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

BY JAMES McCUBREY,‡ JONATHAN M. HOROWITZ, AND REX RISSE$§
From the McArdle Laboratory for Cancer Research, Madison, Wisconsin 53706

Ecotropic murine leukemia viruses (MuLV)¹ 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 F₁ 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 Gpd₁ locus (16). Chen et al. (13) demonstrated that (DBA/2 × RF)F₁ hybrid mice express high levels of ecotropic MuLV, whereas DBA/2, RF, or (RF × DBA/2)F₁ 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.

* Supported by grants CA-22443 and CA-07175 from the National Cancer Institute.
‡ Present address is the Basel Institute for Immunology, Basel, Switzerland.
§ Scholar of the Leukemia Society of America, Inc.
¹ Abbreviations used in this paper: IUdR, Iododeoxyuridine; MuLV, murine leukemia virus; SDS, sodium dodecyl sulfate.
STRUCTURE OF MURINE LEUKEMIA VIRUSES IN RF/J MICE

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 × 10⁵ cells. The following day, some cultures were treated with 20 μg/ml iododeoxyuridine (IUdR) in medium. 2 d later, cultures were washed, and 10⁶ 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 Er-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 μg/ml with predigested 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-isooamyl 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 μg of chromosomal DNA per enzymatic reaction. All reactions were monitored for completeness of digestion by adding a sample of each reaction to 0.5 μg of SV40 or pBR322 DNA. Test reactions were electrophoresed through agarose gels (Seakem Marine Celloids 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. HindIII digested λ DNA was run in parallel with chromosomal digests as molecular weight standards. Gels were stained using a 1 μg/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 6X SSC (0.15 M NaCl, 0.015 M Na₂HPO₄·2H₂O, 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, 2X Denhardt's solution (25), 25 μg/ml yeast RNA, 100 μg/ml denatured salmon sperm DNA, 0.4% SDS, and 3X SSC. Filters were then hybridized with 5 × 10⁶ 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 2X SSC and 1X Denhardt's, then again for 45 min at room temperature with 2X SSC, and finally at 53°C with three changes of a solution of 0.1% SDS and 0.1X 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 X 10⁶ cpm/μg of input probe DNA. α-32P dCTP (600-800 Ci/m 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 F₁ 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 F₁ 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 F₂ 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.** F₁ hybrids of DBA/2 with RF, like other F₁ 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)F₁] 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 ≥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)F₁] 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.
1464 STRUCTURE OF MURINE LEUKEMIA VIRUSES IN RF/J MICE

Table I

| Generation       | Strain                  | Number of positive mice | Percent positive |
|------------------|-------------------------|-------------------------|------------------|
| Parental         | RF/J (RF)*              | 17/17                   | 100              |
|                  | CBA/N (CB)              | 0/4                     | 0                |
|                  | 129/J (1)               | 0/9                     | 0                |
|                  | SWR/J (S)               | 0/24                    | 0                |
|                  | NFS/N (N)               | 0/22                    | 0                |
|                  | DBA/2J (O2)             | 5/10‡                   | 50               |
| F1 Hybrid        | CB × RF                 | 17/17                   | 100              |
|                  | 1 × RF                  | 5/5                     | 100              |
|                  | S × RF                  | 11/11                   | 100              |
|                  | N × RF                  | 1/1                     | 100              |
| Bcl to low-virus | S × (S × RF)*           | 33/71                   | 46               |
| Parental strain  | 1 × (1 × RF)*           | 64/119                  | 54               |
| Bcl to nonparent | (CB × RF) × N           | 16/26                   | 62               |
| Low-virus strain | N × (CB × RF)           | 32/72                   | 44               |
| Total Bc1        |                         | 145/298                 | 50               |
| Bc2 of virus-positive | N × [N × (CB × RF)]   | 15/30                   | 50               |
| Bc1 mice to low-virus | S × [S × (S × RF)]   | 15/29                   | 52               |
| strain           | 1 × [1 × (1 × RF)]      | 5/12                    | 42               |
| Total virus-positive Bc2 |                  | 35/71                   | 49               |
| Bc2 of virus-negative | N × [N × (CB × RF)]   | 0/11                    | 0                |
| Bc1 mice to low-virus | S × [S × (S × RF)]   | 0/7                     | 0                |
| strains          |                         | 0/18                    | 0                |
| F2 with low-virus | (S × RF)F2             | 25/34                   | 71               |
| strains          | (1 × RF)F2              | 39/46                   | 85               |
| Total F2         |                         | 63/80                   | 79               |

* Abbreviations used in Tables I and II.
‡ RF cells showed ≥200 syncytia per 2.5 × 10^5 induced cells, whereas DBA/2 cells showed 0.7 syncytia per 2.5 × 10^5 induced cells.

per 2.5 × 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 F1 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
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 \( R_{jw-2} \) 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) Bcl mice of the \( R_{jw-1}^{-/-} \) genotype, as judged by the results of induction of tail biopsy cells, were crossed to DBA/2 and the progeny of each Bcl 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 Bcl mice failed to generate positive progeny (0/31). These results establish the stable and independent segregation of \( R_{jw-2} \) and \( R_{jw-1} \) and indicate that \( R_{jw-2} \) 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 \( R_{jw-2}^{+/+} \) (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 \( R_{jw-1}^{-/-}, R_{jw-2}^{+/+} \) by use

### 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                    |             |                              |            |
|            | DBA/2 (D2)            | 10/10 (100) |                              |            |
|            | N × (S × RF)          | 8/17 (47)   | 9/17 (53)                   |            |
|            | N × S × RF            | 10/19 (53)  |                              | 9/19 (47)  |
| F1         | D2 × RF               | 5/3 (100)   |                              |            |
| Bc1        | D2 × (D2 × RF)        | 70/291 (24) | 74/291 (25)                  | 147/291 (51) |
| Progeny of | D2 × (S × RF)         | 12/36 (33)  | 12/36 (33)                  | 12/36 (33)  |
| RF 4007    | N × (S × RF)          | 8/17 (47)   | 9/17 (53)                   |            |
| Progeny of | D2 × S × RF           | 10/31 (32)  | 6/31 (19)                   | 15/31 (48)  |
| RF 4008    | N × S × RF            | 10/19 (53)  |                              | 9/19 (47)  |
| Bc2 DBA/2  | D2 × (N × S × RF)*    |             |                              |            |
| Progeny of | 4234                  | 4/8 (50)    | 4/8 (50)                    |            |
| N × SWR × RF | 4233                | 9/9 (100)   |                              |            |
| virus-negative mice | 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.*
of tail biopsy cells yielded progeny embryos of intermediate phenotype when crossed to DBA/2, consistent with a Rjv-2+/− 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 ± 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−/−, Rjv-2+/− 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 (≤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 × (SWR × RF)F1] or [SWR × (SWR × RF)F1] 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+/− Bcl mice were grouped into those that inherited the Fv-1 region (Gpd-1b/b, Fig. 2) from their SWR grandmother or into those that inherited the Fv-1 region from their RF grandfather (Gpd-1a/b, Fig. 2), distinct patterns of virus expression were apparent. The majority (25/29) of the Rjv-1+/−, Gpd-1b/b Bcl mice expressed infectious MuLV with a median titer of 2 × 10^3 infectious U/0.2 ml. The majority (18/27) of Rjv-1+/−, Gpd-1a/b Bcl mice did not express infectious virus; the median titer of those that did was 5 × 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−/−, Rjv-2+/− 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−/−, Rjv-2−/− (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 pC-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 32P-labeled clone pEC-B4. 12 μg 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 λ 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)] Bcl mice, which were phenotyped for *Rjv-1* and *Rjv-2*, or DNA from Bc2 progeny of *Rjv-1−/* [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* 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 × 10⁵ 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.](image-url)
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 fragment |
| Rjv-1**            | 6      | 0      | 0      | 0      | 0           |
| Rjv-1*             | 3      | 0      | 0      | 0      | 0           |
| Rjv-1*, Rjv-2*‡    | 12     | 0      | 0      | 12     | 0           |
| Rjv-1*, Rjv-2*‡    | 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-/- 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-/- 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*+/+ mice carry this sequence. 7 of the 11 Rjv-1-/-, Rjv-2*‡ 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* progeny, the DNA of which carried a 3.8 kb Pvu II ecotropic-hybridizing fragment (data not shown). Unfortunately, the 4 Rjv-2*+/- 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
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 \textit{Rjv-2} to \textit{Pgm-1}, it seems reasonable to conclude that the \textit{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 \textit{Eco RI} fragments of RF DNA hybridized with the ecotropic-specific probe. To determine whether two \textit{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 \textit{Eco RI} ecotropic env-containing fragments were detected in RF DNA, one of which corresponded to \textit{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 \textit{Eco RI} fragments, it is likely that the larger 19-kb fragment is the site of co-migration. From the pattern of junction fragments generated with other enzymes and from the size of the \textit{Eco RI} fragments, we consider it unlikely that two ecotropic proviruses are contained on one \textit{Eco RI} fragment. An alternative explanation is that one ecotropic provirus is contained in a \textit{Eco RI} fragment that is randomly sheared during DNA isolation and is, therefore, not resolved. Other more complicated explanations are also possible.

\textbf{Discussion}

Data from backcross generations of RF mice with several low-virus or virus-negative strains indicated that RF mice transmit a locus (\textit{Rjv-1}) that confers the high virus phenotype after IUdR induction of fibroblastic cells. The pattern of virus induction in mice that carry \textit{Rjv-1} was similar to that of mice that carry the \textit{Akh} loci of AKR. In crosses of RF mice with SWR mice, the inheritance of \textit{Rjv-1} is sufficient to lead to viremia when the suppressive RF \textit{Fv-1} allele is absent. However, \textit{Rjv-1} differs from
STRUCTURE OF MURINE LEUKEMIA VIRUSES IN RF/J MICE

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 Ce-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 1UdR 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 × DBA/2)F1 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.

We thank David J. Grunwald for allowing us to cite the results of his allelism test between RF and NIH-Akv-2 mice, and Carol Sinaiko for her excellent help with tissue cultures. We also thank Wallace Rowe and Janet Hartley for their hospitality during the initial stages of this work.

Received for publication 24 June 1982.

References

1. Rowe, W. P. 1973. Genetic factors in the natural history of murine leukemia virus infection. G. H. A. Clowes Memorial Lecture. Cancer Res. 33:3061.
2. Rowe, W. 1978. Leukemia virus genomes in the chromosomal DNA of the mouse. Harvey lectures. In Harvey Lectures. Academic Press, Inc., New York. 173.
3. Aaronson, S. A., G. Todaro, and E. Scolnick. 1971. Induction of murine leukemia C-type viruses from clonal lines of virus-free BALB/3T3 cells. Science (Wash. D. C.). 174:157.
4. Kozak, C. A., and W. P. Rowe. 1980. In Animal Virus Genetics. B. Fields, R. Jaenisch, and C. F. Fox, editors. Academic Press, Inc., New York. 171.
5. Kozak, C. A., and W. P. Rowe. 1979. Genetic mapping of the ecotropic murine leukemia virus-inducing locus of BALB/c mouse to chromosome 5. Science (Wash. D. C.). 204:69.
6. Ihle, J. N., D. R. Joseph, and J. J. Domotor, Jr., 1979. Genetic linkage of C3H/HeJ and BALB/c endogenous ecotropic C-type viruses to phosphoglucomutase-1 on chromosome 5. Science (Wash. D. C.). 204:71.
7. Rowe, W. P., and C. Kozak. 1980. Germ-line reinsertion of AKR murine leukemia virus genomes in Ako-1 congenic mice. Proc. Natl. Acad. Sci. U. S. A. 77:4871.
8. McCubrey, J., and R. Risser. 1982. Genetic interactions in the induction of endogenous ecotropic murine leukemia viruses in low leukemic mice. Cell. 28:181.
9. McCubrey, J., and R. Risser. 1982. Genetic interactions in the spontaneous production of endogenous ecotropic murine leukemia virus in low leukemic mouse strains. J. Exp. Med. 156:337.
10. Furth, O. B., W. A. Barner, and A. B. Biower. 1940. Studies on resistance to transmissible leukemia in mice by means of parabiosis. Arch. Pathol. 29:163.
11. Duran-Reynals, M. L., F. Lilly, A. Bosch, and K. J. Blank. 1981. The genetic basis of susceptibility to leukemia induction in mice by 3-methylcholanthrene applied percutaneously. J. Exp. Med. 147:459.
12. Law, L. W. 1941. The induction of leukemia in mice following percutaneous application of 9,10-dimethyl-1,2-benzanthracene. Cancer Res. 1:564.
13. Chen, S., F. D. Struuck, M. L. Duran-Reynals, and F. Lilly. 1980. Genetic and non-genetic factors in expression of infectious murine leukemia viruses in mice of the DBA/2 × RF cross. *Cell.* 21:849.

14. Upton, A. C. 1959. Studies on mechanism of leukemogenesis by ionizing radiation. In CIBA Foundation Symposium on Carcinogenesis: Mechanisms of Action. Little, Brown and Co., Boston. 249.

15. Pincus, T., J. Hartley, and W. Rowe. 1971. A major genetic locus affecting resistance to infection with murine leukemia viruses. I. Tissue culture studies of naturally occurring viruses. *J. Exp. Med.* 133:1219.

16. Mayer, A., M. L. Duran-Reynals, and F. Lilly. 1978. *Fv-1* regulation of lymphoma and of thymic ecotropic and xenotropic MuLV expression in mice of the AKR/J × SWR/J cross. *Cell.* 15:429.

17. Mayer, A., F. Duran Struuck, M. L. Duran-Reynals, and F. Lilly. 1980. Maternally transmitted resistance to lymphoma development in mice of reciprocal crosses of the RF/J and AKR/J strains. *Cell.* 19:431.

18. Lowy, D. R., W. P. Rowe, N. Teich, and J. W. Hartley. 1971. Murine leukemia virus: High-frequency activation in vitro by 5-iododeoxyuridine and 5-bromodeoxyuridine. *Science (Wash. D. C.)* 174:155.

19. Chan, H. W., T. Bryan, J. L. Moors, S. P. Staal, W. R. Rowe, and M. A. Martin. 1980. Identification of ecotropic proviral sequences in inbred mouse strains with a cloned subgenomic DNA fragment. *Proc. Natl. Acad. Sci. U. S. A.* 77:5579.

20. Hartley, J. W., and W. P. Rowe. 1975. Clonal cell lines from a feral mouse which lack host-range restrictions for murine leukemia viruses. *Virology.* 65:128.

21. Rowe, W. P., W. E. Pugh, and J. W. Hartley. 1970. Plaque assay technique for murine leukemia viruses. *Virology.* 42:1136.

22. Rowe, W. 1972. Studies of genetic transmission of murine leukemia virus by AKR mice. I. Crosses with *Fv-1* strains of mice. *J. Exp. Med.* 136:1272.

23. Kozak, C., and W. P. Rowe. 1978. Genetic mapping of xenotropic virus-inducing loci in two mouse strains. *Science (Wash. D. C.)* 199:1448.

24. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503.

25. Denhardt, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23:641.

26. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. Mol. Biol.* 113:237.

27. McCubrey, J., and R. Risser. 1982. Allelism and linkage studies of murine leukemia virus activation genes in low leukemic strains of mice. *J. Exp. Med.* 155:1233.

28. Chattopadhyay, S. K., W. P. Rowe, N. M. Teich, and D. R. Lowy. 1975. Definitive evidence that the murine C-type virus inducing locus *Aki-1* is viral genetic material. *Proc. Natl. Acad. Sci. U. S. A.* 72:906.

29. Jenkins, N., N. Copeland, B. Taylor, and B. Lee. 1981. Dilute (d) coat colour mutation of DBA/2J mice is associated with the site of integration of an ecotropic MuLV genome. *Nature (Lond.)* 293:370.

30. Rowe, W., J. W. Hartley, and T. Bremner. 1972. Genetic mapping of a murine leukemia virus-inducing locus of AKR mice. *Science (Wash. D. C.)* 178:860.

31. Kozak, C., and W. P. Rowe. 1980. Genetic mapping of the ecotropic virus-inducing locus *Aki-2* of the AKR mouse. *J. Exp. Med.* 152:1419.

32. Staats, J. 1980. Standardized nomenclature for inbred strains of mice: seventh listing. *Cancer Res.* 40:2083.