structure, suggesting that nondefective revertant viruses are produced by genetic alterations in p15^{gag}.

***Emv-3* Pr65^{gag} is not myristylated.** The Pr65^{gag} precursor of mammalian type-C retroviruses is modified by the covalent attachment of myristic acid to the N-terminal glycine residue of p15^{gag} (20, 49). Myristylation of Pr65^{gag} is required for virus assembly. Using oligonucleotide-directed mutagenesis, Rein et al. (44) either deleted the glycine codon in the Moloney MuLV (Mo-MuLV) p15^{gag} N terminus or converted it to an alanine codon. Upon transfection into mammalian cells, these two mutant genomes directed the synthesis of *gag* proteins, but these proteins were not myristylated. The mutants did not form any detectable virus-specific structures, and the mutant *gag* proteins appeared to remain

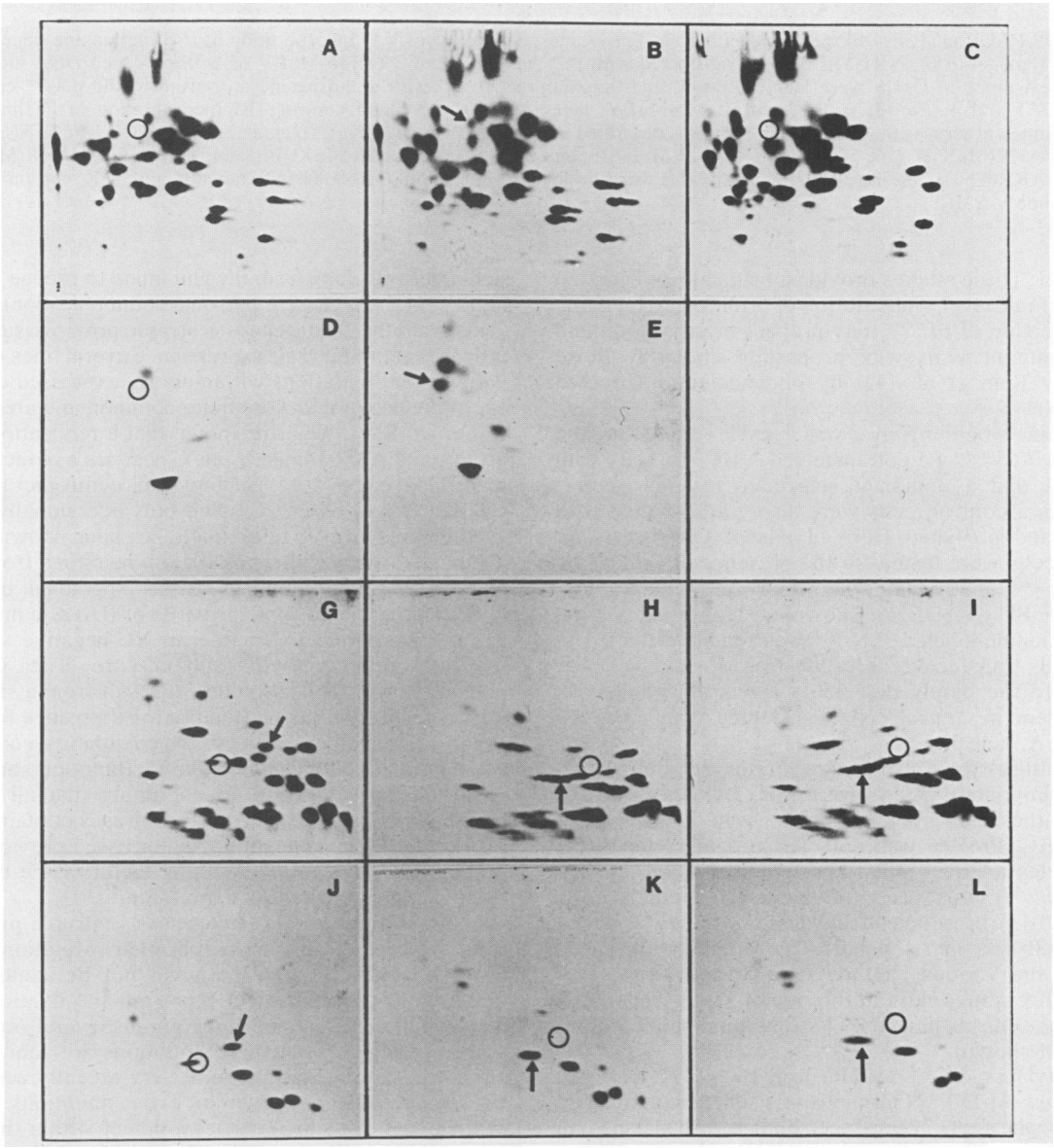

FIG. 4. Peptide maps of *gag* proteins from N(*Emv-3*) viruses. The origin of the viruses and the peptide mapping methods are described in the text. A, [^{3}H]leucine-labeled N(*Emv-3*)2 Pr65^{gag}; B, [^{3}H]leucine-labeled N(*Emv-3*)4 Pr65^{gag}; C, [^{3}H]leucine-labeled N(*Emv-3*)5 Pr65^{gag}; D, [^{3}H]leucine-labeled N(*Emv-3*)2 p15^{gag}; E, [^{3}H]leucine-labeled N(*Emv-3*)4 p15^{gag}; G, [^{3}H]lysine-labeled N(*Emv-3*)2 Pr65^{gag}; H, [^{3}H]lysine-labeled N(*Emv-3*)4 Pr65^{gag}; I, [^{3}H]lysine-labeled N(*Emv-3*)5 Pr65^{gag}; J, [^{3}H]lysine-labeled N(*Emv-3*)2 p15^{gag}; K, [^{3}H]lysine-labeled N[*Emv-3* + AKR(623)]45 p15^{gag}; L, [^{3}H]lysine-labeled N(*Emv-3*)5 p15^{gag}. Peptides that were identified in only some viral isolates are indicated with an arrow. The absence of a peptide is indicated with a circle.

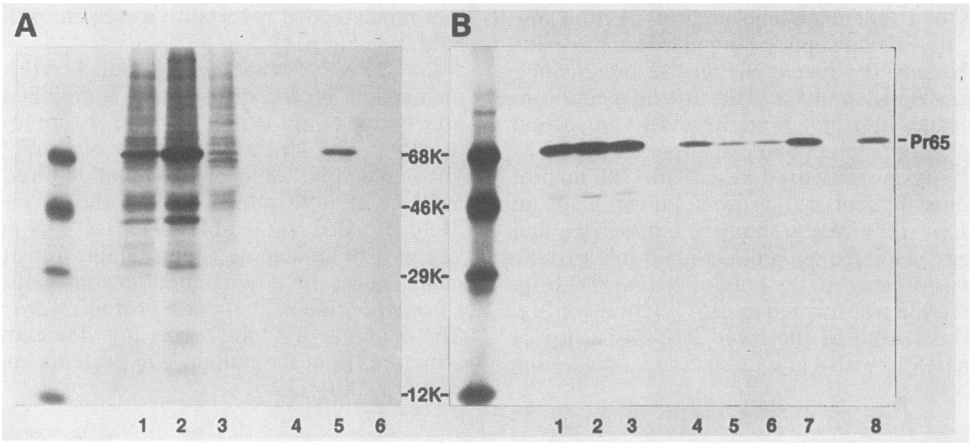

FIG. 5. SDS-PAGE analysis of Pr65^{gag} proteins from *Emv-3* and related viruses. (A) Incorporation of [^{35}S]methionine (lanes 1 to 3) and [^{3}H]myristate (lanes 4 to 6). NIH 3T3 cells cotransfected with pSV2neo and *Emv-3* or Mo-MuLV or with pSV2neo DNA alone and then selected for resistance to G-418 were labeled, lysed, and immunoprecipitated with monospecific antiserum to the p30^{gag} component of Pr65^{gag}. Lanes 1 and 4, *Emv-3*; lanes 2 and 5, Mo-MuLV; lanes 3 and 6, pSV2neo control. (B) Incorporation of [^{3}H]myristate into replication-competent viruses recovered from *Emv-3* as described in the text. Lane 1, N(*Emv-3*)2; lane 2, N(*Emv-3*)4; lane 3, N(*Emv-3*)5; lane 4, N[*Emv-3* + AKR(MuX)]4; lane 5, N[*Emv-3* + AKR(MuX)]13; lane 6, N[*Emv-3* + AKR(MuX)]14; lane 7, N[*Emv-3* + AKR(MuX)]27; lane 8, N[*Emv-3* + AKR(623)]43. Molecular weight standards were: bovine serum albumin, M_r 68,000 (68K); ovalbumin, 46K; carbonic anhydrase, 29K; cytochrome *c*, 12K.

in the cytosol. These studies provide a plausible explanation for the defect in *Emv-3*. Substitution of proline for glutamine in the third codon of p15^{gag} may prevent myristylation and produce a mutant virus with properties similar to those produced by Rein et al. (44) by oligonucleotide-directed mutagenesis.

To determine whether *Emv-3* was capable of directing the synthesis of Pr65^{gag}, we cotransfected NIH 3T3 cells with *Emv-3* DNA and a dominant selectable plasmid vector, pSV2neo. As a control, cells were also cotransfected with Mo-MuLV and pSV2neo DNA. 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 SDS-PAGE. As shown in Fig. 5A, lanes 1 and 2, [^{35}S]methionine-labeled Pr65^{gag} was readily detected in NIH 3T3 cells transfected with *Emv-3* or Mo-MuLV DNA, in contrast to the barely detectable levels of endogenous Pr65^{gag} present in control cells transfected with pSV2neo alone (Fig. 5A, lane 3).

Parallel cultures were also analyzed after metabolic labeling with [^{3}H]myristate. As shown in Fig. 5A, lanes 4 and 5, Pr65^{gag} synthesized by Mo-MuLV was myristylated, whereas *Emv-3* Pr65^{gag} was not. These data confirm that *Emv-3* encodes a Pr65^{gag} that is not myristylated. As expected, N(*Emv-3*) viruses (Fig. 5B, lanes 1 to 3) and N[*Emv-3* + AKR(623)] viruses (lane 8, and data not shown) encoded a Pr65^{gag} that was myristylated. The variability in signal intensity of the various [^{3}H]myristate-labeled viruses appeared to reflect differences in the rate of Pr65^{gag} synthesis in the various cells, as judged by [^{35}S]methionine incorporation (data not shown).

The myristylation defect resulting from the A to C transversion in the *Emv-3* p15^{gag} N terminus was sufficient to account for the poor replication of *Emv-3* seen both in vitro and in vivo. Although unlikely, the studies presented here do not allow us to determine whether other nucleotide substitutions within *Emv-3*, particularly in the 5′ untranslated sequences (Fig. 2), also inhibit *Emv-3* replication. However, data from recent in vitro mutagenesis experiments involving *Emv-3* DNA are consistent with the hypothesis that *Emv-3* carries a single replication defect resulting from the glutamine to proline substitution in p15^{gag} (E. Jørgensen, personal communication).

Several other endogenous ecotropic proviruses contain mutations that inhibit their expression. Several lines of evidence indicate that mutations within poorly expressed endogenous ecotropic proviral loci are quite common and are not limited to *Emv-3*. First, we have shown that a replication-defective provirus of AKR/J mice, *Emv-13*, carries a defect within the p15E-U3 region (12). Second, an ecotropic provirus of C57BL/6 mice, *Emv-2*, which is only occasionally expressed as infectious virus in adult mice, has been shown to contain a defect(s) within the *pol* region resulting from a single nucleotide substitution (R. Risser, personal communication). Third, an ecotropic provirus of BALB/c mice, *Emv-1*, has been reported to encode an XC-negative virus which replicates poorly in vivo and in vitro. The XC-negative phenotype and replication defect result from a single nucleotide substitution at the junction of the genes for the gp70 and p15E envelope proteins which inhibits envelope processing (24; G. Sithanandam and U. Rapp, personal communication). These results do not imply that all poorly expressed endogenous ecotropic proviruses contain mutations that inhibit their replication. As discussed previously, other *cis*-acting as well as *trans*-acting factors have been postulated to regulate provirus expression.

Are mutations within endogenous ecotropic proviral genomes acquired during virus replication? Mutations in endogenous ecotropic proviruses might not be acquired during virus replication but might represent the fixation of mutations acquired after viral integration. Several observations, however, suggest that these mutations are acquired during virus replication. First, several very recently acquired proviruses have been shown to carry mutations. Examples include *Emv-13*, which carries a defect within the P15E-U3 region (12), and Mov-2, Mov-7, and Mov-10 strains, which contain Mo-MuLV proviruses with mutations in the *gag-pol* region (47). Moreover, several studies have suggested that retrovirus replication is especially error prone (7, 39, 52). Recent studies by Dougherty and Temin (14) with a spleen necrosis virus-based retrovirus vector indicate that the mu-

tation rate may be as high as 0.5% per cycle of virus replication. This mutation frequency provides a potent vehicle for rapid retrovirus evolution. Additionally, the *gag* region appears to be uniquely susceptible to mutation (39, 47). The significance of this result with respect to both the mechanism of virus mutation and evolution is unknown.

Origin of E_a and E_b viruses. A plausible model to account for the origin of E_a and E_b viruses can be proposed now that the nature of the defect in *Emv-3* is understood. The nucleotide substitution in p15^{gag} can be corrected either by mutation or by recombination with nondefective endogenous proviral sequences. E_a viruses have apparently corrected the defect by substituting xenotropic sequences in *gag*. The mouse genome contains a large reservoir (>30 copies per haploid mouse genome) of endogenous proviruses that could conceivably contribute these *gag* sequences (23). Viruses similar to E_a viruses have also been generated in vitro by cotransfection of NIH 3T3 cells with *Emv-3* DNA and AKR xenotropic MuLV DNA, designated N[*Emv-3* + AKR-(MuX)] viruses (12). Like N(*Emv-3*) and N[*Emv-3* + AKR(623)] viruses, N[*Emv-3* + AKR(MuX)] viruses encode Pr65^{gag} proteins that are myristylated (Fig. 5B, lanes 4 to 7). Although this substitution clearly produces replication-competent virus, the biological data indicate that E_a viruses replicate poorly in vivo and are nonpathogenic (57). Concordantly, these viruses are predominantly isolated from animals with low or undetectable levels of viremia. Considerable precedent exists to indicate that two or more defective endogenous proviruses can recombine in vivo to generate nondefective virus, as in the proposed origin of E_a viruses. Treatment of mice that carry two defective Mo-MuLV proviruses, Mov-7 and Mov-10, with 5-azacytidine efficiently activates these silent genomes, and nondefective viruses are produced (25). Enhanced production of ecotropic MuLV in BALB/c × C57BL/6 hybrids has been shown to result from the interaction of two loci that most likely represent the ecotropic virus structural genes, *Emv-1* and *Emv-2*, carried by these strains (33). The spontaneous generation of replication-competent virus in CWD/J mice has been shown to result from recombination of two endogenous ecotropic proviruses, *Emv-1* and *Emv-3* (55). Similarly, recombination between defective endogenous proviruses has also been reported to occur in chickens (8).

E_b viruses are virtually indistinguishable from the prototypic ecotropic virus of AKR mice and could arise by mutation, recombination, or both. The origin(s) of these viruses will be determined only when the nucleotide sequence of the *gag* region of several E_b isolates is known.

The origin of E_c viruses remains an enigma. Peptide mapping data have been unable to document the source of the parental viruses that generate E_c viruses. This leaves open the possibility that E_c viruses do not originate from *Emv-3* or other DBA/2 endogenous proviruses but are transmitted horizontally as infectious agents in the DBA/2 strain. A satisfactory explanation for the origin of E_c viruses must await the development of specific nucleic acid probes and rigorous analysis of the endogenous proviral sequences carried by DBA/2 mice.

Induction of *Emv-3* expression by chemical carcinogens. By knowing the nature of the defect in *Emv-3*, we can also hypothesize how chemicals such as 5-azacytidine and DMBA affect *Emv-3* expression. 5-Azacytidine has been shown to demethylate genes in vitro, including endogenous retroviral genes, and to induce transcription (10, 17, 22, 38). Although 5-azacytidine may activate *Emv-3* transcription, this locus encodes a mutant virus that is probably incapable of forming virus particles. 5-Azacytidine should therefore be relatively ineffective at inducing *Emv-3* replication, as observed. The efficient induction of E_b viruses by DMBA may be related to its mutagenic effect on DNA. DMBA is known to form large adducts with guanine and adenine residues, resulting in numerous unspecified mutations (4, 13). For example, over 90% of all DMBA-induced skin carcinomas in mice have been shown to contain an activated *Ha-ras-1* oncogene resulting from an A to T transversion in the second base of codon 61 (5, 43). Similar activating mutations may be induced in *Emv-3* by DMBA. Any nucleotide substitution within codon 3 of *Emv-3* p15^{gag} that results in a Pro to Gln substitution would produce a replication-competent provirus. Amplification of this revertant virus by many cycles of virus replication would ultimately lead to overt viremia in mutagenized animals. In addition, sequence analysis of the p15^{gag} sequences of several mammalian retroviruses suggests that amino acids other than glutamine can be encoded at codon 3 and still produce a functional myristylated *gag* precursor (proline is not among these amino acids [50]). In this regard, it is interesting that Towler et al. (56) recently purified from yeast cells the myristyl transferase that catalyzes the addition of myristic acid to the N-terminal glycine residues of many eucaryotic proteins. This enzyme appears to have a very similar specificity to that in mammalian cells. By synthesizing a number of peptide substrates, these authors determined the structural information that is necessary for efficient myristylation. While a number of amino acid substitutions are permitted at codon 3, a proline substitution virtually eliminated myristylation. This plasticity in *gag* sequences increases greatly the number of nucleotide substitutions in codon 3 that could conceivably produce replication-competent virus. The structure of viruses isolated from DMBA-treated mice is consistent with this model.

Analysis of the p15^{gag} sequences of E_b isolates recovered from DMBA-treated DBA/2 mice should make it possible to determine whether *Emv-3* expression is activated by mutations within codon 3. If this is the case, this type of approach may ultimately provide a very useful in vivo experimental system for studying the effects of chemical mutagens at the DNA level.

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