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Genetics

A unique sequence related to the ecotropic murine leukemia virus is associated with the *Fv-4* resistance gene

(resistance to Friend virus complex/Asian mice)

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ABSTRACT **Several strains of laboratory and wild-derived mice from Japan carry the dominant allele at the *Fv-4* locus (*Fv-4*^{r}) that is responsible for resistance to infection by exogenous ecotropic murine leukemia virus. We have used blot hybridization with a probe specific for the ecotropic viral envelope to show that a unique envelope-reactive sequence is present in the Japanese mouse *Mus musculus molossinus* and in four independently derived partially congeneic strains carrying *Fv-4*^{r}. Analysis of 31 backcross mice shows complete concordance between inheritance of this fragment and resistance to Friend virus complex-induced erythroblastosis. Inheritance of this sequence also suppresses spontaneous expression of the endogenous ecotropic viruses carried by *M. m. molossinus*. Restriction enzyme analysis shows that the *Fv-4*^{r}-associated sequence is different from the full-length ecotropic proviruses of laboratory mice. Infectious virus cannot be induced from mice carrying only the *Fv-4*^{r}-associated sequence. Examination of other wild-derived mice resistant to Friend virus complex shows that *Mus cervicolor cervicolor* also contains the *Fv-4*^{r} sequence. Our data indicate that a unique or incomplete provirus containing ecotropic envelope-related sequences is responsible for *Fv-4*-mediated resistance to both exogenous and endogenous ecotropic virus in various Asian mice.**

A number of genetic loci have been described that interfere with the rapid induction of erythroblastosis in mice by Friend virus complex. Alleles at the *Fv-1* locus restrict the replication-competent ecotropic murine leukemia virus (MuLV) component of Friend complex (1), and the recessive allele at the *Fv-2* locus prevents the replication of the defective spleen focus-forming virus (2). More recently, Suzuki identified a locus called *Fv-4* in FRG (formerly G) strain mice (3). Mice carrying the dominant resistance allele at this locus (*Fv-4*^{r}) do not develop spleen foci or splenomegaly after inoculation with Friend complex, and they do not support the growth of N- or NB-tropic MuLV. Genetic studies have localized *Fv-4* to chromosome 12 in FRG mice and the Japanese wild mouse *Mus musculus molossinus* (4, 5). Other feral mice from Asia and California show similar resistance to endogenous ecotropic virus (5, 6), and preliminary data suggest that the resistance gene of California wild mice, *Akvr-1*, is allelic with *Fv-4* (7).

The mechanism by which the *Fv-4*^{r} allele restricts the replication of ecotropic virus has not been determined. Recent data have shown that resistance is associated with a cell surface antigen found in *Fv-4*^{r} lymphoid cells (8) and that this antigen may be related to the MuLV envelope glycoprotein, gp70 (9). This suggests that *Fv-4*^{r} controls the expression of endogenous retroviral sequences.

In this study, we used blot hybridization with an ecotropic envelope-specific probe to identify a unique sequence in the DNAs of *Fv-4*^{r} mice, and we show that this sequence segregates in Mendelian crosses with *Fv-4*-mediated resistance to endogenous and exogenous virus. Our data also show that infectious virus cannot be induced from mice carrying only the *Fv-4*^{r}-associated ecotropic sequence and that the structure of this sequence differs from that of the ecotropic proviruses found in various laboratory mice. Finally, we describe the distribution of this sequence in other wild-derived mice resistant to Friend complex virus.

MATERIALS AND METHODS

Mice. Partially congeneic BALB/c mice (C4W) carrying the wild mouse *Fv-4*^{r} locus were a kind gift of T. Odaka (Institute of Medical Sciences, University of Tokyo, Tokyo) (5). T. Odaka also provided tissue samples from the BALB/c colony used to construct the congeneic mice, from C4W animals inbred after three additional backcross generations (C4W-9), and from an independently derived BALB/c congeneic mouse (Hz) (10). Tissues were also provided from FRG (formerly G) strain mice and congeneic mice carrying the *Fv-4*^{r} locus of FRG mice on an AKR background. The *M. m. molossinus* mice (colony III) were obtained from T. Roderick (The Jackson Laboratory). Other wild mice were kindly provided by M. Potter (National Cancer Institute). NS.*Hx*^{2N} is a partial congeneic mouse bred in our laboratory carrying a chromosome 5-linked locus for hemimelic extra toes. All other animals were obtained from the Small Animal Section, Veterinary Research Branch, Division of Research Sciences, National Institutes of Health. Hybrid mice were bred in our laboratory.

Friend Susceptibility. NB-tropic Friend virus complex was originally obtained from F. Lilly (Albert Einstein College of Medicine, Bronx, NY) and was serially passaged in BALB/c mice. Animals were tested for susceptibility to Friend complex disease after retro-orbital inoculation of 0.2 ml of a 1% filtered spleen extract that contained 3×10^4 spleen focus-forming units/ml and 3×10^6 XC plaque-forming units (pfu)/ml. Mice were killed 2–4 weeks after inoculation, their livers were removed for DNA extraction, and their spleens were weighed. Single-cell suspensions of each spleen were plated as infectious centers on SC-1 cells (11). Four or five days later, ecotropic virus was scored in these cultures by the XC test (12).

DNA Extraction and Blot Hybridization. High molecular weight DNA was extracted from the liver or from spleen tissue of hemi-splenectomized mice as described (13). DNAs were digested with restriction enzymes, electrophoresed on 0.6% agarose gels, and transferred to nitrocellulose membranes as described (14). The recombinant plasmid (pEc-$_{env}$) containing ecotropic MuLV envelope (*env*)-specific DNA sequences (13) was labeled with ^{32}P by nick-translation (15). Nitrocellulose membranes were hybridized as described (16) and then exposed to Kodak AR film for 3–7 days at −70°C with DuPont Lighting Plus intensifier screens.

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Abbreviations: MuLV, murine leukemia virus; pfu, plaque-forming units; *env*, viral envelope gene; IdUrd, 5-iododeoxyuridine; 5-aza-Cyd, 5-azacytidine; kb, kilobase(s); MCF, mink cell focus-forming virus; gp70, major viral envelope glycoprotein.

Virus Induction and Assay. Tail biopsy tissue of adult mice was tested for infectious ecotropic virus. Cell-free extracts were inoculated into cultures of SC-1 cells (11) and assayed for virus by the XC test 5–6 days later (12).

Tissue cultures were prepared from tail biopsy tissue of weanling mice as described (17). When the cultures were in subconfluent growth, 20 μg of 5-iododeoxyuridine (IdUrd) per ml or 2 μg of 5-azacytidine (5-azaCyd) per ml was added for 48 hr. The cultures were then washed and overlaid with SC-1 cells (for ecotropic virus) or cells of the mink lung line CCL64 (for xenotropic virus) to amplify viral titers. The clarified fluid from the mink cultures was tested for xenotropic virus by focus formation on the mink S^+L^- line of Peebles (18). For ecotropic virus, cultures were passaged at 12 days and at weekly intervals thereafter and were monitored directly for ecotropic virus by the XC test. In some cases, cell cultures that were negative after several weeks were also examined by immunofluorescence using a fluorescein-conjugated anti-MuLV antibody (19).

RESULTS

Ecotropic *env*-Related Sequences in *Fv-4*^{r} Mice. DNAs from BALB/c, FRG, the three BALB.*Fv-4*^{r} congeneic mice (C4W, C4W-9, Hz), and *M. m. molossinus* (III) were digested with *Pst* I and examined by blot hybridization for ecotropic *env*-specific fragments (Fig. 1). All of these DNAs contained the 8.2-kilobase (kb) fragment generated by *Pst* I cleavage of full-length ecotropic proviral DNA (20). The *Fv-4*^{r} mice contained an additional 4.1-kb fragment that annealed to the ecotropic *env*-specific probe. DNAs from the BALB/c mice used to construct the congeneic mice lacked this 4.1-kb fragment. Similarly, the AKR.*Fv-4*^{r} congeneic mice contained both 8.2- and 4.1-kb *Pst* I fragments, whereas the AKR mouse DNA contained only the 8.2-kb fragment (data not shown).

Mendelian Analysis of C4W Mice. In an effort to correlate the presence of this unique 4.1-kb *Pst* I fragment with *Fv-4*-mediated resistance to exogenous and endogenous virus, *Fv-4*^{r} mice were crossed and backcrossed with mice of *Fv-4*-sensitive, ecotropic virus-negative strains. In one experiment, C4W mice were mated with NS.*Hx*^{2N} females and F_1 males were backcrossed to NFS/N females. Thirty-one backcross mice were inoculated with Friend complex virus. Within 2 weeks, 12 mice developed enlarged spleens (>0.5 g) with high titers of XC virus (2–10 × 10^5 pfu/10^7 cells). Nineteen animals were resistant; they had normal-sized spleens (<0.3 g) containing little or no recoverable virus (0–10^2 pfu/10^7 cells).

Table 1. Segregation of ecotropic *env*-specific sequences in NFS × (NS.*Hx*^{2N} × C4W) backcross mice

Mice	Phenotype Hx	Phenotype Fv-4	Ecotropic *env*-specific *Pst* I fragments, kb	No. of mice
Parent				
C4W	+	r	8.2, 4.1	
NS.*Hx*^{2N}	Hx	s	—	
Backcross	Hx	s	—	6
	+	s	8.2	6
	Hx	r	4.1	7
	+	r	8.2, 4.1	12

Blot hybridization of DNAs from all 31 backcross mice showed that the two *env*-specific *Pst* I fragments of the C4W parent (8.2 and 4.1 kb) assorted independently of one another (Table 1 and Fig. 2). As expected, all mice with the Hx phenotype lacked the 8.2-kb fragment, consistent with the close linkage between this locus and the proviral locus *Cv* on BALB chromosome 5 (21). The smaller 4.1-kb *env*-reactive fragment was present in 19 mice and absent in 12. Inheritance of this fragment showed complete concordance with the resistance phenotype, indicating that this ecotropic sequence is integrated at or near the *Fv-4* locus (Table 1).

Mendelian Analysis of *M. m. molossinus*. Partially inbred *M. m. molossinus* mice were crossed and backcrossed with virus-negative NFS mice. Progeny were tested for spontaneous and induced expression of endogenous ecotropic virus and then inoculated with Friend virus complex to follow inheritance of *Fv-4*^{r}. Analysis of first backcross mice showed that 75% were virus inducible, suggesting that these mice

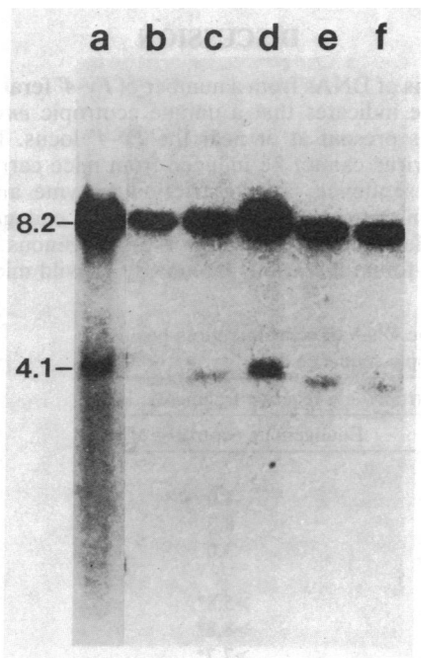

FIG. 1. Blot hybridization of *Pst* I-digested mouse liver or spleen DNAs. Lanes: a, *M. m. molossinus*; b, BALB/c; c, C4W; d, C4W-9; e, Hz; and f, FRG. Fragments are shown in kb.

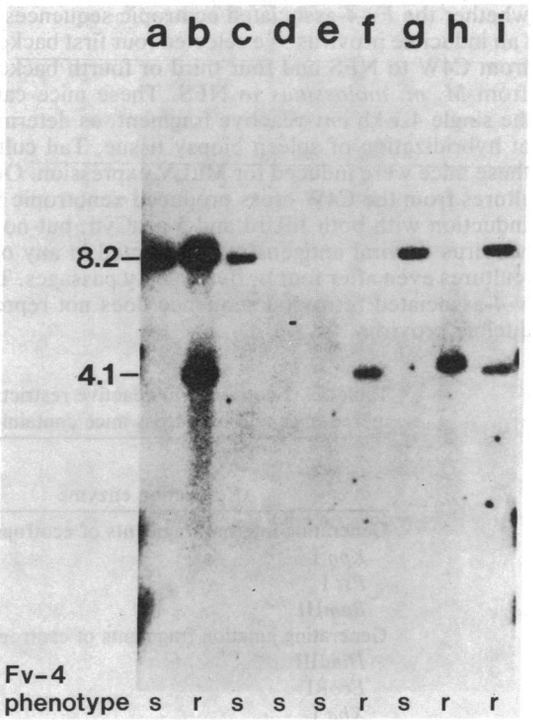

FIG. 2. Blot hybridization of *Pst* I-digested mouse liver DNAs. Lanes: a, BALB; b, C4W; c–i, mice from the backcross NFS × (NS.*Hx*^{2N} × C4W). Fragments are shown in kb.

Table 2. Expression of endogenous ecotropic virus in the cross NFS × (NFS × *M. m. molossinus*)

Mice	Number positive for virus/number tested (%)	
	Induced	Spontaneous
All	79/105* (75)	14/61 (23)
Fv-4$^{r/s}$	10/13 (77)	0/29 (0)
Fv-4$^{s/s}$	5/9 (56)	14/32 (44)

The XC test was used to score ecotropic virus in the tail extracts of adult mice and in cultured tail fibroblasts 2 weeks after IdUrd induction. Different mice were tested for induced and spontaneous expression.
*22 of these 105 mice were randomly selected for *Fv-4* typing.

carry two virus inducibility loci (Table 2). Both *Fv-4*$^{r/s}$ and *Fv-4*$^{s/s}$ segregants responded to induction, indicating that *Fv-4*^{r} does not inhibit virus induction. In contrast, spontaneous virus expression was detected in the tail extracts of only 14 of the 61 backcross mice (24%) tested. None of these 14 positive mice inherited the *Fv-4*^{r} allele. These data show that spontaneous expression of the endogenous ecotropic viral loci of *M. m. molossinus* mice is restricted by *Fv-4*^{r}.

Genomic DNA of *M. m. molossinus* mice carries multiple copies of the ecotropic viral genome (22). To test for the association between resistance to Friend complex and endogenous proviral sequences, backcross mice carrying the *Fv-4*^{r} allele were identified by their resistance to Friend complex and mated with NFS mice. At the third backcross generation, six animals were tested for susceptibility to Friend complex and examined for ecotropic proviral DNA. Southern blot hybridization revealed a 4.1-kb *Pst* I fragment in the three mice resistant to Friend virus. Two of these resistant mice had no other ecotropic-reactive fragments. Thus, resistance to Friend complex is associated with inheritance of this unique ecotropic sequence in *M. m. molossinus* mice.

Virus Inducibility. Many of the endogenous ecotropic viral sequences of inbred and wild mice are complete viral genomes that can be expressed as infectious virus. To determine whether the *Fv-4*-associated ecotropic sequences represent an inducible provirus, we selected four first backcross mice from C4W to NFS and four third or fourth backcross mice from *M. m. molossinus* to NFS. These mice carried only the single 4.1-kb *env*-reactive fragment, as determined by blot hybridization of spleen biopsy tissue. Tail cultures from these mice were induced for MuLV expression. One of the cultures from the C4W cross produced xenotropic virus after induction with both IdUrd and 5-azaCyd, but no XC-positive virus or viral antigens were detected in any of the other cultures even after four or five weekly passages. Thus, the *Fv-4*-associated retroviral sequence does not represent an inducible provirus.

Restriction Enzyme Analysis. Both the *Pst* I cleavage pattern and the lack of virus expression suggest that the *Fv-4*^{r}-associated sequence may represent a grossly rearranged or deleted provirus. Therefore, DNA samples from backcross mice lacking all but the 4.1-kb fragment were digested with other restriction enzymes. Blot hybridization results summarized in Table 3 show that the restriction fragments generated by five enzymes are substantially smaller than expected for digestion of full-length ecotropic proviral DNA. The 17.3-kb *Kpn* I fragment is much larger than the internal 4.0-kb *Kpn* I fragment generally obtained from endogenous ecotropic provirus, suggesting that at least one of the internal *Kpn* I sites is missing in the *Fv-4*^{r}-associated sequence. These data show that the *Fv-4*^{r}-associated ecotropic sequence is distinct from the full-length ecotropic proviruses found in laboratory mice. Comparison with published restriction maps of various wild mouse ecotropic virus isolates (23) also shows marked differences. Therefore, the *Fv-4*^{r} sequence may represent a fragmented provirus or a provirus with unique internal restriction sites.

Distribution of *Fv-4*^{r} in Wild Mouse Populations. Odaka and his colleagues have previously shown that other Asian wild mice show resistance to Friend complex (5). Therefore, various wild-derived mice were screened for resistance to Friend complex and the presence of ecotropic MuLV-related sequences. Six different wild-derived mice were identified that were resistant to induction of splenomegaly and had little or no Friend ecotropic virus in their spleens 2–4 weeks after inoculation. DNAs from these resistant mice were examined by blot hybridization for endogenous ecotropic-reactive sequences. Results showed that the *Fv-4*-associated 4.1-kb fragment was present in *Mus cervicolor cervicolor* but not in resistant mice of *Mus pahari*, *Mus caroli*, *Mus cookii*, *Mus hortulanus* (Fig. 3), and *Mus cervicolor popaeus* (not shown). The five mice lacking the *Fv-4*^{r} fragment contained no other ecotropic *env*-related sequences, whereas *M. c. cervicolor* carried additional ecotropic fragments, including the 8.2-kb fragment expected for full-length ecotropic proviruses. These results indicate that *M. c. cervicolor* has the *Fv-4*^{r} gene but that neither the 4.1-kb fragment of *Fv-4*^{r} nor any other endogenous ecotropic sequence is associated with resistance in the other wild mice.

DISCUSSION

Our analysis of DNAs from a number of *Fv-4*^{r} feral and laboratory mice indicates that a unique ecotropic *env*-reactive sequence is present at or near the *Fv-4*^{r} locus. Infectious ecotropic virus cannot be induced from mice carrying only the *Fv-4*^{r} sequence, and restriction enzyme analysis of DNAs from these animals shows that this endogenous sequence differs substantially from the endogenous ecotropic proviruses found in various laboratory or wild mice.

Table 3. Ecotropic *env*-reactive restriction fragments in genomic DNA of ecotropic virus-positive inbred mice and backcross mice containing only the *Fv-4*^{r} ecotropic sequence

Restriction enzyme	Ecotropic *env*-reactive fragments, kb	
	Fv-4^{r}	Endogenous ecotropic MuLV
Generating internal fragments of ecotropic MuLV		
Kpn I	17.3	4.0
Pst I	4.1	8.2
*Bam*HI	0.75	3.0
Generating junction fragments of ecotropic MuLV		
*Hin*dIII	9.3	>5.8*
*Eco*RI	5.2	>8.8*
Xba I	4.1	>7.7*
Sac I	2.6	>5.5*

*The minimal length represents the size of the proviral sequences in cell-virus junction fragments. Ecotropic MuLV fragment sizes have been described (23).

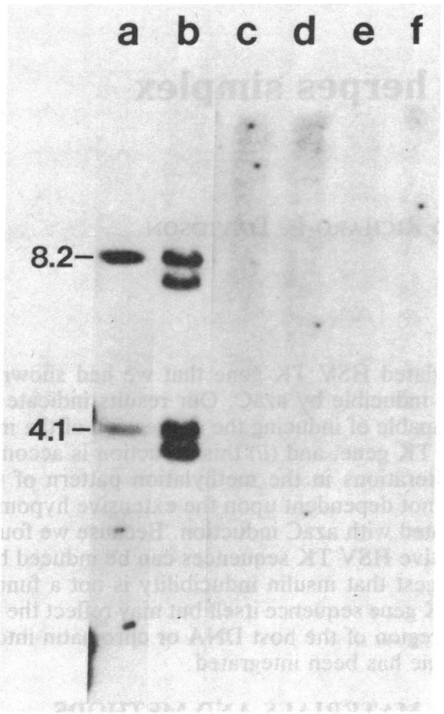

FIG. 3. Blot hybridization of *Pst* I-digested mouse liver DNAs. Lanes: a, C4W; b, *M. c. cervicolor*; c, *M. pahari*; d, *M. cookii*; e, *M. caroli*; and f, *M. hortulanus*. Fragments are shown in kb.

The concordant inheritance of this unique ecotropic sequence and *Fv-4* resistance suggests that *Fv-4* may be analogous to several other resistance genes. In the chicken, three loci designated *ev-3*, *ev-6*, and *ev-9* represent defective endogenous proviruses that control the expression of high levels of viral envelope glycoproteins. Inheritance of any one of these genes reduces susceptibility to exogenous avian viruses of the same subgroup (24). In the DBA mouse, resistance to infection with mink cell focus-forming (MCF) MuLVs correlates with the presence of a cell surface MCF virus-related glycoprotein (25). In both of these systems, resistance to exogenous infection was attributed to the binding of retroviral envelope glycoproteins to cell surface receptors.

Consistent with the hypothesis that *Fv-4* resistance is similarly mediated by viral interference, previous studies have identified an *Fv-4*-associated cell surface glycoprotein immunologically related to viral gp70s (8, 9). This antigen is presumably the product of the *Fv-4*^{r}-associated ecotropic envelope sequences identified in this report. If the model of *Fv-4* resistance by interference is correct, the specificity of the resistance should parallel the specificity of binding between the *env* glycoproteins and viral cell surface receptors. In the mouse, unique receptors have been described for ecotropic, amphotropic, and MCF MuLVs (26–28). Consistent with our identification of the *Fv-4*^{r}-associated sequence as ecotropic *env*-related, *Fv-4* resistance is known to interfere with ecotropic but not amphotropic MuLVs (8).

The presence of the *Fv-4* gene has survival value for any mouse exposed to infectious ecotropic viruses. These viruses have long been associated with a variety of neoplastic and neurologic diseases, and virus integration into germline cells can also result in mutational events that alter normal development (29). The wild-derived mice in which we have identified the *Fv-4*^{r} sequence are naturally exposed to infectious ecotropic virus. Thus, both *M. m. molossinus* and *M. c. cervicolor* contain multiple endogenous ecotropic proviruses, and infectious virus with ecotropic host range has been isolated from these mice (30, 31). Wild mice of Lake Casitas, California, carry a resistance locus (*Akvr-1*) that appears to be allelic with *Fv-4*; these mice also harbor infectious ecotropic virus (32). These mice must be tested by blot hybridization to determine if they also carry the *Fv-4*^{r} *env*-related sequence.

In addition to mice that contain both *Fv-4*^{r} and infectious ecotropic virus, we also identified five wild-derived mouse populations that show resistance to Friend virus but that lack any endogenous ecotropic *env*-related sequences. Preliminary results further indicate that these mice lack any sequences homologous to *env*-specific segments of xenotropic or MCF MuLVs (unpublished data). Thus, viral interference cannot account for resistance in these animals. The basis for the *in vivo* restriction to exogenous MuLV in these mice must be investigated.

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