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Transgenic *Fv-4* Mice Resistant to Friend Virus

TERESA I. LIMJOCO,[1] PETER DICKIE,[1] HIDETOSHI IKEDA,[2] AND JONATHAN SILVER[1]*

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892,[1] and Aichi Cancer Center Research Institute, Nagoya, Aichi-ken 464, Japan[2]

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***Fv-4* is a mouse gene that confers resistance to infection with ecotropic retroviruses. A candidate *Fv-4* gene was cloned previously and found to resemble the 3′ half of a murine leukemia virus (MuLV). To study the effect of this gene in vivo, we generated two transgenic mouse strains carrying the *Fv-4 env* gene under control of its presumed natural promoter, a cellular sequence unrelated to retroviruses. Transgenic progeny expressed a 3-kb *Fv-4 env* RNA in all of the organs and tissues examined, as well as an *Fv-4* envelope antigen on the surface of thymocytes and spleen cells, similar to mice carrying the natural *Fv-4* gene. One of the two transgenic strains (designated Fv4-2) expressed three to nine times as much transgene RNA and protein as the other strain (Fv4-11). When challenged with a Friend virus complex containing up to 10^4 XC PFU of Friend MuLV, Fv4-2 mice were completely resistant to development of splenomegaly and had no detectable ecotropic virus in the spleen or blood, confirming that the cloned *Fv-4* gene is responsible for resistance to ecotropic MuLV in vivo. In contrast, Fv4-11 mice were only partially resistant, developing viremia and splenomegaly at the highest inoculum dose but recovering from viremia several weeks after inoculation with 10-fold less virus. The phenotype of recovery from viremia in Fv4-11 mice was unexpected and suggests that low levels of expression of the *Fv-4* gene enhance the effectiveness of the immune response.**

Natural resistance to subgroups of retroviruses has been found as a polymorphic trait in several experimental species (28). In mice, one of the strongest genetically determined resistance traits was first identified in Japan (32) and designated *Fv-4* because it was the fourth Friend virus resistance trait characterized. The same resistance gene was discovered independently in wild mice in California (12, 24). *Fv-4* resistance is inherited as a simple Mendelian dominant trait mapped to mouse chromosome 12 (17, 25). After breeding of *Fv-4* resistance onto laboratory mouse strains, it was shown that inheritance of *Fv-4* is associated with expression of a cell surface antigen cross-reactive with ecotropic murine leukemia virus (MuLV) envelope glycoprotein gp70 (15, 16, 38). *Fv-4* resistance also cosegregated with a replication-defective, ecotropic virus-related proviral sequence (7, 22), presumably the gene that encodes the envelope-related cell surface antigen.

The *Fv-4*-associated endogenous provirus was cloned, and its structure was found to be that of the 3′ portion of a provirus extending from the end of the *pol* gene through a complete *env* gene to a 3′ long terminal repeat (7, 14). No 5′ long terminal repeat sequence was identified upstream of the envelope sequence. Mouse cells transfected with this putative *Fv-4* gene expressed a circa 3-kb *Fv-4 env* RNA, the same size as that in mice carrying the *Fv-4* resistance trait. High levels of expression depended on the presence of sequences upstream of the *Fv-4 env* coding sequence, suggesting that these sequences contain the natural promoter for the *Fv-4 env* gene (18). Various segments of the upstream DNA from the clone were subsequently tested for promoter-enhancer activity when placed upstream of a reporter gene transfected into NIH 3T3 cells, and one putative promoter-containing *Hin*dIII-*Bam*HI segment was identified (13a). A subportion of the comparable region cloned from an *Fv-4*^{s} mouse (map positions 4.3 to 6.2; Fig. 1 of reference 19) was also shown to have promoter activity.

NIH 3T3 cell lines stably transfected with the cloned *Fv-4 env* gene expressed a cell surface *Fv-4*^{r}-specific MuLV envelope protein and were variably resistant to superinfection by ecotropic MuLVs (18). The variability in resistance to ecotropic retroviruses in vitro was not surprising because the *Fv-4*-encoded phenotype is only variably expressed in tissue culture cells derived from *Fv-4*-resistant mice (26, 36, 37).

To confirm that the cloned *Fv-4*-associated retrovirus is responsible for the resistance phenotype in vivo and to investigate whether appropriate control elements are contained within the putative promoter segment of the genomic clone, we made transgenic mice with a derivative of the *Fv-4* clone and tested them for expression of transgene RNA, *Fv-4*-associated envelope protein, and resistance to infection with Friend MuLV.

MATERIALS AND METHODS

Transgene DNA construct. Plasmid pUC19-FL2.3-Fv4 was constructed by cloning a 2.3-kb putative promoter-containing region from the *Fv-4*^{r} genomic clone (*Hin*dIII-*Bam*HI fragment from map positions 3.9 to 6.2 kb [19]) into the *Sma*I site of pUC19. This fragment was then excised as a 2.3-kb *Eco*RI-*Hin*dIII fragment and cloned into pUC19 upstream of a 4.9-kb *Hin*dIII-*Eco*RI fragment (map positions 12.3 to 17.2 kb [19]) containing the deleted *pol*, complete *env*, and 3′ long terminal repeat sequences of the endogenous *Fv-4*-associated retrovirus (Fig. 1). The 7.2-kb *Fv-4* cassette produced by digestion with *Eco*RI was gel purified for microinjection.

Transgenic mice. Single-cell embryos from inbred FVB/N mice (Harlan Sprague-Dawley) were microinjected with the 7.2-kb FL2.3-Fv4 fragment as previously described (13) and implanted in pseudopregnant FVB/N recipients. Founder mice were tested by Southern blotting and polymerase chain reaction (PCR). Mice positive for the transgene were bred and maintained as hemizygotes in two lineages.

DNA isolation and analysis. DNA was extracted from mouse tails by a salting-out method by using a kit from

* Corresponding author.

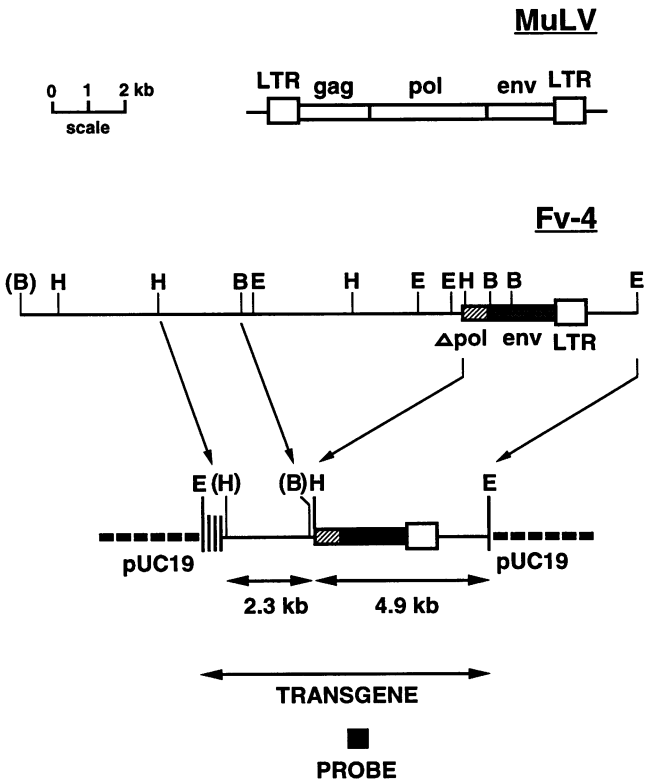

FIG. 1. Structures of the MuLV provirus and the *Fv-4* gene. The MuLV-like sequence of *Fv-4* is aligned with the MuLV sequence above it (18). The 4.9-kb *Hin*dIII-*Eco*RI retroviral fragment was cloned together with the 2.3-kb *Hin*dIII-*Bam*HI 5′-flanking sequence into pUC19, and digestion with *Eco*RI produced the 7.2-kb transgene (see Materials and Methods). A 539-bp PCR fragment specific for *Fv-4 env* (see text) was used as a probe. H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI. LTR, long terminal repeat.

Stratagene (La Jolla, Calif.) and digested with *Hin*dIII. Ten micrograms of DNA was electrophoresed through a 0.8% agarose gel, transferred to nylon membrane (Hybond; Amersham, Arlington Heights, Ill.), and hybridized to a ^{32}P-labelled *Fv-4 env* fragment at 68°C for 2 h by using a commercial buffer (Quik-hyb; Stratagene). The 539-bp *Fv-4* envelope probe fragment was generated by PCR from cloned *Fv-4* DNA by using primers and the conditions given below. Filters were washed twice at room temperature with 1× SSC (1× SSC is 150 mM NaCl plus 15 mM Na citrate)–0.1% sodium dodecyl sulfate (SDS) and twice at 68°C with 0.1× SSC–0.1% SDS, rinsed with 2× SSC, and exposed to XAR film (Kodak, Rochester, N.Y.).

Genomic DNA was analyzed by PCR for the presence of the *Fv-4* envelope gene by using oligonucleotides 4718-1 (5′GTGACAGGAGGGTTAGCGCATAAAG-3′) and 4718-3 (5′TTTGGGGGCCTGCGGGGATGAC-3′), which are specific for a 539-bp sequence within *Fv-4 env*. PCR was done at 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min for 30 cycles with a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.).

RNA isolation and analysis. Total RNA was extracted from fresh organs and tissues with a commercial kit (Stratagene), and 10 to 20 μg was fractionated in 1.0% agarose–0.02 M MOPS [3-(*N*-morpholino)propanesulfonic acid]–0.66 M formaldehyde gels. Northern (RNA) blots were prepared by transferring RNA to nylon membranes (Hybond; Amersham) and hybridized and washed as described for DNA analysis. Filters were stripped with boiling 0.1× SSC–0.1% SDS for 30 min and then rehybridized to a [^{32}P]kinase-labelled mouse β-actin oligonucleotide (Clontech, Palo Alto, Calif.) at 55°C for 2 h, washed twice with 1× SSC–0.1% SDS at room temperature, and exposed to XAR film (Kodak).

Virus stock. An NB-tropic Friend virus complex (virus stock A) was kindly provided by C. Kozak (National Institutes of Health, Bethesda, Md.). Virus stock B was prepared by inoculating a 6-week-old FVB/N mouse intraperitoneally with 0.1 ml (10^5 XC PFU) of virus stock A. Three weeks later, the grossly enlarged spleen was removed and prepared as a cell-free 10% (wt/vol) homogenate with cold phosphate-buffered saline, filtered through a 0.45-μm membrane (Corning, Corning, N.Y.), and stored at −70°C.

Virus challenge. Mice 1 to 6 months old were inoculated intraperitoneally with 0.1 or 0.5 ml of serial dilutions of stock virus. After 1 to 5 weeks, spleens were harvested and prepared as 10% (wt/vol) cell-free filtered extracts. In time course experiments, 3 to 5 μl of tail blood was drawn at various times after infection and mixed with 0.5 ml of Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville, Md.). Virus titers in spleen extracts and blood samples were determined by the XC plaque assay (29).

Immunofluorescence assay for expression of *Fv-4 env*. Cell suspensions were prepared with phosphate-buffered saline from freshly harvested thymuses of 5-month-old mice, washed twice with Hanks balanced salt solution supplemented with 0.1% sodium azide and 0.1% bovine serum albumin, incubated with a 1:100 dilution of anti-*Fv-4* envelope monoclonal antibody (20) for 20 min on ice, washed twice, and labelled with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (GIBCO-BRL, Gaithersburg, Md.). Cell samples were analyzed for fluorescence intensity on an EPICS Profile flow cytometer (Coulter Corp., Hialeah, Fla.).

RESULTS

Establishment of transgenic lines. FVB/N mice were chosen for these experiments because fertilized eggs from this strain have large pronuclei, which facilitates microinjection. Single-cell embryos from FVB/N mice were injected with DNA consisting of the *Fv-4*-associated truncated provirus under the control of putative regulatory sequences found upstream of this provirus in a genomic clone (Fig. 1 and Materials and Methods). Potential founders were screened by PCR analysis of tail DNA, and positive mice were further analyzed by Southern blotting. *Hin*dIII-digested DNA from one founder, designated Fv4-11, contained a single *Fv-4 env*-reactive fragment (data not shown). This fragment, approximately 9 kb long, was present in single-copy amounts on the basis of comparison to a diluted *Fv-4* plasmid DNA control. Founder Fv4-11 transmitted the transgene to 43 of his 85 offspring, which is consistent with his being nonmosaic. This transgene was maintained by backcrossing to parental FVB/N mice; the resulting strain was designated Fv4-11.

Southern analysis of *Hin*dIII-digested DNA from another founder, Fv4-2, gave three fragments (data not shown). These three fragments were apparently due to closely linked transgenes because 13 of 13 transgenic descendants of founder Fv4-2 showed the same pattern of three bands, and the frequency of transmission was that expected for a single gene (91 of 187 backcross progeny). The pattern of three

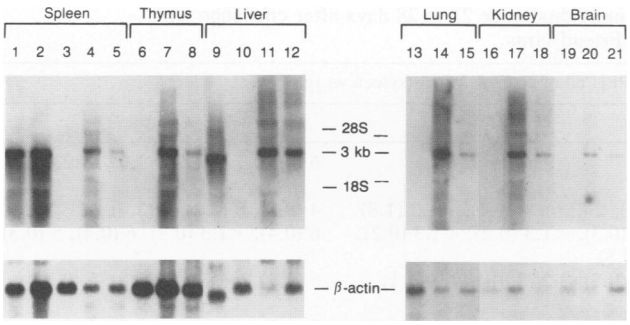

FIG. 2. Northern blots showing expression of RNA in various tissues from nontransgenic control, Fv4-2, Fv4-11, and *Fv-4*^{r}-carrying wild mice. Lanes: 1, *M. musculus molossinus*; 2 and 9, *M. musculus castaneus*; 3, 6, 10, 13, 16, and 19, nontransgenic controls; 4, 7, 11, 14, 17, and 20, Fv4-2; 5, 8, 12, 15, 18, and 21, Fv4-11. *M. musculus molossinus* and *M. musculus castaneus* are wild strains of mice carrying the *Fv-4*^{r} allele. RNA from an *M. musculus castaneus* liver sample (lane 9) ran slightly faster than the other RNAs (seen in rRNA bands after ethidium bromide staining), probably because of a difference in the salt content of this sample.

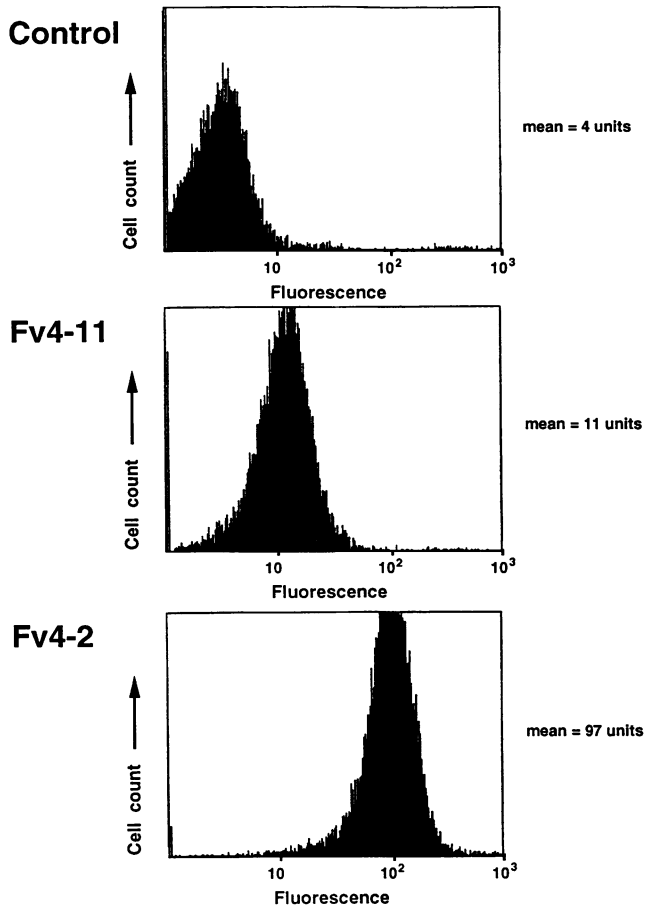

FIG. 3. Fluorescence-activated cell sorter analysis of cell surface expression of *Fv-4 env* antigen. The *x* axis represents the relative intensity of fluorescence, and the *y* axis shows the number of cells. Cell surface *Fv-4 env* was detected by using an anti-*Fv-4 env* monoclonal antibody (20).

bands could result from integration of multiple copies of the transgene at the same chromosomal site. On the basis of signal intensity from diluted *Fv-4* plasmid DNA, we estimate that Fv4-2 heterozygous mice contain three or four copies of the transgene.

RNA expression. Northern analysis of transgenic mice showed a 3-kb RNA species similar in size to the transcript present in *Fv-4*^{r} wild mice (*Mus musculus molossinus* and *M. musculus castaneus*; Fig. 2, lanes 1, 2, and 9). This RNA species was detected in all of the tissues sampled, including spleen, thymus, liver, lung, kidney, and brain (Fig. 2, lanes 4 and 5, 7 and 8, 11 and 12, 14 and 15, 17 and 18, and 20 and 21), as well as heart, skeletal muscle, and small intestine tissues (data not shown). The level of expression of *Fv-4 env* RNA in the Fv4-2 samples was consistently higher than in the Fv4-11 samples after normalization for the amount of RNA loaded (as determined by hybridization to β-actin). From densitometric scans of autoradiographs, we estimated that Fv4-2 mouse tissues contained three- to sevenfold more *Fv-4 env* RNA than did Fv4-11 mouse tissues.

***Fv-4 env*-encoded protein expression.** By using an *Fv-4* envelope-specific monoclonal antibody (20), we found that Fv4-2 mouse thymocytes express about nine times more cell surface *Fv-4* antigen than do Fv4-11 thymocytes (Fig. 3). Expression of cell surface *Fv-4* was uniform on thymocytes. Spleen cells from Fv4-2 and Fv4-11 mice also expressed the *Fv-4*-encoded envelope antigen, although the levels were about one-half to one-third of those on thymocytes (data not shown).

Resistance of transgenic mice to Friend virus complex. The Friend virus complex consists of a replication-competent virus which can be scored in an XC assay and a defective spleen focus-forming virus which is responsible for rapid-onset erythroblastosis and splenomegaly (8, 11, 31). Fv4-2 mice were completely resistant to Friend virus infection in our experiments. None of 33 Fv4-2 mice inoculated with the Friend virus complex developed splenomegaly or had detectable levels of XC-positive virus in the spleen or blood when tested from 1 to 5 weeks after infection (combined experiments shown in Table 1 and Fig. 4). XC titers for the infected Fv4-2 mice were less than 20 to 100 PFU/ml of blood or spleen extract (which corresponded to no syncytia in our assays), versus about 10^6 PFU/ml for infected nontransgenic control mice.

In contrast, Fv4-11 mice were only partially resistant. When given high inoculum doses (10^5 XC PFU of Friend virus), these mice developed grossly enlarged spleens (>1 g) with levels of virus in the spleen approximately as high as those of nontransgenic controls (Table 1, row 2). At lower inoculum doses (10^3 to 10^4 XC PFU), 9 of 11 Fv4-11 animals were negative for virus in the spleen at 3 to 5 weeks after infection, whereas 11 of 12 nontransgenic controls had large amounts of virus at these times (10^4 to 10^6 XC PFU/ml of spleen extract) (Table 1, rows 3 to 5). Interestingly, two of the Fv4-11 mice with little or no detectable virus in the spleen extract at 21 days after inoculation nevertheless had gross splenomegaly (Table 1, row 4; XC titers, 160 and <20 PFU/ml for spleen weights of 2.9 and 3.1 g, respectively). Persistence of splenomegaly despite recovery from viremia has been observed in other mouse strains infected with the Friend virus complex (4, 5).

To investigate the partial resistance of Fv4-11 mice more carefully, we tested inoculated mice for viremia at approximately weekly intervals (Fig. 4). Most Fv4-11 mice became viremic but then recovered from viremia, with levels of XC plaque-forming virus approximating those in nontransgenic

TABLE 1. Virus titers in spleens and spleen weights of individual mice 21 to 38 days after challenge with decreasing doses of Friend virus

Virus stock	Dose (XC PFU)[a]	Day of sacrifice	XC titer, log PFU/ml of spleen extract (spleen wt [g])		
			Fv4-2	*Fv4-11*	Control
A	10^5, i.p.	29	<1.3 (0.3), <1.3 (0.3), <1.3 (0.3), <1.3 (0.3)		6 (0.5), 7 (2.1), 7 (1.6), 7 (2.0)
A	10^5, i.v.	25		4 (2.1), 2 (2.6), 4 (1.1), <1.3 (1.8)	4 (0.8), 6 (2.2), 6 (3.4)
A	10^4, i.p.	38	<1.3 (0.2), <1.3 (0.2), <1.3 (0.5), <1.3 (0.2)	<1.3 (0.3), <1.3 (0.2), <1.3 (0.2), 6 (0.5)	6 (0.4), <1.3 (0.2), 6 (0.4), 5 (0.3)
B	10^4, i.p.	21	<1.3 (0.2), <1.3 (0.2), <1.3 (0.2), <1.3 (0.1)	<1.3 (0.2), 2.2 (2.9), <1.3 (3.1)	6 (1.3), 6 (1.4), 5 (1.6), 6 (0.3)
B	10^3, i.p.	21	<1.3 (0.1), <1.3 (0.1), <1.3 (0.1), <1.3 (0.1)	<1.3 (0.2), <1.3 (0.2), <1.3 (0.2), <1.3 (0.2)	5 (0.3), 4 (0.3), 6 (0.7), 5 (0.4)

[a] i.p., intraperitoneally; i.v., intravenously.

mice at 1 week and less than those of controls at 2 weeks and no detectable virus by 3 to 4 weeks after infection (Fig. 4, middle panel). In contrast, none of the Fv4-2 mice had detectable viremia (Fig. 4, lower panel), whereas most of the nontransgenic mice remained viremic throughout the experiment (Fig. 4, upper panel). It is of note that 3 of the 13 nontransgenic mice cleared their viremia and 2 significantly reduced their titers by 6 weeks after infection.

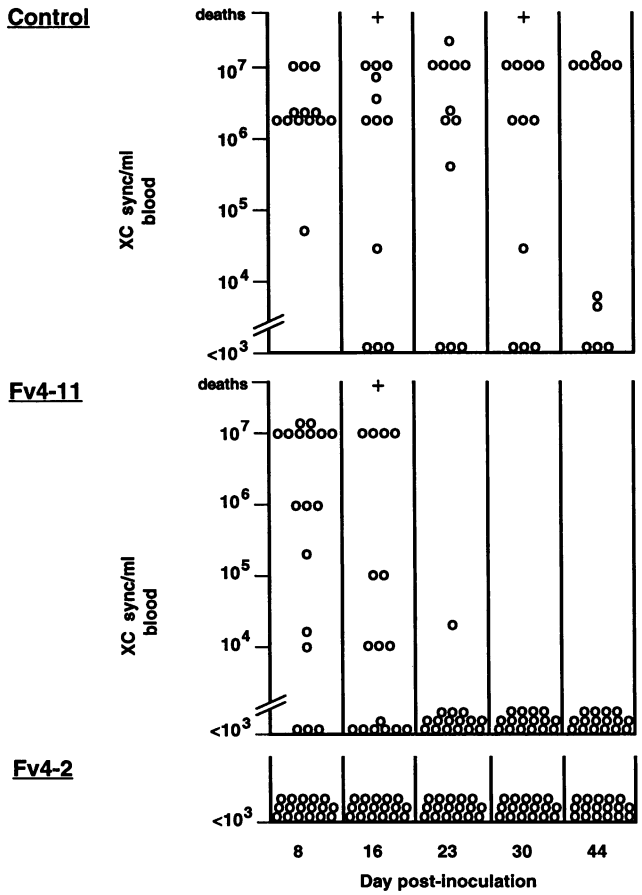

FIG. 4. Time course experiment. Thirteen control, 17 *Fv4-2*, and 17 *Fv4-11* mice were infected with Friend virus, and blood samples were assayed for virus at approximately weekly intervals. About one-half of each group received 10^4 and the other half received 10^5 XC PFU of stock virus intraperitoneally. Since there was no significant difference in the virus titers between mice that received high- or low-dose inocula, these groups were combined when each graph was plotted.

DISCUSSION

These experiments provide formal proof that the cloned defective *Fv-4* provirus is responsible for resistance to ecotropic MuLV in vivo. This result is not surprising in view of the facts that resistance cosegregated with an expressed ecotropic virus-related envelope gene and transfection of this gene into NIH 3T3 cells led to resistant cell lines (18). However, expression of *Fv-4* resistance in tissue culture is notoriously variable (37); the degree of resistance is much weaker than in vivo (26, 36); the pattern of resistance in *Fv-4* heterozygous cells is peculiar, reportedly recessive in vitro but dominant in vivo (37); and single-cell clones from resistant cell cultures may be sensitive (26, 37). Differences between in vivo and in vitro results could be secondary to variable or fluctuating expression of *Fv-4 env* (or *Fv-4 env* relative to the ecotropic virus receptor) in tissue culture cells compared with hematopoietic cells in vivo. Whatever the explanation, because of inconsistencies between in vivo and in vitro results, confirmation of resistance at the whole-mouse level was warranted.

Previous results also left uncertain the location of the cellular promoter for *Fv-4*. Tissue culture experiments showed that a 2.3-kb *Hin*dIII-*Bam*HI fragment contained sequences with promoter-enhancer activity in NIH 3T3 cells (13a), but how these sequences would function in vivo was unknown. Our results suggest that the 2.3-kb fragment contains the relevant regulatory sequences, but they do not fully answer this question because of the difference in expression level between Fv4-2 and Fv4-11 strains. Differences in expression between the two strains could be secondary to effects of the integration site, the transgene copy number, or even possible transgene rearrangement in Fv4-2 mice. Analysis of expression in other transgenic strains should clarify the strength of the 2.3-kb putative *Fv-4* promoter. Preliminary results obtained with a third *Fv-4* transgenic strain indicate that it is strongly resistant, like Fv4-2 mice. Because of its apparent promiscuity, the *Fv-4* promoter could be useful to drive expression of other genes in transgenic mice. We are currently investigating the use of this promoter to drive expression of the human immunodeficiency virus type 1 envelope gene.

The correlation between degree of resistance to Friend virus and level of expression of the *Fv-4* envelope in Fv4-2

and Fv4-11 mice strengthens the conclusion that *Fv-4* envelope expression is responsible for resistance. A similar correlation between the level of *Fv-4 env* expression and resistance has been seen in vitro (20).

The mechanism of *Fv-4* resistance is presumed to be related to the phenomenon of viral interference in vitro. When tissue culture cells are chronically infected with one retrovirus, they are resistant to superinfection with the same retrovirus or related retroviruses (27, 30, 34). The likely reason is that endogenously synthesized viral envelope protein may compete with exogenous virus for the virus receptor. An endogenously synthesized envelope may bind to the virus receptor intracellularly, thereby blocking its transport to the cell surface. Biochemical evidence in support of this model comes from studies of human immunodeficiency virus in which the envelope precursor binds to CD4, the human immunodeficiency virus receptor, in the endoplasmic reticulum (6). Related studies with reticuloendotheliosis virus showed that envelope mutants which remain predominantly in the endoplasmic reticulum can still cause superinfection interference (9).

The ecotropic virus receptor was recently cloned (2) and found to function as a dibasic amino acid transporter in xenopus oocytes (21, 35). Because of the possibility that the *Fv-4*-encoded envelope protein would bind to the receptor and interfere with amino acid transport, we analyzed the urine of Fv4-2 and Fv4-11 mice for abnormalities in amino acid levels; no abnormalities were detected. Our transgenic Fv4-2 and Fv4-11 mice were healthy, even as homozygotes. Therefore, the ecotropic virus receptor may be a nonessential gene in mice.

Our finding of recovery from viremia in Fv4-11 mice was unexpected, because the level of viral interference would not be expected to increase with time after infection. Recovery from viremia is more consistent with an immune mechanism. Recovery from Friend viremia in other mouse strains is associated with production of neutralizing antibodies which can confer resistance to Friend virus after passive transfer (4, 5, 10). The facts that 3 (of 13) nontransgenic FVB/N mice infected with Friend virus recovered from viremia and 2 more had dramatically reduced titers 6 weeks after inoculation (Fig. 4) suggest that the immune response of the FVB/N strain is fairly strong. Reduced infectibility on a cellular level in FVB/N mice carrying the Fv4-11 mouse transgene might allow the immune response to contain what would otherwise be an overwhelming infection.

The ability to recover from viremia with Friend virus is inherited as a single dominant gene (*Rfv-3*) in crosses involving C57BL mice (4, 5, 10). This is formally equivalent to the effect of the Fv4-11 mouse transgene in FVB/N mice. Although interference has been presumed to operate independently of the immune response (23) and *Rfv-3* resistance has been presumed to be due to an immune mechanism, the similarity between the Fv4-11 mouse transgene and *Rfv-3* suggests a deeper relationship. For example, could *Rfv-3* resistance be due to an endogenous retrovirus or other gene which decreases infectibility in some cells and thereby allows the immune response to clear the infection?

Although we stress that we have no direct evidence for a role of the immune system in recovery of Fv4-11 mice, the suggestion of synergy between cellular resistance and the immune response has interesting implications for gene therapy approaches to retrovirus infection. (i) It suggests that even low levels of expression of an interfering envelope gene introduced by gene therapy might tip the balance in favor of an effective immune response. (ii) It raises the question of whether genetically engineered interference targeted to lymphocytes—cells capable of mounting an immune response—might suffice for recovery from infection. We plan to use the *Fv-4* transgenic strains to make various types of radiation chimeras to investigate this question.

The phenomenon of inherited, viral envelope-mediated resistance is quite general and is seen with other classes of viruses and even in species as disparate as plants (3). Gene therapy experiments have shown that this phenomenon can be used to engineer resistance to natural pathogens in commercially important crops (1, 33). Because envelope-based resistance is so widespread in nature and is potentially manipulable, we believe that envelope-related therapeutic approaches to human retroviral infections, such as AIDS, should be vigorously pursued.

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