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Mechanism of Chemical Activation of Expression of the Endogenous Ecotropic Murine Leukemia Provirus *Emv-3*†

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DBA/2 mice carry a single endogenous ecotropic murine leukemia provirus, *Emv-3*. This provirus is defective; it is very poorly expressed in young DBA/2 mice. The defect in *Emv-3* is caused by a single base substitution in codon 3 of p15^{gag}. The resulting amino acid substitution inhibits myristylation of the *gag* precursor and subsequent virus assembly. Despite this defect, percutaneous treatment of DBA/2 mice with the carcinogen and mutagen 7,12-dimethylbenz[*a*]anthracene (DMBA) induces ecotropic murine leukemia virus replication in virtually all treated mice. We hypothesized that this induction is the result of a DMBA-induced reverse mutation in codon 3 of p15^{gag} which allows for efficient myristylation. We tested this hypothesis by isolating ecotropic viruses from DMBA-treated mice and determining the DNA sequences of selected regions of p15^{gag}, including codon 3. In support of the above-described model, all of the viruses examined contained single nucleotide substitutions in codon 3. In addition, most of the replication-competent viruses that were sequenced appeared to result from simple mutation of *Emv-3* rather than recombination with other endogenous murine leukemia viruses. These studies may provide a basis for development of a sensitive assay for the mutagenic activity of a variety of chemical carcinogens in vivo.

Endogenous ecotropic murine leukemia virus (MuLV) proviral sequences have been extensively studied both for their ability to induce lymphomas and as a model system for eucaryotic gene expression. DBA/2 mice carry a single endogenous ecotropic MuLV provirus, *Emv-3*, that is causally associated with the recessive dilute (d^v) coat color mutation (4, 9, 11). *Emv-3* is not expressed in young mice and is expressed in only approximately one-third of adult mice, which suggests that it is defective. DNA transfection and marker rescue experiments (3) have indicated that *Emv-3* is defective in the *gag* region of the genome. DNA sequencing, peptide mapping, and [^{3}H]myristate labeling experiments (5) have further localized this defect to a single nucleotide substitution in codon 3 of p15^{gag}. This sequence difference results in substitution of proline (CCG) for glutamine (CAG), which prevents myristylation of the Pr65^{gag} amino terminus. Since myristylation is required for virus assembly at the plasma membrane (17), virus replication is effectively halted. When codon 3 is mutated in vitro from proline to glutamine, Pr65^{gag} is myristylated and infectivity in NIH 3T3 transfection assays is increased, nearly to the level observed with the prototypic ecotropic provirus AKR(623) (12).

Percutaneous treatment of young DBA/2 mice with 7,12-dimethylbenz[*a*]anthracene (DMBA) induced ecotropic virus expression in approximately 95% of the mice (14). Virus isolates from DMBA-treated and untreated mice have been divided into two major classes, E_a and E_b, largely on the basis of electrophoretic patterns of virion proteins (15, 25). E_b viruses, which are the most frequently isolated class, appear to have ecotropic *gag* and *env* regions. In contrast, E_a viruses have an ecotropic *env* region and a xenotropic *gag* region, suggesting that they result from recombination between endogenous xenotropic viruses and *Emv-3*. DMBA treatment increases the ratio of E_b to E_a isolates (25).

We hypothesized that the increase in viremia and E_b virus isolation caused by DMBA treatment is the result of reverse mutations in codon 3 of p15^{gag}, causing substitution of glutamine, or another amino acid residue which allows for myristylation, for proline. To test this hypothesis, we determined the p15^{gag} sequences of viruses obtained from both DMBA-treated and untreated DBA/2 mice.

MATERIALS AND METHODS

Animals and cells. DBA/2J mice were raised in our breeding colony at the National Cancer Institute-Frederick Cancer Research Facility. *Mus dunni* fibroblasts (13) were a gift from Marilyn Lander (Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, Md.) and were grown in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum.

DMBA treatment. DBA/2J mice (5 to 6 weeks of age) were treated on their shaved backs with 50 μl of DMBA at 2 mg/ml in acetone twice weekly for 7 weeks (14).

Isolation and growth of viruses. Splenocytes (10^5, 10^6, or 10^7) from treated or untreated mice were cocultivated with *M. dunni* fibroblasts at approximately 50% confluence in 100-mm-diameter dishes. After 2 days, the cells were passaged at a dilution of 1:10. Subsequently, the cells were put through three or four additional 1:10 passages twice weekly to allow for virus replication and to remove the DBA/2J splenocytes. Virus replication was initially detected by syncytium formation in the *M. dunni* fibroblasts. After syncytia were observed, culture fluids were filtered through Millex-GS 0.22-μm-pore-size syringe filters (Millipore Corp.) and placed on fresh *M. dunni* fibroblast monolayers to eliminate contamination with DBA/2J splenocytes. Previously isolated viruses (15, 25) were grown on *M. dunni* fibroblasts and passaged as described above.

PCR amplification of viral sequences from crude lysates of

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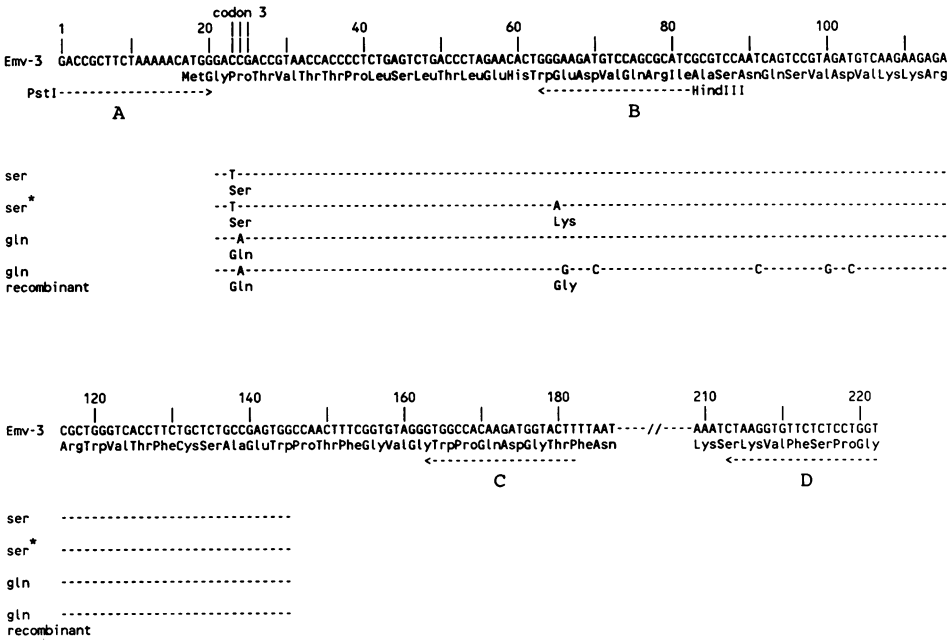

FIG. 1. p15^{gag} DNA sequences from *Emv-3* and viruses isolated from DMBA-treated and untreated DBA/2 mice, determined by direct sequencing of PCR products. Primers A, B, C, and D, used in PCR amplification, are shown as arrows; the arrowheads represent the 3′ ends of the primers, and primers B, C, and D represent the complement of the strand shown. The dashes indicate identical sequences.

infected cells. Virus-infected *M. dunni* fibroblasts were washed with phosphate-buffered saline and lysed with water (18). The lysate was boiled for 15 min, and a small portion of the lysate (representing DNA from 1×10^4 to 5×10^4 cells) was amplified by polymerase chain reaction (PCR; 19) by using synthetic oligonucleotide primers A and B (see Fig. 1). In every experiment, lysates from mock-infected *M. dunni* fibroblasts were prepared and amplified in parallel with infected cells as controls for possible contamination.

Cloning and sequencing of PCR products. Amplified DNA was chloroform extracted twice, cleaved with *Pst*I and *Hin*dIII, and electrophoresed on 4% NuSieve agarose (FMC Corp.) gels. The 92-base-pair (bp) fragment expected as a result of the amplification was excised, eluted, and ligated to *Pst*I-*Hin*dIII-cleaved M13mp18. Recombinant bacteriophage that resulted from transformation of *Escherichia coli* JM105 by the resulting plasmids were purified, and DNA was extracted and sequenced by the dideoxy-chain termination method (20).

Direct sequencing of PCR products. Crude lysates prepared as described above were amplified by asymmetric PCR (7, 10) with primers A and D (See Fig. 1) and a 100-fold excess of primer A. The PCR products were dialyzed against 10 mM Tris–1 mM EDTA by using Centricon 30 columns (Amicon Corp.). Dideoxy sequencing was performed by using an internal primer (primer C; see Fig. 1) end labeled with ^{32}P by T4 polynucleotide kinase by using the Sequenase kit (United States Biochemical). The conditions recommended by the manufacturer of the kit were used, except that the labeling step was omitted. Water was substituted for the labeling mixture, and the annealed template and primer were mixed directly with the termination mixtures.

RESULTS AND DISCUSSION

Generation and sequencing of DMBA-induced viral isolates. Twelve DBA/2J mice were treated with DMBA as described in Materials and Methods. The mice were killed by CO_2 inhalation at various times (see below) after completion of DMBA treatment, and the spleens were removed. Splenocytes were cocultivated with *M. dunni* fibroblasts, which are permissive for ecotropic MuLV replication but carry no detectable endogenous MuLV sequences (11). Virus replication was initially detected by syncytium formation in the *M. dunni* cells and later by PCR amplification of ecotropic viral sequences. Splenocytes from three DMBA-treated mice euthanized 4 weeks after the completion of treatment did not yield detectable virus upon cocultivation. In contrast, when mice were euthanized 8 to 12 weeks after the completion of treatment, upon observation of loss of weight, hunched backs, and/or enlarged lymph nodes, splenocytes from nine of nine mice yielded virus upon cocultivation. Virus was also isolated from splenocytes of one of six age-matched, untreated DBA/2J mice tested. These results are consistent with previous studies of Nexø and Ulrich (14), who isolated virus from splenocytes of 4 of 14 untreated DBA/2Fib mice versus 17 of 19 DMBA-treated DBA/2Fib mice by cocultivation with SC-1 cells.

Crude lysates were prepared from the infected cells (see Materials and Methods), and a small portion of the lysate was amplified by PCR with primers A and B (Fig. 1). These primers contained *Pst*I and *Hin*dIII restriction endonuclease cleavage sites at their 5′ ends to allow cloning and sequencing in phage M13. A 92-bp fragment (representing 50 bp of the primer sequence and 42 bp of p15^{gag}) was observed after amplification of lysates from all of the cultures that exhibited syncytia; this fragment was not observed when lysates from cultures without syncytia were amplified. The 92-bp fragment was also not observed when DNA from mock-infected *M. dunni* cells was prepared and amplified in parallel with infected cultures. This negative control was performed with all preparations of lysates and with all PCR amplifications. The 92-bp fragments from each of the crude lysates were

TABLE 1. Nucleotide sequence of the region spanning p15^{gag} codon 3 in viruses expressed in DMBA-treated DBA/2J mice, determined from cloned PCR products

Virus	Sequence[a]	No. of isolates	Expected myristylation
Parental (*Emv-3*)	MetGlyProThrValThrThrProLeuSerLeu ATGGGACCGACCGTAACCACCCCTCTGAGTCTG	0	–
Revertant	Ala ------G----------	0	+
Revertant	Thr ------A----------	0	?
Revertant	Ser ------T----------	7	+
Revertant	Arg ------G----------	0	?
Revertant	Gln ------A----------	2	+
Revertant	Leu ------T----------	0	+

[a] Viruses carrying nucleotide substitutions in the third base of codon 3 would encode proline and presumably be replication defective. Dashes indicate sequences identical to that of *Emv-3*.

cloned in M13mp18. Three independent M13 clones from each lysate were subsequently sequenced. The results are shown in Table 1. Seven of nine lysates from DMBA-treated mice yielded sequences that encoded serine at codon 3, while two encoded glutamine at codon 3. The rest of the 42-bp viral sequence was identical to *Emv-3*. All three sequences from each lysate were identical, suggesting that we examined clonal isolates. This lack of heterogeneity may have resulted from competition during virus replication in the animals, in culture, or in both. Alternatively, the frequency of DMBA-induced virus activation may have been low, and we may have observed single events.

Glutamine is found at p15^{gag} codon 3 in all of the MuLVs sequenced (8, 22). Therefore, the two glutamine viruses that we identified may have resulted from recombination with other endogenous MuLVs rather than by mutation.

Direct sequencing of DMBA-induced and uninduced viral isolates. To further address the question of mutation versus recombination, we again amplified viruses from each of the crude lysates by using primers A and D (Fig. 1), which would allow us to examine a larger region (130 bp) of the gene for p15^{gag}. We reasoned that a recombinant virus would likely differ in sequence from *Emv-3* within this larger region. Instead of sequencing independent M13 subclones from each cell lysate as before, we performed direct sequencing by using an internal primer (primer C; Fig. 1) end labeled with ^{32}P by T4 polynucleotide kinase (see Materials and Methods). This obviated the need to sequence multiple clones to identify heterogeneity within PCR products obtained from a single crude lysate. It also eliminated the probability of identifying sequence differences that may result from errors introduced during PCR amplification.

To obtain a larger sample of viruses and to further determine the origin of *Emv-3* viruses expressed spontaneously in DBA/2J mice, we also grew, amplified, and directly sequenced viruses obtained from DMBA-treated and untreated DBA/2Fib mice in a previous series of experiments performed by Ulrich and Nexø (25). Three of the viruses examined had been characterized as E_b viruses; however, most were uncharacterized. A summary of the sequencing results from both groups of viruses is shown in Table 2.

Some viral isolates appear to be recombinants. While heterogeneity was also not observed among viruses from a single lysate, three of the six glutamine-containing viruses (Table 2) contained several sequence differences with respect to *Emv-3* (Gln recombinants; Fig. 1), which suggests that they were recombinant viruses. PCR amplification and direct sequencing of DBA/2J genomic DNA yielded an approximately equimolar mixture of this sequence and that of *Emv-3*, confirming that DBA/2J mice carry an endogenous virus with this sequence. PCR amplification and direct sequencing of genomic DNA from DBA/2J d^{+18J}/d^{+18J} mice, which lack *Emv-3* (4), yielded the Gln recombinant sequence (data not shown).

Second nucleotide substitution observed in some serine viruses. Among the 17 serine-containing viruses analyzed, 12 had sequences identical to that of *Emv-3*, consistent with the hypothesis that these viruses were derived by mutation rather than recombination. Five of the viruses (designated Ser* in Fig. 1) were identical to *Emv-3*, with the exception of a single nucleotide difference at position 65. This nucleotide change results in substitution of Lys for Glu. When the only viral isolate obtained from six untreated mice was sequenced, it also had the Ser* sequence. PCR amplification and direct sequencing of DBA/2J genomic DNA failed to identify an endogenous virus with this sequence, suggesting that these viruses are not recombinants but that this substitution resulted from a second mutation during virus replication in the mice or in cell culture. Lysine has been observed at this position in other replication-competent MuLVs, including Moloney MuLV and Rauscher MuLV (26). Both

TABLE 2. Summary of direct sequencing of DBA/2 virus isolates

Source	DMBA treatment	No. of nonrecombinant viruses		No. of Gln (CAG) recombinant viruses
		Ser (TCG)[a]	Gln (CAG)	
This study	+	7 (2)	1	1
	–	1 (1)	0	0
Ulrich and Nexø	+	7	2	0
	–	2 (1)	0	2
Combined	+	14 (3)	3	1
	–	3 (2)	0	2

[a] The numbers in parentheses are numbers of Ser* viruses (see the text and Fig. 1).

viruses replicate to high titers in vivo and in vitro, suggesting the possibility that this amino acid substitution confers a selective growth advantage on the Ser* viruses.

Effect of DMBA treatment on the types of viruses isolated. The viruses from DMBA-treated mice were predominantly nonrecombinant, with Ser or Gln at codon 3, including the three that had previously been characterized as E_b isolates by peptide analysis. These data are consistent with the hypothesis that E_b viruses are the result of simple mutation of *Emv-3* (serine and nonrecombinant glutamine viruses) and that E_a viruses are the result of recombination between *Emv-3* and xenotropic endogenous viruses.

In contrast, the viruses isolated from untreated mice were more evenly split between recombinant and nonrecombinant viruses (Table 2). Ulrich and Nexø (25) observed that DMBA treatment increases the ratio of E_b to E_a virus isolates, which is consistent with our observations.

Only two of the six possible base substitutions were observed at codon 3. Although there are six possible base changes at codon 3 that would result in an amino acid substitution (Table 1), we observed only two, serine and glutamine. This propensity for serine and glutamine substitutions may simply reflect the specificity of the mutagen. Alternatively, these may be the only two amino acid substitutions that allow for efficient virus replication. In support of the first view, alanine has been found in a position analogous to that of $p15^{gag}$ codon 3 in other myristylated proteins, such as human immunodeficiency virus *gag*, picornavirus capsid protein VP4, and cytochrome b_5 reductase (16, 21). In support of the second view, threonine and arginine have not been observed in any myristylated protein. Furthermore, in the in vitro *Saccharomyces cerevisiae N*-myristoyltransferase system of Towler et al. (23, 24), synthetic polypeptides with leucine in position 2, corresponding to $p15^{gag}$ codon 3, were myristylated, but with a decreased V_{max} (6 to 10% relative to polypeptides with serine or glutamine at position 2). Mutations to leucine may occur in our system; however, they may be lost during virus replication after competition with viruses containing serine or glutamine substitutions in codon 3.

Serine has not been found at codon 3 of $p15^{gag}$ in any endogenous or exogenous MuLV genome sequenced, strongly suggesting that the serine viruses result from mutation of *Emv-3* $p15^{gag}$ codon 3 rather than by recombination with other endogenous proviruses. It is likely that these mutations are due to the direct action of DMBA on the *Emv-3* provirus, although it is conceivable that DMBA induces *Emv-3* replication by some unknown mechanism and that the nucleotide substitutions occur later during virus replication.

Relationship of the mutations observed to the mechanism of DMBA action. Metabolites of DMBA have been shown to form adducts with adenine and guanine residues (1, 6) and to cause transversions in vivo in mouse skin tumors in H-*ras* (2). Whether the preponderance of C→T transitions over C→A transversions observed here is due to any specificity of DMBA or to a selective advantage of viruses with serine at codon 3 remains to be determined. If the former is true, then other mutagens that have different mutational specificities may produce a different spectrum of codon 3 mutations.

Ecotropic virus induction in the DBA/2 mouse as a general in vivo mutagenesis assay. In this study, we have shown that most of the viruses induced by DMBA treatment of DBA/2 mice have a mutation in codon 3 of *Emv-3* $p15^{gag}$ which restores the myristylation site for the *gag* precursor protein. This system potentially provides a convenient model for mammalian in vivo mutagenicity testing for transitions and transversions of C-G base pairs. In its simplest form, DBA/2J mice can be treated with a putative mutagen, aged for several weeks, and subsequently tested for viremia by radioimmunoassay, followed by sequencing of viral isolates after PCR amplification. Since activation of *Emv-3* can be caused only by a limited range of mutations (codon 3 contains only G-C base pairs), the system can be extended by generation of transgenic mice carrying other defective viral genomes which can be activated by mutagens with different specificities. This strategy may offer even greater sensitivity, since transgenes are usually integrated as multiple tandem copies (27), providing more potential targets for mutagenesis.

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