Linkage of the Fv-2 Gene to a Newly Reinserted Ecotropic Retrovirus in Fv-2 Congenic Mice

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Restriction enzyme and Southern gel analyses were used to determine the number and location of endogenous ecotropic retroviruses in the germ line of several mouse strains congenic at the Fv-2 gene locus. A new endogenous ecotropic provirus was observed in the germ line of B6.S (Fv-2^ss) mice, in addition to the resident provirus found in its congenic partner C57BL/6 (Fv-2^r). This new provirus was similar in structure to the C57BL provirus. The SIM strain of mice, the donors of the Fv-2^a allele in B6.S mice, does not contain ecotropic proviruses, suggesting that the new provirus in the B6.S mouse strain arose by germ-line reintegration during the construction of this strain. Mendelian segregation analysis indicated that this new provirus was linked to the Fv-2 gene locus on chromosome 9. In three other Fv-2^a congenic mouse strains—B10.C (47N), B6.C (H-7^a), and C57BL/6J Trf^a, Bgs^—no additional ecotropic endogenous viruses were detected, suggesting that the reinsertion event that occurred during the construction of B6.S is not essential for the acquisition of the Fv-2^a phenotype in the C57BL genetic background. Although numerous reports of germ-line insertions of ecotropic virus in high-virus mouse strains have been received, the present results provide definitive evidence that similar germ-line amplifications of endogenous ecotropic virus can occur in a low-virus mouse strain.

The germ line of many inbred strains of mice contains multiple copies of DNA sequences homologous to the genomes of murine leukemia viruses (MuLV; 25). These endogenous retroviruses can be divided into two classes on the basis of their host range: ecotropic MuLVs, which replicate only in murine cells, and xenotropic MuLVs, which can infect heterologous but not mouse cells. Various inbred strains of mice differ in the frequency with which ecotropic virus can be isolated. In general, high-virus strains, such as AKR and C58, contain multiple endogenous ecotropic proviruses (7-9, 33), whereas low-virus strains, including C57BL, BALB/c, and C3H/HeJ, contain a single endogenous ecotropic provirus (9, 14, 16-18).

Many of these endogenous proviruses are located at diverse chromosomal locations, suggesting that these viruses can stably integrate into the germ line at many different sites. Several recent reports have demonstrated that different sublines of the high-virus AKR strain are polymorphic with respect to the number and location of endogenous proviruses (6, 12, 23, 25, 34, 37), indicating that the amplification of these sequences is occurring as the result of exogenous infection in vivo, followed by stable integration into the germ line. Similar observations have been made during the construction of congenic mice involving the high-virus AKR strain of mice (30). In contrast, the number and site of integration of the endogenous ecotropic provirus in low-virus strains appear to be stable among different mouse sublines (16, 23).

During the analysis of the mouse Fv-2 gene locus, which is the major determinant of susceptibility or resistance to Friend spleen focus-forming virus (19), we reported (20) differences in the expression of endogenous-retrovirus-related sequences in the Fv-2 congenic mouse strains C57BL/6 (B6; Fv-2^r) and B6.S (Fv-2^a). These mice differ at the Fv-2 gene locus and a small linked segment of chromosome 9 (1, 2). The B6.S strain carries the Fv-2^a (susceptibility) allele, whereas the parental B6 strain is homozygous Fv-2^r (resistant). The observation that these congenic mice differ with respect to the expression of endogenous retroviral sequences and a recent unexpected finding that the endogenous ecotropic virus locus of C3H mice appears to segregate with the Fv-2 locus in C57BL/6J × C3H/HeJ recombinant inbred mouse strains (15) prompted us to examine the number and location of ecotropic MuLV proviruses in the germ line of several independently constructed pairs of Fv-2 congenic mice. We report here that B6.S...
mice contain a new endogenous ecotropic provirus that resulted from a germ-line reintegration event during the construction of this strain. In addition, we present evidence that this new provirus is closely linked to the Fv-2 locus in these mice.

MATERIALS AND METHODS

Mice. Four pairs of mouse strains congenic at the Fv-2 locus were used. The construction of B6.S (Fv-2<sup>s</sup>) mice congenic with C57BL/6JUt (Fv-2<sup>r</sup>) was described by Axellad et al. (2). The donor of the Fv-2<sup>r</sup> allele in these congenics was the SIM (Fv-2<sup>s</sup>) strain. The above mice are maintained at the Ontario Cancer Institute. Other mice congenic at the H-7 histocompatibility locus, which is closely linked to Fv-2, were B10.C (47N) (31) and B6.C (H-7<sup>s</sup>) (3) and their H-7<sup>s</sup> congenic partners C57BL/10Sn and C57BL/6J, respectively. These mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. The fourth congenic strain used was C57BL/6J Tfr<sup>d</sup>, Bgs<sup>d</sup>, which is congenic at the Bgs gene locus (4). Bgs is also closely linked to Fv-2, and the Bgs congenic mice carry the Fv-2<sup>r</sup> allele of the donor CBA/J mice. These mice were kindly provided by V. Chapman (Roswell Park, Buffalo, N.Y.).

Cells and viruses. The polycythemia-inducing Friend MuLV complex (FV-P) was obtained by harvesting the medium of an NIH/3T3 virus-producing cell line, as previously described (5).

Treatment of mice with acetylphenylhydrazine and Friend virus. During these experiments, we found that some B6 × B6.S F1 mice did not develop polycythemia or splenomegaly under conditions that caused disease in the sensitive B6.S parent. This semidominance of the Fv-2<sup>r</sup> allele in heterozygous mice has been documented by others (24). To overcome this partial resistance, we injected 5- to 7-week-old mice with 10 times more virus or sensitized them to FV-P virus by a single 0.25-ml intraperitoneal injection of 1-acetyl-2-phenylhydrazine (6 mg/ml). The acetylphenylhydrazine was first dissolved in 1 ml of ethanol and then diluted in phosphate-buffered saline. Four to five days after acetylphenylhydrazine treatment, the mice were injected with FV-P intravenously. Although occasionally we have observed moderate splenomegaly in C57BL/6J (Fv-2<sup>s</sup>) mice as a result of phenylhydrazine treatment, none of these Fv-2<sup>r</sup> mice has developed spleen foci or polycythemia after injection with Friend virus.

DNA extraction. DNA was extracted by a modification of the method of Gross-Bellard et al. (11). The organs were homogenized in a glass tissue homogenizer containing 5 ml of buffer A (Tris-hydrochloride [pH 8.0], 10 mM NaCl, 10 mM EDTA). Protease K (500 μg/ml) and sodium dodecyl sulfate (1%) were added, and the homogenate was incubated overnight at 37°C. The solution was extracted with phenol and chloroform and dialyzed against TE buffer (10 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA). RNA was digested with RNase A (50 mg/ml) and RNase T1 (1 U/ml) at 37°C for 4 h. The DNA was again extracted with phenol and chloroform and dialyzed against TE buffer. It was concentrated by ethanol precipitation, pooled on a glass rod, suspended in TE buffer, and stored at 4°C.

Analysis of DNA by restriction endonuclease digestion and gel electrophoresis. DNA (20 μg) was digested with restriction enzymes overnight as specified by the suppliers (Bethesda Research Laboratories, Gaithersburg, Md.; Boehringer Mannheim Corp., Montpellier, Quebec, Canada). The DNA samples were electrophoresed on 0.8-cm-thick 0.6% agarose gels in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA) at 20 V for 18 to 42 h. The DNA in the gels was acid depurinated by the technique of Wahl et al. (36) before transfer to nitrocellulose filters, as described by Southern (32). The filters were hybridized with 1 × 10⁶ cpm of nick-translated ecotropic-virus-specific probe per ml (0.5 × 10⁶ to 1 × 10⁷ cpm/μg) in 50% formamide and 10% dextran sulfate for 16 h at 42°C and washed as described previously (36).
Nick translation. The entire plasmid containing the 400-base-pair ecotropic-virus-specific fragment of AKR MuLV DNA (9; kindly provided by D. Lowy, National Institutes of Health, Bethesda, Md.) was nick translated by modification of the technique of Rigby et al. (26). DNA was nick translated at a concentration of 1.5 to 3.0 ng of buffer per μl. The DNA was treated with DNase I (3 pg/μl) for 15 min at 15°C before the addition of DNA polymerase I. One hundred microcuries of [α-32P]dCTP per 20 μl (3.200 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was used.

RESULTS

Association of a new endogenous ecotropic virus in Fv-2 congenic B6.S mice. Four different pairs of mice were used to determine whether Fv-2 congenic strains differ in their number or location of endogenous ecotropic viruses. The mouse strain B6.S, congenic with C57BL/6 (B6) mice, was obtained by serial intercrosses and backcrosses with the Friend virus-sensitive SIM strain (2). B6 mice are Fv-2", whereas B6.S mice are Fv-2'. Strains B6.C (H-7*) and B10.C (47N) were made by the substitution of the BALB/c H-7 histocompatibility locus onto the B6 and C57BL/10Sn (B10) genetic backgrounds, respectively (3, 31). Because Fv-2 and H-7 are closely linked, B6.C (H-7*) and B10.C (47N) mice also carry the Fv-2' allele of BALB/c mice. Similarly, strain C57BL/6J Trph, Bgsd is congenic with B6 and was constructed by transferring the Bgs gene of CBA/J mice onto the B6 background. Because of the close linkage of the Bgs and Fv-2 loci, these mice also carry the Fv-2' allele of CBA/J mice.

To examine the number of ecotropic proviruses in these Fv-2 congenic mice, we digested cellular DNA with the restriction endonuclease PvuII and analyzed it by the method of Southern with the ecotropic-virus-specific probe. Digestion with this enzyme resulted in three ecotropic-virus-containing fragments: an internal viral fragment and both 5' and 3' virus-cell junction fragments (Fig. 1). Only one of these PvuII-generated fragments, the 3' virus-cell junction fragment, was detected by the ecotropic-specific probe.

Figure 2 shows that PvuII digestion of cellular DNA from the Fv-2 congenic mice B6.C (H-7*); B10.C (47N); C57BL/6J Trph, Bgsd; C57BL/10Sn; and C57BL/6J resulted in a single ecotropic virus fragment with a size of 5.2 kilobases (kb), indicating that these mice contain a single ecotropic virus in their germ line. This PvuII fragment is similar in size to the endogenous virus (Emv-2) found in C57BL and C57BR sublines (16). In contrast, the digestion of B6.S DNA with PvuII yielded two ecotropic virus fragments with sizes of 5.2 and 15.3 kb (Fig. 2), a result that suggests that these mice may contain two ecotropic endogenous viruses. SIM mice, which were the donors of the Fv-2' allele to B6.S mice (2), do not appear to contain an endogenous ecotropic virus (Fig. 2).

Structure of the ecotropic B6.S virus. To determine whether the new ecotropic virus of B6.S mice was of genome length and structurally related to the resident provirus in B6 mice (Fig. 1), we digested cellular DNA with restriction endonucleases that produce internal viral fragments and analyzed it by Southern gel electrophoresis. The digestion of B6 DNA with the restriction endonucleases BamHI, PstI, or KpnI yielded ecotropic-virus-specific fragments with sizes of 3.3, 8.2, and 4.4 kb, respectively (Fig. 3). Identical results were obtained when cellular DNA from the Fv-2 congenic B6.S strain was digested with these enzymes (Fig. 3). These results suggest that the additional 15.3-kb ecotropic virus fragment observed in B6.S mice after digestion with PvuII was derived from a complete ecotropic provirus rather than a subgenomic fragment representing only a portion of the viral genome. In addition, these results indicate that the two proviruses in B6.S mice have similar, if not identical, structures.

Germ-line location of the new ecotropic virus in B6.S mice. To determine whether the new ecotropic virus was located in the germ line of B6.S mice, we performed two types of experiments. First, cellular DNA from a number of different organs was examined for the presence of the new ecotropic virus. For these experiments, we
chose to digest cellular DNA with the restriction endonucleases HindIII or XbaI. The digestion of cellular DNA with these enzymes, followed by Southern gel analysis with the ecotropic-virus-specific probe, resulted in the production of 3' and 5' virus-cell junction fragments, respectively (Fig. 1). In agreement with previous results (16), the digestion of B6 spleen or kidney DNA with HindIII resulted in a single virus-cell junction fragment of 7 kb (Fig. 4A). In contrast, the digestion of B6.S spleen or kidney DNA with HindIII resulted in two virus-specific fragments of 7 and 10 kb (Fig. 4A). Similar results were observed with liver DNA (data not shown).

Figure 4B shows the results of digesting spleen or kidney DNA of B6 and B6.S mice with the restriction endonuclease XbaI. The digestion of cellular DNA with this enzyme revealed a single 5' virus-cell junction fragment with a size of 12 kb in B6 mice (Fig. 4B), in agreement with previous findings (16), whereas two 5' virus-cell junction fragments of 12 and 18 kb were observed in B6.S mice. Thus, these results confirm and extend the findings presented above that digestion with the restriction endonuclease PvuII of DNA from B6.S mice yielded two ecotropic viruses, one identical to that found in B6 mice and an additional provirus located at a different site in the B6.S germ line.

To determine whether this new ecotropic virus locus is segregating in the B6.S mouse strain, we examined the DNA from a number of individual B6.S mice for the presence of the new ecotropic virus. Kidney DNA from 17 B6.S mice was digested with HindIII and analyzed by Southern gel analysis. All of the individual B6.S mice examined contained two ecotropic viruses in their DNA (data not shown). Taken together, these results suggest that the Fv-2 congenic B6.S mouse strain contains a new ecotropic virus in its germ line.

**Linkage of Fv-2 with the new endogenous virus in B6.S mice.** To test whether the new ecotropic virus in B6.S mice was genetically linked to the Fv-2 locus, we crossed B6 with B6.S mice and looked for segregation in the F2 generation of the 10-kb HindIII virus fragment with Friend virus sensitivity. If the two loci are unlinked, one would expect an independent assortment of these two dominant markers. Typical results of Southern gel analysis of DNA from some (B6 × B6.S) F1 and F2 mice are shown in Fig. 5. The digestion with HindIII of DNA from F1 mice resulted in the two proviral fragments of 7 and 10 kb, as expected. The F2 mice exhibited two distinct patterns after Southern gel analysis with the ecotropic-virus-specific probe: DNA from 29 out of 38 (76%) mice examined contained the 10-kb HindIII fragment, whereas that from the remaining 9 mice did not. These results show that the additional ecotropic provirus in B6.S mice is segregating in a dominant Mendelian manner.

Table 1 presents the detailed results of the segregation analysis for 38 F2 mice. These mice were analyzed for their Fv-2 genotype by measuring their sensitivity to Friend virus and the presence or absence of the 10-kb HindIII ecotropic virus fragment. The new ecotropic virus in B6.S mice cosegregates with the Fv-2' allele (Table 1). The fact that some recombinant mice could be detected (3 of 38) indicates that the new
ecotropic provirus and Fv-2 are not allelic. These results also indicate that the new ecotropic provirus in B6.S is closely linked to the Fv-2 gene locus.

**DISCUSSION**

The results presented in this study indicate that a germ-line reinsertion of an endogenous ecotropic provirus occurred during the construction of B6.S, an Fv-2' mouse strain congenic with B6 (2). The provirus involved in this reinsertion event appears to be the resident ecotropic provirus found in B6 mice because SIM mice, the donor of the Fv-2' allele, do not appear to contain an endogenous ecotropic provirus. Furthermore, the structure of the new provirus appears to be identical to that of the endogenous ecotropic virus found in B6 mice, as judged by the size of the internal restriction enzyme fragments of the two viruses.

The observation that the B6 ecotropic provirus has reinserted at a new location in its Fv-2 congenic partner B6.S provides a further example of the polymorphism in the number and location of endogenous retroviruses that can arise in inbred strains of mice within a few generations. Previous examples of such germ-line reinsertion events involved the high-virus AKR mouse strain (6, 12, 23, 25, 30, 34, 37).

In addition, germ-line reinsertions of B-tropic virus in C57BL mice congenic at the H-2 locus (10, 22) and in early generations of BXH-2 (C57BL/6J × C3H/HeJ) recombinant inbred mice have been reported (15). In these instances, the C57BL mice were crossed to mouse strains that show an earlier spontaneous induction of virus than do C57BL mice. The H-2 congenics were made by crosses to the AKR high-virus strain (10) and the C57BR strain (22), which can produce virus at 1 to 3 months of age (28). C3H, one of the progenitors of the BXH-2 strains, shows an immune response to ecotropic virus early in life and at high titers (13). This is in contrast to C57BL mice, which do not express B-tropic virus or produce high-titer antibody to ecotropic viruses until 6 months of age (13, 21, 29). We could not detect N- or B-tropic virus released from the spleen cells of 3- to 5-month-old B6 or B6.S mice by the XC infectious center plaque assay (unpublished data). In addition, no spontaneous ecotropic virus induction has been detected in the SIM strain of mice (A. Axelrad, personal communication). Thus, the present results provide a definitive example of a germ-line insertion event involving low-virus mouse strains.

Germ-line reinsertions of retroviral genomes could theoretically occur by at least two distinct mechanisms: the integration of a newly synthesized DNA copy of the viral genome after virus infection or the transposition of a copy of the resident provirus by mechanisms similar to that used by other transposable genetic elements present in procaryotes and lower eucaryotes. Because the structure of the newly acquired proviral DNA copies would be identical regardless of which of these two routes of insertion of proviral DNA was used, it is not possible on the basis of the present results to distinguish between these two mechanisms. The finding that a germ-line reinsertion of an endogenous MuLV can occur even in a low-virus mouse strain such as C57BL/6 raises the possibility that viral gene expression and viremia may not be prerequisites for such an amplification event.

Mendelian segregation analysis has demonstrated that the new ecotropic provirus in B6.S mice is closely linked to the Fv-2 gene on
TABLE 1. Linkage analysis of Fv-2 with the new endogenous ecotropic provirus in B6.S mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>No. of mice</th>
<th>Spleen wt range (g)</th>
<th>Spleen foci</th>
<th>Ecotropic provirus (10-kb HindIII fragment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parents</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td>3</td>
<td>0.1-0.12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B6.S</td>
<td>3</td>
<td>0.45-3.63</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F1</td>
<td>16</td>
<td>0.11-2.4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F2</td>
<td>19</td>
<td>0.19-2.3</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>8</td>
<td>0.17-0.66</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.1-0.21</td>
<td>-</td>
<td>-</td>
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<td></td>
<td>3</td>
<td>0.1-0.14</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>1</td>
<td>0.33</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>2</td>
<td>0.11</td>
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\[ a \chi^2 = 19.7; \ p < 0.001. \]

b Of the F2 mice with the Fv-2\' phenotype, 27 inherited the new ecotropic provirus, and 1 did not; of the F2 mice with the Fv-2\# phenotype, 2 inherited the new ecotropic provirus, and 8 did not.

c These mice were treated with phenylhydrazine as described in the text.

Chromosome 9. The B6.S congenic mouse strain was constructed by 14 repeated backcrosses of the Fv-2\# allele of SIM mice onto the C57BL/6 background, selecting at each backcross generation for the Fv-2\# allele by progeny testing (2). Thus, the germ-line reinsertion of the new B6.S provirus could have occurred at any stage in the construction of the B6.S strain and would still be maintained because of its close linkage to the Fv-2\# allele.

Congenic mice are widely used to analyze biological, biochemical, and immunological aspects of individual genes on identical genetic backgrounds. Such mice are assumed to be identical everywhere except in a very small chromosomal region contiguous to the selected gene. One implication of the present results is that such an assumption may not, in general, be valid. The presence of endogenous retroviruses, and perhaps other transposable genetic elements, that can reinsert into new sites in the germ line on a contemporary time-scale suggests that such mice may possess additional genotypic and phenotypic differences that are unrelated to either the selected gene or closely linked loci in the background strain.

The finding that the Fv-2 congenic mouse strain B6.S contains a new ecotropic virus that is genetically linked to Fv-2 raises the question as to whether such a reinsertion event is essential for the acquisition of the Fv-2\# phenotype in C57BL mice. Two observations suggest that it is not. First, SIM mice, which were the donors of the Fv-2\# allele in B6.S mice (2), do not contain an endogenous ecotropic provirus, as detected with the ecotropic-specific probe used in these experiments. Secondly, three other Fv-2 congenic mice constructed on the C57BL background—C57BL/6 Trf\#, Bgs\#; B6.C (H-7); and B10.C (47N)—do not appear to contain new ecotropic endogenous viruses.

Although the Fv-2 gene locus was first identified as the major host gene controlling susceptibility to Friend spleen focus-forming virus (19), more recent studies by Axelrad and his colleagues have provided evidence that this gene, or a gene very closely linked to Fv-2, also plays a central role in regulating the proportion of normal erythroid progenitor cells (burst-forming unit-erythroid) that are in the S phase of the cell cycle in uninfected mice (35). Thus, the Fv-2 locus appears to be a hematopoietic regulatory gene involved in an early step in erythropoiesis. Although the new ecotropic provirus and the Fv-2 locus are closely linked, recombination between these two loci was observed. Nevertheless, depending on the molecular distance separating them, it may still be possible to clone this hematopoietic regulatory locus or other genes in this region of chromosome 9 by using this integrated proviral locus as a starting point in chromosome walking experiments.

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LITERATURE CITED


