

STRUCTURE AND EXPRESSION OF ENDOGENOUS  
ECOTROPIC MURINE LEUKEMIA VIRUSES  
IN RF/J MICE\*

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Ecotropic murine leukemia viruses (MuLV)<sup>1</sup> are integrated into multiple nonallelic sites in the chromosomes of the high leukemic mouse strains AKR, C3H/Fg, and C58 and are expressed in high titers of infectious MuLV throughout life (1, 2). MuLV of a similar biologic and serologic type have been recovered from cell cultures of the low leukemic strains BALB/c and C3H/He after treatment with halogenated pyrimidines (3) and also have been shown to be genetically transmitted (4). In the case of these two low virus strains that shared a common ancestor during inbreeding, evidence supports the contention that the ecotropic MuLV-inducing loci of these two strains are located at a single site on chromosome 5 (5, 6).

Recent studies in this laboratory have documented another distinguishing feature of high and low leukemic mouse strains—the relative inducibility of ecotropic MuLV. Ecotropic virus-inducing genes of high leukemic mice display a high frequency of MuLV production after treatment of cells *in vitro* with halogenated pyrimidines, whereas virus-inducing genes of low leukemic mice show a much lower frequency of MuLV production (7, 8). Such studies (8, 9) also have demonstrated that virus-linked genes can interact to increase the frequency of MuLV induction and spontaneous expression *in vivo* in specific F<sub>1</sub> hybrids of low leukemic mice.

It was, therefore, of interest to examine endogenous MuLV transmission by RF mice, a strain that was derived in parallel with AKR (10) and shows a moderate spontaneous incidence of leukemia late in life (11). RF mice are very sensitive to lymphoma induction by polycyclic hydrocarbons (11–13) or x-rays (14). Genetic studies indicated that cells of RF mice are restrictive to many N-tropic MuLV strains (15), and in crosses of RF with AKR, RF mice transmit a leukemia-suppressive gene tightly linked to the *Gpd-1* locus (16). Chen et al. (13) demonstrated that (DBA/2 × RF)F<sub>1</sub> hybrid mice express high levels of ecotropic MuLV, whereas DBA/2, RF, or (RF × DBA/2)F<sub>1</sub> mice do not, and Mayer et al. (17) demonstrated that RF mice transmit a single dominant gene for ecotropic MuLV expression as well as a maternal factor that suppresses MuLV expression. In the present study, we examined ecotropic MuLV expression in RF mice after induction of mouse embryo or tail biopsy cells with halogenated pyrimidines (18) and the genetic transmission of proviral genomes

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† *Abbreviations used in this paper:* IUdR, iododeoxyuridine; MuLV, murine leukemia virus; SDS, sodium dodecyl sulfate.

by DNA hybridization experiments with a molecularly cloned ecotropic-specific probe (19). Our studies indicate that RF mice transmit two dominant genes of different phenotype for ecotropic MuLV induction and three ecotropic proviral genomes.

### Materials and Methods

*Mice.* Mice of the following strains were purchased from The Jackson Laboratory, Bar Harbor, ME: AKR, DBA/2, SWR, 129, and RF. CBA and NFS mice were provided by Dr. Carl Hansen, Small Animal Unit, National Institutes of Health, Bethesda, MD. All crosses were performed at the McArdle Laboratory or Laboratory of Viral Disease, NIH, Bethesda, MD.

*Virus Induction.* Individual embryos from 14–18-d pregnant females or tail tissues from 1–14-d-old mice were removed and tissue cultures prepared as described (8). Confluent cultures were replated onto multiple 60-mm plates, each of which received  $2-3 \times 10^5$  cells. The following day, some cultures were treated with 20  $\mu\text{g}/\text{ml}$  iododeoxyuridine (IUdR) in medium. 2 d later, cultures were washed, and  $10^5$  SC-1 cells (20) were added in medium containing 3% fetal bovine serum and antibiotics. 8–10 d later, plates were developed in the standard XC plaque test (21), and MuLV-induced syncytia scored microscopically. Plates that did not receive IUdR showed no syncytia.

*Virus Expression In Vivo.* Virus expression in vivo was monitored by titration of virus from tail extracts (22).

*Isoenzyme Typing.* The isoenzyme markers *Pgm-1*, *Gpi-1*, *Gpd-1*, *Mod-1*, and *Es-3* were typed by standard procedures (23).

*DNA Extraction and Purification.* Spleens were surgically removed from appropriate mice and immediately frozen in liquid nitrogen. Individual spleens were ground to a fine powder, and the ground material was resuspended in 4.6 ml of STE buffer (100 mM NaCl, 20 mM Tris-base, pH 7.4, 10 mM EDTA), and brought to 200  $\mu\text{g}/\text{ml}$  with pre-digested Proteinase K (EM Biochemicals, Darmstadt, West Germany) and 0.4% sodium dodecyl sulfate (SDS). After incubation of the solution for 1 h at 37°C, an equal volume of buffered phenol (Mallinkrodt Inc., Science Products Div., St. Louis, MO) was added and used in an initial round of protein extraction, followed by two rounds of extractions using equal volumes of phenol and chloroform-isoamyl alcohol (24:1). Aqueous phases were carefully removed using wide-bore pipettes, and, after the addition of two volumes of ice cold 100% ethanol, DNA was spooled out of solution. DNA was resuspended in 10 mM Tris-base, pH 7.4, 0.1 mM EDTA, and stored at 4°C.

*Restriction Endonuclease Digestion and Gel Electrophoresis.* Restriction enzymes were acquired from New England Biolabs, Beverly, MA, and used under buffer and reaction conditions described by the supplier. In general, a twofold excess of enzyme was used over recommended amounts to digest 12  $\mu\text{g}$  of chromosomal DNA per enzymatic reaction. All reactions were monitored for completeness of digestion by adding a sample of each reaction to 0.5  $\mu\text{g}$  of SV40 or pBR322 DNA. Test reactions were electrophoresed through agarose gels (Seakem Marine Colloids Div., FMC Corp., Rockland, ME) in a Tris-acetate buffer, stained with ethidium bromide, and photographed under ultraviolet light. Those reactions judged to be complete were raised to 10 mM EDTA, 10 mM Tris, pH 8.1, 10% sucrose, 0.01% bromphenol blue, then electrophoresed through agarose gels until the bromphenol blue dye front had migrated 17 cm. *Hind* III digested  $\lambda$  DNA was run in parallel with chromosomal digests as molecular weight standards. Gels were stained using a 1  $\mu\text{g}/\text{ml}$  solution of ethidium bromide and photographed under shortwave ultraviolet light.

*DNA Transfer to Nitrocellulose and Filter Hybridization.* DNA was transferred to nitrocellulose *in situ* as follows: gels were soaked in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 60 min, then in neutralization buffer (0.5 M Tris-base, pH 7.5, 1.5 M NaCl) for 120 min, DNA was then allowed to transfer to nitrocellulose (Schleicher and Schuell) under a stream of 6 $\times$  SSC (0.15 M NaCl, 0.015 M  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ , pH 7.0) essentially by the method of Southern (24) for 48 h. Filters were baked *in vacuo* at 80°C for 3 h and preannealed for 18–24 h in a 41°C incubator using 5 ml of a buffer containing: 50% formamide, 20 mM Hepes, pH 7.4, 2 $\times$  Denhardt's solution (25), 25  $\mu\text{g}/\text{ml}$  yeast RNA, 100  $\mu\text{g}/\text{ml}$  denatured salmon sperm DNA, 0.4% SDS, and 3 $\times$  SSC. Filters were then hybridized with  $5 \times 10^6$  cpm of nick translated ecotropic

virus-specific probe in 5 ml of preannealing buffer for 40–48 h at 41°C. Hybridized filters were washed by the following protocol: each filter was rinsed for 15 min at room temperature with 2× SSC and 1× Denhardt's, then again for 45 min at room temperature with 2× SSC, and finally at 53°C with three changes of a solution of 0.1% SDS and 0.1× SSC. Filters were exposed to Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY) supplemented with a Dupont Cronex Lightning-Plus intensifying screen (Dupont Instruments, S & P Div., Wilmington, DE). Films were placed at –70°C for up to 5 d before development.

*Nick Translation of Ecotropic-specific Probe DNA.* The ecotropic specific DNA was kindly provided as plasmid pEC-B4 by Dr. Malcolm Martin; its construction and characterization have been reported elsewhere (19). Nick translation reactions were performed essentially as reported by Rigby et al. (26). After phenol-chloroform extraction and ethanol precipitation of labeled probe DNA, specific activities were found to be between 1.5 and  $2.5 \times 10^8$  cpm/ $\mu$ g of input probe DNA.  $\alpha$ - $^{32}$ P dCTP (600–800 Ci/mole; New England Nuclear, Boston, MA) was used exclusively as label for all nick translation reactions.

## Results

*Virus Activation in Crosses of CBA/N, 129, SWR, and NFS with RF: Identification of Rjv-1.* Several hundred (range of 200 to confluent) MuLV-induced syncytia were observed when cell cultures of RF or various RF F<sub>1</sub> hybrids were treated with IUdR, co-cultivated with SC-1 cells, and assayed for *in situ* UV-XC plaque formation. In contrast, parallel tests with cells from CBA/N, NFS, 129, or SWR mice yielded no syncytia, and 0–2 syncytia were observed on DBA/2 cells. Thus, the pattern of activation of ecotropic MuLV by IUdR seen in RF cultures behaved as a dominant, highly penetrant trait in F<sub>1</sub> hybrids. One-half (145/288) of the mice from the first backcross (Bcl) generation to virus-negative strains shared the virus activation phenotype of the RF grandparent, a result consistent with the transmission of a single dominant virus-inducing gene from RF mice. Progeny tests of Bcl animals and tests of individual F<sub>2</sub> cultures confirmed this segregation pattern in that virus-positive Bcl animals transmitted the virus-inducing phenotype to 50% (35/71) of their progeny (Table I). From these results, we conclude that in crosses with CBA/N, NFS, 129, and SWR, RF mice transmit a single dominant gene for high (AKR-like) ecotropic MuLV activation. We shall refer to this locus as *Rjv-1* in accordance with established nomenclature.

*Virus Activation in Crosses of DBA/2 with RF: Identification of Rjv-2.* F<sub>1</sub> hybrids of DBA/2 with RF, like other F<sub>1</sub> hybrids with RF, yielded cultures that showed several hundred syncytia after IUdR treatment (Table II). However, the pattern of virus induction found in cultures of [DBA/2 × (DBA/2 × RF)F<sub>1</sub>] Bcl embryos was more complex than that found in the previous crosses. As demonstrated by the data in Fig. 1, Bcl embryos derived from crosses of DBA/2 with RF yielded cultures that showed the parental phenotypes of RF and DBA/2 as well as an intermediate number of syncytia upon IUdR induction, a phenotypic pattern not observed in backcross embryos from crosses of 129 or SWR with RF performed in parallel tests (Fig. 1). Because we observed 0–2 *in situ* syncytia on induction of DBA/2 embryo cultures and consistently observed  $\geq 200$  plaques on induction of embryo cultures from crosses of 129 or SWR with RF, we selected these two values for the *in situ* syncytia values assigned to the DBA/2 parental class and the segregation of *Rjv-1*, respectively. Based on this classification, we find that 51% (147/291) of the [DBA/2 × (DBA/2 × RF)F<sub>1</sub>] embryos showed the *Rjv-1* phenotype, 24% (70/291) showed the DBA/2 parental phenotype, and 25% (74/291) showed an intermediate phenotype of 5–200 syncytia

TABLE I  
*Induction of Infectious Ecotropic MuLV in Crosses of RF to CBA/N, NFS/N, SWR, and 129 Mice*

Generation	Strain	$\frac{\text{Number of positive mice}}{\text{Number of total mice}}$	Percent positive
Parental	RF/J (RF)*	17/17‡	100
	CBA/N (CB)	0/4	0
	129/J (I)	0/9	0
	SWR/J (S)	0/24	0
	NFS/N (N)	0/22	0
	DBA/2J (D2)	5/10‡	50
F <sub>1</sub> Hybrid	CB × RF	17/17	100
	1 × RF	5/5	100
	S × RF	11/11	100
	N × RF	1/1	100
Bc1 to low-virus Parental strain	S × (S × RF)*	33/71	46
	1 × (1 × RF)*	64/119	54
Bc1 to nonparental Low-virus strain Total Bc1	(CB × RF) × N	16/26	62
	N × (CB × RF)	32/72	44
		145/288	50
Bc2 of virus-positive Bc1 mice to low-virus strain Total virus-positive Bc2	N × [N × (CB × RF)]	15/30	50
	S × [S × (S × RF)]	15/29	52
	1 × [1 × (1 × RF)]	5/12	42
		35/71	49
Bc2 of virus-negative Bc1 mice to low-virus strains	N × [N × (CB × RF)]	0/11	0
	S × [S × (S × RF)]	0/7	0
		0/18	0
F <sub>2</sub> with low-virus strains Total F <sub>2</sub>	(S × RF)F <sub>2</sub>	25/34	71
	(1 × RF)F <sub>2</sub>	39/46	85
		63/80	79

\* Abbreviations used in Tables I and II.

‡ RF cells showed  $\geq 200$  syncytia per  $2.5 \times 10^5$  induced cells, whereas DBA/2 cells showed 0.7 syncytia per  $2.5 \times 10^5$  induced cells.

per  $2.5 \times 10^5$  induced cells (Table II). These data indicated the independent segregation of a second RF ecotropic MuLV-inducing gene, which we shall refer to as *Rjv-2*. Cells of mice that carry *Rjv-2* alone had a lower frequency of MuLV expression after IUdR treatment than cells of mice that carry *Rjv-1*. Induction of virus from *Rjv-2* was apparently dependent on a gene(s) from DBA/2 for its expression; the 129, NFS, or SWR genetic backgrounds were insufficient for the detection of *Rjv-2* in this test system.

The existence of a second virus-inducing gene in RF was confirmed in two types of crosses. Two different male RF mice were mated to females of the SWR strain, and the male F<sub>1</sub> offspring were crossed to NFS and to DBA/2 females. Individual backcross mice were tested for virus induction with tail biopsy cells and classified according to their phenotype (Table II). The results of these crosses confirmed that RF mice carried two MuLV induction loci of different phenotype because an intermediate induction

TABLE II  
*Induction of Ecotropic MuLV by IUdR Treatment of Tissue Cultures from Crosses of DBA/2 with RF Mice*

Generation	Strain	Virus induction phenotype (syncytia/culture)		
		0-2 (DBA/2)	5-200 (Intermediate)	≥200 (RF)
Parental	RF			8/8 (100)
	DBA/2 (D2)	10/10 (100)		
F <sub>1</sub>	D2 × RF			5/5 (100)
Bc1	D2 × (D2 × RF)	70/291 (24)	74/291 (25)	147/291 (51)
	Progeny of RF 4007*			
	D2 × (S × RF)	12/36 (33)	12/36 (33)	12/36 (33)
	N × (S × RF)	8 /17 (47)		9/17 (53)
	Progeny of RF 4008*			
	D2 × S × RF	10/31 (32)	6/31 (19)	15/31 (48)
N × S × RF	10/19 (53)		9/19 (47)	
Bc2 DBA/2	D2 × (N × S × RF)*			
Progeny of N × SWR × RF virus-negative mice	4234	4/8 (50)	4/8 (50)	
	4233	9/9 (100)		
	4224	8/8 (100)		
	4236	14/14 (100)		
	4240	7/12 (58)	5/12 (42)	
	4219	7/11 (64)	4/11 (36)	

\* Performed with tail biopsy tissue cultures.

phenotype was observed when (SWR × RF) mice were crossed to DBA/2 mice, and an intermediate phenotype was not observed when (SWR × RF) mice were crossed to NFS. We further infer from the data of these crosses that the DBA/2 gene(s) necessary for expression of the *Rjv-2* virus-positive phenotype is dominant in its action because individual outcross mice had only one DBA/2 gene complement. The independent segregation of a low inducible gene was confirmed when six individual NFS × (SWR × RF) Bc1 mice of the *Rjv-1*<sup>-/-</sup> genotype, as judged by the results of induction of tail biopsy cells, were crossed to DBA/2 and the progeny of each Bc1 mouse were examined for MuLV induction (Table II). Of the six backcross mice, three generated virus-positive progeny in approximately the proportion expected (observed: 13 positive and 18 negative; expected: 15.5 of each class), and the remaining three Bc1 mice failed to generate positive progeny (0/31). These results establish the stable and independent segregation of *Rjv-2* and *Rjv-1* and indicate that *Rjv-2* requires a gene(s) of DBA/2 for its detection.

One problem with these experiments is the high proportion of noninducible progeny obtained from mice expected to carry *Rjv-2*<sup>+/-</sup> (40/97 observed vs. 24/97 expected). It is likely that this discrepancy reflects the use of tail biopsy cells rather than embryo cells in the test culture. Two mice that were phenotyped as *Rjv-1*<sup>-/-</sup>, *Rjv-2*<sup>-/-</sup> by use

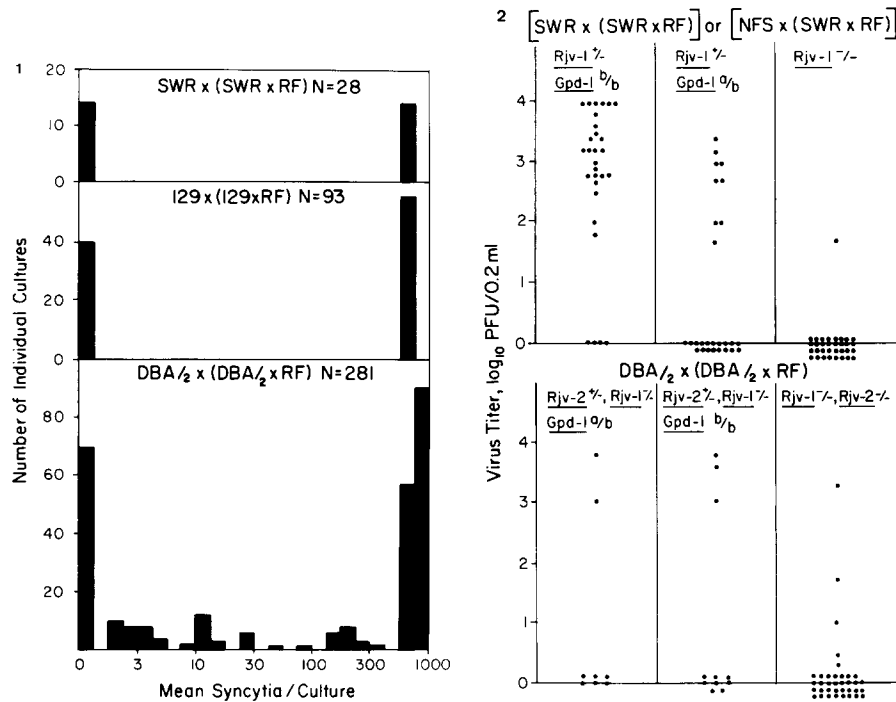


FIG. 1. Number of *in situ* virus-producing cells after IUDR treatment of embryo cultures from individual backcross mice. The backcrosses and numbers of individuals examined are indicated in each panel. Only data accumulated from experiments performed in parallel are included in this figure; more extensive data on 129 and SWR crosses are reported in Table I.

FIG. 2. Spontaneous virus production in tail tissue of backcross mice. Mice from the indicated crosses were analyzed for inheritance of *Rjv-1* and *Rjv-2*, judged from induction tests with tail biopsy cells of newborn mice, for inheritance of *Gdp-1* isoenzyme in kidney extracts and for expression of infectious MuLV in 0.2 ml of a 10% wt/vol extract of tail tissue from 2–4-mo-old mice. Thus, each dot reflects a mouse analyzed for all three traits.

of tail biopsy cells yielded progeny embryos of intermediate phenotype when crossed to DBA/2, consistent with a *Rjv-2*<sup>+/-</sup> genotype.

**Genetic Linkage of *Rjv* Loci.** The linkage of *Rjv-1* to several mouse chromosomes known to carry endogenous viruses in other strains has been examined by determining whether *Rjv-1* was coordinately inherited with the genetic markers of the RF grandparent found on chromosomes 7, 4, 8, 5, and 11. No association of *Rjv-1* inheritance with any of these markers was observed in the 32–84 individuals tested for these traits, nor was *Rjv-1* allelic to the *Akv-2* virus-inducing gene of AKR mice (D. J. Grunwald, unpublished observations).

We also examined the linkage of *Rjv-2* to genetic markers on chromosomes 7, 4, 5, and 11 in the 24 individual mice that carried only *Rjv-2* and were available for tests of isoenzymes. Positive association ( $P = 0.05$ ) between inheritance of *Rjv-2* and *Pgm-1*, which is located on chromosome 5, was observed. 4/24 of the segregating mice were recombinant for *Pgm-1* and *Rjv-2*, a result that places *Rjv-2* at  $16 \pm 7$  centimorgans from *Pgm-1*. Because we recently identified a gene of BALB/c mice on chromosome 5 that interacts with a gene of C57BL/6 mice to enhance ecotropic virus induction (8, 27), we crossed an *Rjv-1*<sup>-/-</sup>, *Rjv-2*<sup>+/-</sup> mouse to C57BL/6. Of the 20 progeny

obtained, 9 yielded cultures of the more inducible phenotype, a result that indicates that virus production can be induced from *Rjv-2* in conjunction with a gene(s) of B6 as well as in conjunction with a gene of DBA/2.

*Spontaneous Virus Expression in Tail Tissue from Rjv-1, Rjv-2 Genotyped Backcross Mice.* When RF mice were tested for spontaneous virus expression in vivo, they were found to be quite low, and in crosses with AKR, RF mice transmitted a gene linked to *Gpd-1* that suppresses ecotropic virus expression (16). Although RF mice carried a highly efficient ecotropic virus-inducing locus *Rjv-1*, only low titers of ecotropic MuLV were recovered from tail extracts of RF mice ( $\leq 10$  infectious U/0.2 ml). To explore the role of *Rjv* loci and other MuLV regulatory genes on spontaneous virus expression by these mice, individual [NFS  $\times$  (SWR  $\times$  RF)F<sub>1</sub>] or [SWR  $\times$  (SWR  $\times$  RF)F<sub>1</sub>] Bcl animals were examined for the presence of infectious MuLV in tail extracts, a test for viremia (22).

Clearly, mice that have not inherited *Rjv-1* did not express significant titers of MuLV in vivo (Fig. 2, top right panel), although one-half of these mice would be expected to have inherited *Rjv-2*. Among Bcl mice that had inherited *Rjv-1*, considerable heterogeneity in spontaneous virus expression was apparent (Fig. 2, top left and middle panels). When these *Rjv-1*<sup>+/-</sup> Bcl mice were grouped into those that inherited the *Fv-1* region (*Gpd-1*<sup>b/b</sup>, Fig. 2) from their SWR grandmother or into those that inherited the *Fv-1* region from their RF grandfather (*Gpd-1*<sup>a/b</sup>, Fig. 2), distinct patterns of virus expression were apparent. The majority (25/29) of the *Rjv-1*<sup>+/-</sup>, *Gpd-1*<sup>b/b</sup> Bcl mice expressed infectious MuLV with a median titer of  $2 \times 10^3$  infectious U/0.2 ml. The majority (18/27) of *Rjv-1*<sup>+/-</sup>, *Gpd-1*<sup>a/b</sup> Bcl mice did not express infectious virus; the median titer of those that did was  $5 \times 10^2$  infectious U/0.2 ml. These results indicate that the *Rjv-1* locus confers higher levels of MuLV production on mice in the absence of inhibitory genes linked to the *Gpd-1* locus, presumably *Fv-1*. Thus, the *Fv-1* allele of RF may partially suppress expression of the RF endogenous ecotropic virus (Fig. 2) in addition to suppressing the endogenous ecotropic virus of AKR (16), and the *Fv-1* allele of RF may in part account for the low amounts of ecotropic MuLV recovered from RF mice.

The expression of infectious ecotropic virus in vivo has also been examined in mice from DBA/2 backcross populations, where induction of *Rjv-2* was also detected. Although only 19 *Rjv-1*<sup>-/-</sup>, *Rjv-2*<sup>+/-</sup> segregants were analyzed for both spontaneous virus expression in vivo and *Gpd-1* inheritance, it was apparent that *Rjv-2* did not frequently lead to viremia. Of these 19 segregants, 14 produced no ecotropic MuLV in tail extracts, and the virus-negative mice appeared to be equally distributed among *Gpd-1* types. This low level of expression was not significantly different from that seen in backcross mice genotyped as *Rjv-1*<sup>-/-</sup>, *Rjv-2*<sup>-/-</sup> (5/36 vs. 5/19). These data indicate that inheritance of *Rjv-2*, unlike inheritance of *Rjv-1*, does not lead to high levels of MuLV expression in vivo.

*Ecotropic Proviral Genomes in RF Mice.* Previous studies (29, 19) on AKR mice and NIH mice congenic for *Akv* virus-inducing loci indicated that MuLV-inducing genes co-segregated with ecotropic proviral nucleotide sequences. To determine whether ecotropic proviral genomes segregated with *Rjv* loci, we examined the DNA of RF and *Rjv* segregating mice by the procedure of Southern (24), using a molecularly cloned probe that recognizes a 500 base pair region of the *env* gene of ecotropic MuLV and does not hybridize to xenotropic MuLV (19).

To determine whether the ecotropic proviruses of RF mice are of genome length and are similar to the endogenous ecotropic viruses of AKR mice (Fig. 3), RF DNA was digested with the restriction endonucleases *Pst* I, *Kpn* I, or *Bam* HI and analyzed by the method of Southern with the ecotropic MuLV-specific probe. The autoradiogram in Fig. 4 demonstrates that ecotropic virus fragments of 8.9 kb, 4.4 kb, and 3.3 kb were obtained after digestion of RF DNA with those enzyme. Thus, these results are consistent with the presence of intact ecotropic proviral sequences in the genomic DNA of RF mice and do not indicate the presence of subgenomic fragments with homology to the ecotropic virus envelope gene fragment.

The number of ecotropic proviruses in the genome of RF mice was established by digestion of RF DNA with *Pvu* II, an enzyme that cleaves twice within the provirus on the 5' side of the ecotropic-specific sequence. Digestion with *Pvu* II will yield two proviral fragments with adjoining cellular DNA sequences. If the sites of integration of the proviruses are different, junction fragments would be expected to differ in molecular size due to differences in the restriction endonuclease sites within the adjoining cellular DNA. The ecotropic-specific probe detects one of these fragments for each integrated provirus, the 3' virus-cell junction fragment in the case of *Pvu* II.

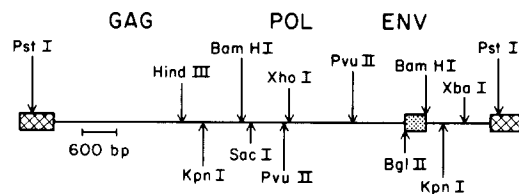


FIG. 3. Restriction endonuclease map of endogenous ecotropic MuLV. The genes for viral core antigens, GAG, DNA polymerase, POL, and envelope glycoproteins, ENV, are illustrated as well as the proviral LTR (long terminal repeats) found at the termini of viral DNA. The ecotropic virus-specific fragment derived from the envelope gene and carried by clone pEC-B4 is represented by the shaded box (19).

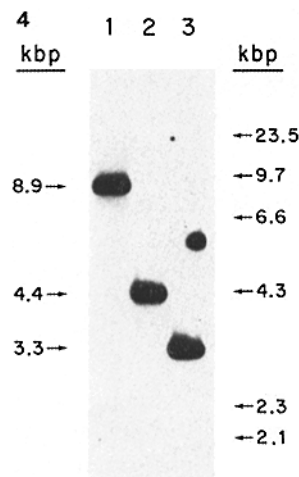


FIG. 4. Internal organization of ecotropic proviruses of RF/J mice. Spleen DNA from an RF/J mouse was digested with *Pst* I, *Kpn* I, or *Bam* HI, electrophoresed in a 0.7% agarose gel, as described in Materials and Methods, and hybridized with  $^{32}$ P-labeled clone pEC-B4. 12  $\mu$ g of DNA was digested with each enzyme: (lane 1) *Pst* I digestion; (lane 2) *Kpn* I digestion; (lane 3) *Bam* HI digestion. *Hind* III-digested  $\lambda$  DNA size standards are given to the right of the figure.



Digestion of RF DNA with *Pvu* II and Southern analysis with this probe revealed three fragments of 6.4, 4.6, and 3.8 kb, a result that indicates that three ecotropic *env* genes are present in RF DNA (Fig. 5). Combined with the results of RF DNA digestion with *Pst* I, *Kpn* I, and *Bam* HI, we conclude that each of the three *env* genes is associated with an intact ecotropic proviral genome.

To determine which ecotropic proviral genomes are associated with which *Rjv* loci, DNA from [DBA/2 × (DBA/2 × RF)] Bc1 mice, which were phenotyped for *Rjv-1* and *Rjv-2*, or DNA from Bc2 progeny of *Rjv-1*<sup>-/-</sup> [NFS × (SWR × RF)] mice, which were phenotyped for *Rjv-2*, were analyzed for ecotropic sequences by the Southern procedure using the enzyme *Pvu* II (Fig. 5). The results of the analysis of segregating mice, presented in Table III, demonstrate that the *Rjv-1*<sup>+</sup> phenotype cosegregates with the presence of the 6.4 and 4.6 kb *Pvu* II fragments (*P* < 0.01). In 15 Bc1 mice, these two junction fragments segregate together. This result suggests that these proviruses may be genetically linked. The 3.8 kb *Pvu* II fragment segregates independently of the *Rjv-1* phenotype. Thus, if the *Rjv-1* function is carried within an ecotropic proviral sequence, it must be encoded by one or both of the genomes

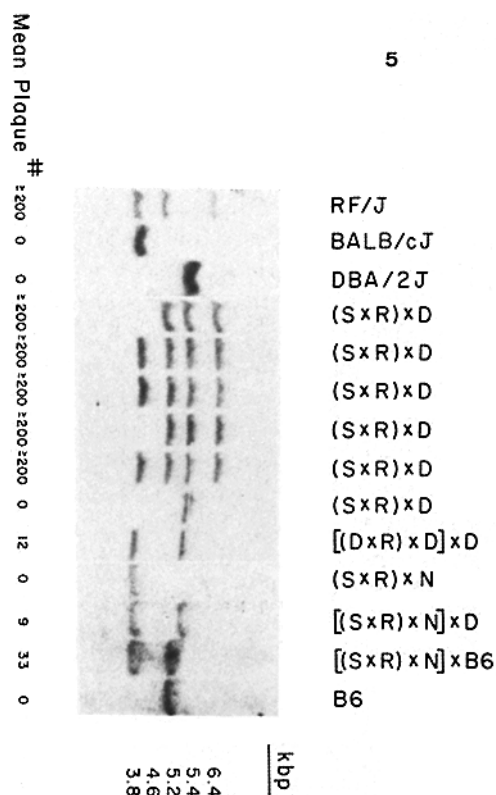


FIG. 5. Segregation of ecotropic proviruses in RF/J backcross mice. Spleen DNA from RF/J proviral segregants were digested with *Pvu* II and analyzed as in Fig. 4. Below each lane are mean plaque values recorded for  $2.5 \times 10^5$  tail biopsy cells from the same mouse induced with IUdR. DNA was isolated from segregants resulting from the crosses performed as indicated above each lane. Mouse strain abbreviations are as follows: S, SWR/J; N, NFS/N; D, DBA/2J; B6, C57BL/6J, R, RF/J.

TABLE III  
*Association of Rjv Virus-Inducing Loci with Ecotropic Proviral Cellular DNA  
 Junction Fragments*

Phenotype	Number of mice carrying an RF Pvu II fragment				
	Total	6.4 kb	4.6 kb	3.8 kb	No frag- ment
<i>Rjv-1</i> <sup>+</sup> *	6	6	6	3	0
<i>Rjv-1</i> <sup>-</sup> *	9	0	0	5	4
<i>Rjv-1</i> <sup>-</sup> , <i>Rjv-2</i> <sup>+</sup> ‡	12	0	0	12	0
<i>Rjv-1</i> <sup>-</sup> , <i>Rjv-2</i> <sup>-</sup> ‡	11	0	0	4	7

\* DNA from mice of the [DBA/2 × (DBA/2 × RF)] Bc1 generation was analyzed.

‡ DNA from mice of the [DBA/2 × (DBA/2 × RF)] Bc1, and DBA/2 or B6 × [*Rjv-1*<sup>-/-</sup> [NFS × (SWR × RF)] Bc2 population was analyzed.

identified by the *Pvu* II 6.4 or 4.6 kb fragments. DBA/2 mice carry an ecotropic provirus genome that yields a 5.4 kb 3' junction fragment upon digestion with *Pvu* II and hybridization with the ecotropic-specific probe (29); this band is apparent in the DNA samples from DBA/2 backcross mice (Fig. 5). B6 mice carry an ecotropic provirus that yields a 5.2 kb 3' junction fragment upon digestion with *Pvu* II and hybridization with the ecotropic-specific probe, and this fragment is apparent in DNA from the B6 × [*Rjv-1*<sup>-/-</sup> NFS × (SWR × RF)] mouse.

The *Rjv-2* phenotype cosegregates with the ecotropic genome detected in the 3.8 kb *Pvu* II fragment in that all *Rjv-2*<sup>+/-</sup> mice carry this sequence. 7 of the 11 *Rjv-1*<sup>-/-</sup>, *Rjv-2*<sup>-/-</sup> mice lack the 3.8 kb *Pvu* II ecotropic *env* gene fragment. The remaining four DBA/2 × [NFS × (SWR × RF)] mice that contained this fragment yet showed no syncytia on induction probably result from false-negative results of IUdR induction tests and not recombination between *Rjv-2* and proviral sequences. The induction tests of these four mice were done with tail biopsy cells, and, as noted earlier, these cells tend to be less sensitive to induction than mouse embryo cells. Two other examples of possible false negatives were encountered in these experiments. On progeny tests with DBA/2 these false negative mice yielded *Rjv-2*<sup>+</sup> progeny, the DNA of which carried a 3.8 kb *Pvu* II ecotropic-hybridizing fragment (data not shown). Unfortunately, the 4 *Rjv-2*<sup>-/-</sup> provirus-positive mice presented in Table III were no longer available for breeding experiments.

In conclusion, *Rjv-1* cosegregates with two proviral genomes, represented by 6.4 and 4.6 kb *Pvu* II junction fragments, and *Rjv-2* cosegregates with the proviral genome identified by a 3.8 kb *Pvu* II junction fragment.

*Structure of Rjv-1 and Rjv-2 Ecotropic Proviral Genomes.* The single ecotropic provirus of BALB/c mice yields a similar 3.8 kb junction fragment after digestion of BALB/c DNA with *Pvu* II and hybridization with the ecotropic-specific probe (Fig. 6). We examined the possibility that the *Rjv-2* provirus was located in cellular DNA sequences similar to those found adjacent to the BALB/c provirus. This was done by cleaving RF DNA and BALB/c DNA with the enzymes *Eco* RI, *Xba* I, *Hind* III, *Sac* I, or *Pvu* II and resolving the DNA fragments in parallel by the Southern procedure. The results of that experiment, presented in Fig. 6, demonstrate that digestion of DNA

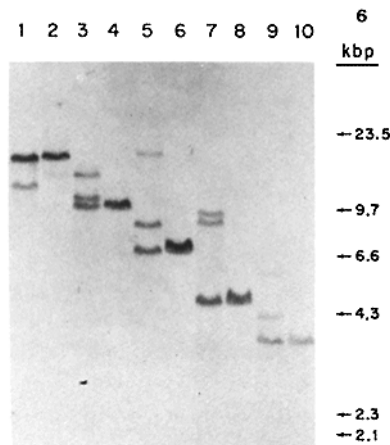


FIG. 6. Comparison between integration sites of RF/J and BALB/c ecotropic proviruses using five restriction enzymes. Spleen DNA were digested and analyzed as in Fig. 4. RF/J DNA was electrophoresed in lanes 1, 3, 5, 7, and 9; BALB/c DNA was electrophoresed in lanes 2, 4, 6, 8, and 10. (lanes 1 and 2) *Eco* RI digestion; (lanes 3 and 4) *Xba* I digestion; (lanes 5 and 6) *Hind* III digestion; (lanes 7 and 8) *Sac* I digestion; (lanes 9 and 10) *Pvu* II digestion.

with each enzyme yields an ecotropic *env*-containing fragment from RF and from BALB/c that co-migrate. Therefore, from the patterns of junction fragments generated with five restriction endonucleases and the genetic linkage of *Rjv-2* to *Pgm-1*, it seems reasonable to conclude that the *Rjv-2* provirus is located at a position similar to that found for the BALB/c ecotropic provirus.

It is also apparent in Fig. 6 that only two *Eco* RI fragments of RF DNA hybridized with the ecotropic-specific probe. To determine whether two *Eco* RI fragments had by chance co-migrated, similar DNA samples were electrophoresed through a 0.4% agarose gel and analyzed by the same procedure. Under conditions that resolved fragments differing by 50 bp, only two *Eco* RI ecotropic *env*-containing fragments were detected in RF DNA, one of which corresponded to *Rjv-2*. Although the results of this experiment do not exclude co-migration of ecotropic *env*-containing fragments, they do indicate that such fragments must be quite close in size. Judging by the difference in intensity exhibited by the two *Eco* RI fragments, it is likely that the larger 19-kb fragment is the site of comigration. From the pattern of junction fragments generated with other enzymes and from the size of the *Eco* RI fragments, we consider it unlikely that two ecotropic proviruses are contained on one *Eco* RI fragment. An alternative explanation is that one ecotropic provirus is contained in a *Eco* RI fragment that is randomly sheared during DNA isolation and is, therefore, not resolved. Other more complicated explanations are also possible.

### Discussion

Data from backcross generations of RF mice with several low-virus or virus-negative strains indicated that RF mice transmit a locus (*Rjv-1*) that confers the high virus phenotype after IUdR induction of fibroblastic cells. The pattern of virus induction in mice that carry *Rjv-1* was similar to that of mice that carry the *Akv* loci of AKR. In crosses of RF mice with SWR mice, the inheritance of *Rjv-1* is sufficient to lead to viremia when the suppressive RF *Fv-1* allele is absent. However, *Rjv-1* differs from

*Akv-1* or *Akv-2* (30, 31) in its chromosomal location. In addition, the arrangements of restriction endonuclease sites in cell DNA adjoining *Akv-1* or *Akv-2* (19) differ from those found adjacent to any of the ecotropic proviruses of RF mice. Thus, although RF mice are related in pedigree to AKR and share many isoenzyme and alloantigenic markers with AKR (32), the locations of the ecotropic proviruses in these two strains are clearly different.

Results from crosses of RF with DBA/2 demonstrated the segregation of a second virus-inducing locus in addition to *Rjv-1*. This second locus, termed *Rjv-2*, differs from *Rjv-1* both in phenotype and chromosomal position. Genetic studies indicated that *Rjv-2* is located on chromosome 5, as is the BALB/c virus-inducing gene *Cv-1* (5, 6), and segregates independently from *Rjv-1*. Judged by the size similarity of DNA fragments generated with five different restriction endonucleases, this proviral genome is located in cellular DNA similar to that found at the integration site of the endogenous BALB/c ecotropic provirus. Such a result is not expected because RF mice are not related to BALB/c in pedigree (32). Coincident proviral genome integration events presumably reflect contamination of RF at some point by BALB/c or two independent virus integration events that resulted in a similar integration site, a process not thought to take place under most conditions.

The phenotype of *Rjv-2* also differed from that of *Rjv-1*. A lower frequency of virus-producing cells was observed after IUdR induction of cells that contained only *Rjv-2* than of cells that contained *Rjv-1*. Indeed, detection of *Rjv-2* depended upon a dominant gene(s) supplied by DBA/2 or B6 because virus induction from *Rjv-2* was not observed in crosses with the ecotropic virus-negative strains SWR, 129, NFS, or CBA/N. Moreover, inheritance of *Rjv-2* did not lead to high levels of ecotropic MuLV expression in vivo.

Recent work with other mouse strains indicates that systems of genetic interaction for virus induction are by no means unique to *Rjv-2* (8). Experiments in this laboratory (8, 9) have demonstrated that BALB/c and C57BL/6 mice each carry a single locus, *Inc-1* and *Inb-1* respectively, that in combination enhance virus induction in vitro and spontaneous expression of MuLV in vivo. Enhanced virus induction was not observed in crosses of BALB/c or C57BL/6 with 129, SWR, or NFS. In view of the similar locations of *Rjv-2* and the BALB/c gene *Inc-1* (27) and the similar locations in cell DNA of the ecotropic proviruses associated with these loci, it is possible that *Rjv-2* and *Inc-1* are identical and encoded by ecotropic proviral sequences. To date, however, very little virus induction has been observed in (BALB/c  $\times$  DBA/2) $F_1$  cells, and so additional genetic factors or subtle differences between *Inc-1* and *Rjv-2* may exist.

The existence of specific loci that lead to different levels of MuLV expression after induction indicates a more complex biology than was initially suspected for endogenous ecotropic MuLV. Whether these different patterns of expression result from different chromosomal locations of the proviruses or differences within specific ecotropic proviruses remains to be determined.

### Summary

High leukemic mouse strains possess proviral genomes that are more inducible for virus expression by halogenated pyrimidines than the proviral genomes harbored by low leukemic mice. We investigated the induction and arrangement of ecotropic proviruses in RF mice, a strain of mouse that develops a moderate incidence of

leukemia late in life. We found that RF mice, unlike either high or low leukemic inbred strains, carried both a gene for high efficiency virus induction (*Rjv-1*) and a gene for low efficiency virus induction (*Rjv-2*). Virus induction from mice that contained *Rjv-2* alone was observed only in crosses with two other strains that carried ecotropic proviruses, i.e., DBA/2 and C57BL/6, and not in crosses performed with mice that lacked ecotropic proviruses, i.e., 129, SWR, and NFS. Inheritance of the *Rjv-1* gene frequently resulted in viremia when a virus-suppressive gene(s) of RF (most likely *Fv-1*) was not present in the same individual. *Rjv-1* and *Rjv-2* virus induction genes co-segregated with ecotropic proviruses integrated in different cellular DNA sequences. *Rjv-2*, the less inducible ecotropic provirus of RF mice, is located in cellular DNA sequences very similar to those found adjacent to the ecotropic provirus of BALB/c. These results document a second system of virus interaction or complementation and demonstrate that ecotropic proviruses of different phenotypes can be found within an individual mouse strain.

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