RESISTANCE TO FRIEND LEUKEMIA VIRUS IN TRANSGENIC MICE EXPRESSING THE NATIVE *FV-4* GENE

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Summary. – *Fv-4* is a truncated ecotropic retrovirus gene that codes for an envelope protein under control of a cellular promoter. It confers resistance to ecotropic murine leukemia viruses. Transgenic mice were derived using the native *Fv-4* gene as the construct for microinjection. Two founder mice were derived. In both founder lines, there was no detectable expression of the transgene or resistance to Friend murine leukemia virus (FrMLV) in hemizygotes. In one line, the resistance was observed in homozygotes with Fv-4 RNA formation in the thymus but not in the spleen or in other tissues. In the other founder line, a homozygous male was identified. Double integrants, derived from breeding this homozygous male to homozygous females from the other founder line, were also resistant. These results indicate that the native gene confers the resistance in homozygous transgenic mice or double integrants derived from different founders but not hemizygotes.

Key words: Fv-4; transgenic mice; retroviral envelope gene; viral interference; disease resistance; Friend murine leukemia virus

Introduction

Transgenic livestock resistant to infectious diseases have been the goal of several research groups (Muller and Brem, 1994). To gain a better understanding of the usefulness of strategies designed to produce disease resistance, transgenic mice have been important models. Among the approaches employed, viral interference has been shown to be a mechanism that may be exploited to specifically produce resistance to viral diseases. Important contributions to this field have come from the study of the mouse *Fv-4* gene, which is a defective endogenous retroviral sequence that confers resistance to ecotropic murine leukemia viruses (Suzuki, 1975; Ikeda and Sugimura, 1989; Dandekar *et al.*, 1987). The retroviral genetic regions represented by this gene include a truncated polymerase (pol) gene, complete envelope (env) gene, and 3'-LTR. The putative promoter is in the 5' cellular flanking region, 6 to 8.3 kb upstream of the retroviral sequences (Limjoco *et al.*, 1993). Wild mice heterozygous for this gene are completely resistant to the ecotropic FrMLV, but cells cultured from these mice are only resistant in the homozygous state (Yoshikura and Odaka, 1978). The mechanism of resistance appears to be a blocking of cell surface receptors for the virus by endogenous *Fv-4* envelope gene protein. Support for this theory has been strengthened by the observation that Fv-4 protein can be exported into the extracellular space and block the binding of FrMLV to mouse thymocytes or spleen cells (Kitagawa *et al.*, 1995, 1996). A plasmid expression vector in which approximately 10 kb (80%) of cellular sequences 5' to the retroviral sequences were removed, leaving the putative promoter juxtaposed to the retroviral DNA, has been used to derive two founder lines of transgenic mice (Limjoco *et al.*, 1993). In one of the founder lines, hemizygous mice were almost completely resistant to FrMLV, but in the other line there was significant susceptibility to FrMLV-induced erythroleukemia.

The purpose of this study was to evaluate transgenic animals harboring the native *Fv-4* gene with all flanking cellular sequences and to determine if increased or more consistent expression of *Fv-4* gene in transgenic mice could be achieved.

Abbreviations: FFU = focus-forming unit; FrMLV = Friend murine leukemia virus; IC = infectious center

Materials and Methods

Derivation of transgenic mice. Transgenic mice were derived by established protocols (Hogan *et al.*, 1994). The construct used for microinjection (Fig. 1) was a complete molecular clone of *Fv-4* gene and flanking cellular sequences (Ikeda and Sugimura, 1989) including the putative promoter in lambda phage. Transgenic mice were identified by DNA dot blot and Southern blot analyses (Ausubel, 1991). Homozygosity was confirmed in mice offspring by mating them to non-transgenic mice and evaluating resulting litters by DNA dot blot hybridization with ^{32}P-labelled lambda phage DNA as probe. The offspring that produced only transgenic pups in these matings were considered homozygous. Double integrants were obtained by breeding homozygous mice from two different founder lines. Copy numbers of transgenes were determined by DNA dot blot analysis using a known amount of *Fv-4* construct DNA added to nontransgenic mouse DNA for comparison.

Evaluation of mice infected with FrMLV. The virus infection was performed on FVB/N nontransgenic, hemizygous, homozygous, and double integrant mice, that were 10 to 25 weeks of age. All mice except sham-inoculated controls were infected with 4 x 10^4 focus forming units (FFU) of the Lilly-Steeves B-tropic strain (Morrison *et al.*, 1986) of FrMLV (family *Retroviridae*, genus *Mammalian type C retrovirus*) including the spleen focus-forming virus. The virus stock was a 20% homogenate of spleen from an experimentally infected BALB/c mouse in phosphate-buffered saline pH 7.2. The titer of this stock was 5 x 10^7 FFU/ml. Spleen weight and virus titers in spleen were determined following necropsy at 21 days post infection. Virus assays on each spleen were performed by incubating 10^6 spleen cells with *Mus dunni* cells as described previously (Sitbon *et al.*, 1985). Virus titers are given as the number of infectious centers (IC) per 10^6 spleen cells.

RNA analysis of mouse tissues. To determine the level of expression of *Fv-4* gene, RNA dot blot hybridization was performed (Ausubel, 1991) by placing 10 – 20 μg total RNA extracted from thymus of 6-week-old mice onto a nitrocellulose membrane using a 96-well manifold. Samples from individual mice were analyzed in triplicate. The blots were hybridized with cloned Fv-4 probe (Dandekar *et al.*, 1987). A duplicate blot was hybridized with a rat cyclophylin gene probe (Calvalho *et al.*, 1996; Danielson *et al.*, 1988; Saito *et al.*, 1994). Probes were labelled with ^{32}P and autoradiographed after hybridization. The autoradiographs were analyzed using a computerized densitometer (NIH Image Processing and Analysis Program Version 1.95 for Macintosh) which gave densitometric values (in units) for each sample. Background values were subtracted and the average value obtained for triplicate samples using the Fv-4 probe was divided by the average value for triplicate samples using the cyclophylin probe, thus normalizing the *Fv-4* gene expression in relation to cyclophylin gene expression. For Northern blot analysis, RNA was glyoxylated and processed as described previously (Ausubel, 1991).

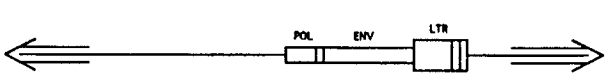

Fig. 1
***Fv-4* gene construct used to derive transgenic mice**
Fv-4 sequences (boxes), cellular sequences including putative promoter (single line), and lambda phage sequences (triple line).

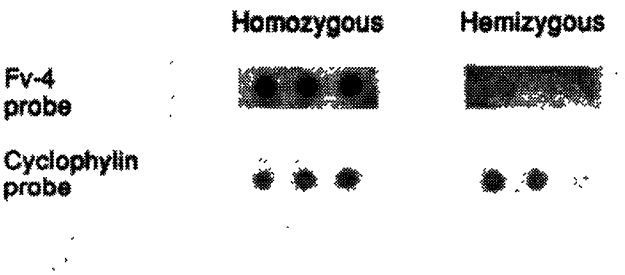

Fig. 2
Dot blot analysis of RNAs from transgenic mouse tissues
Autoradiograph of dot blot of triplicate samples of total RNAs (20 mg) from thymuses of homozygous and hemizygous mice hybridized with the cyclophylin and Fv-4 probes.

Statistical analysis of spleen weights and virus titers in infected mice was done using analysis of variance and the Fischer's exact test (Ostle and Malone, 1988). To analyze RNA dot blots, analysis of variance and the Kruskal-Wallis test (Ostle and Malone, 1988) were used. Because of the wide variation in virus titers among nontransgenic mice, natural logarithm or square root transformations of data points were used in the analysis of variance.

Results

Two transgenic founder lines were derived, designated 3300 and 3305. Copy numbers were 1 copy/cell for the 3305 line and 2 copies/cell for the 3300 line. Homozygous male and female mice were derived from both lines, but female 3300 mice failed to produce offspring when mated to a homozygous 3300 male. The level of of Fv-4 RNA was studied in 3305 homozygous and hemizygous mice (Fig. 2). Thymus RNA from four homozygous 3305 mice and four hemizygous 3305 mice were used. Thymus RNAs from four nontransgenic mice served as controls. The densitometric values (in units) derived using the Fv-4 probe divided by the values (in units) obtained with the cyclophylin probe, averaged for each group, were as follows: homozygous – 3.31 (range 1.24 – 9.07), hemizygous – 0.32 (range 0.10 – 0.71), nontransgenic – 0.51 (range 0.40 – 0.59). Statistically significant differences ($P < 0.05$) could be demonstrated between homozygous mice and the other two groups but not between hemizygous and nontransgenic mice. In contrast to thymus samples, there were no differences between spleen samples in any of the groups

of mice (P >0.05). To prove that the lack of *Fv-4* gene expression in the spleen was not due to breakdown of RNA in the dot blot, and to examine expression in other tissues, Northern blot analysis of heart, kidney, lung, liver, and spleen RNA from a homozygous 3305 mouse was performed with both the cyclophylin and Fv-4 probes (Fig. 3). Cyclophylin RNA, which appears as a band of approximately 800 b, was detected in all five tissues but there was no of Fv-4 RNA (2700 b) in any of them.

Spleen weights of infected homozygous 3305 and double integrant (3305/3300) mice were significantly (P <0.05) lower than those of infected hemizygous 3300, hemizygous 3305 and nontransgenic mice, but they were not significantly different from those of uninfected nontransgenic mice (Table 1). The spleens of two of the homozygous 3305 mice were heavier than 0.31 g (the highest spleen weight in the uninfected group). Virus titers in the spleens of infected homozygous and double integrant mice were lower than those of other infected groups (P <0.02). Although the virus was present in some of the double integrant mice, its titers were low. Hemizygous mice from both founder lines were not different from nontransgenic mice in their resistance characteristics. No differences were found between any of the other infected groups.

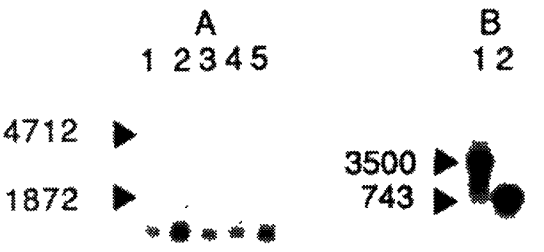

Fig. 3
Northern blot analysis of RNAs from homozygous transgenic mouse tissues
A: Autoradiograph of blot of RNAs from homozygous 3305 mouse hybridized with the cyclophylin and Fv-4 probes. Lanes 1-5: total RNA from heart, kidney, liver, lung and spleen, respectively.
B: Control. Autoradiograph of blot of cyclophylin (lane 2) and Fv-4 (lane 1) DNAs hybridized with the cyclophylin and Fv-4 probes. Arrowheads and numbers on the left margins indicate size standards.

Discussion

Although there was clear resistance to FrMLV in homozygous 3305 and double integrant mice, some of these mice had enlarged spleens or small amounts of the virus detectable in the spleen, indicating that the resistance was not complete. This was also seen in previous experiments with hemizygous transgenic mice having a recombinant *Fv-4* construct (Limjoco *et al.*, 1993). In that work, in which most of the flanking cellular sequences were removed, almost complete resistance to FrMLV in hemizygous mice was observed in one founder line (similar to our homozygous and double integrant mice), while in the other, significant susceptibility to the virus was observed. The level of resistance was related to the level of Fv-4 RNA in thymus and to a lesser extent in the spleen as well as in other tissues, as determined by Northern blot analysis of total RNA from these tissues (Limjoco *et al.*, 1993). The inconsistency of *Fv-4* gene expression in these transgenic mice justified our study of transgenic mice using the native *Fv-4* gene. The construct used in our experiments retained all of the cellu-

Table 1. Challenge of transgenic *Fv-4* mice with FrMLV

Mice	No. of mice in group	Mean spleen weight[a] (g)	No. of mice with spleen weight over 0.31 g	% of mice with detectable virus	Mean FrMLV titer in spleen[a] (IC/10^6 cells)
Homozygous 3305	20	0.24 (1)	2	0	0 (3)
Hemizygous 3305	12	0.60 (2)	5	66	3000 (4)
Double integrant 3300/3305	12	0.18 (1)	0	33	2 (3)
Hemizygous 3300	16	1.04 (2)	11	70	1400 (4)
Nontransgenic infected	19	0.83 (2)	15	78	3400 (4)
Nontransgenic uninfected	13	0.15 (1)	0	–	–

[a]Means followed by different numbers in parentheses are significantly different (P <0.05).

lar sequences intervening between the putative promoter and retroviral sequences, but the expression could only be detected in the thymus of homozygous mice. Hemizygous mice had no detectable level of Fv-4 RNA. Furthermore, this lack of expression in hemizygous mice matched the lack of resistance to FrMLV in these mice, indicating that the absence of Fv-4 RNA was not an artifact of our dot-blot hybridization procedure. The native *Fv-4* gene therefore does not result in its greater or more consistent expression in transgenic mice. Although this may simply be a function of the proximity of *Fv-4* gene to the promoter, there is also the possibility that the native gene may have other elements that reduce the level of expression, or that other genetic elements not included in our construct are necessary for efficient expression. Experiments with double integrant mice demonstrated that the resistance was not solely due to the integration site of the transgene.

The Fv-4 RNA level in thymus was high in our homozygous mice, but in hemizygous mice, it was not different from nontransgenic mice. This was in contrast to the Fv-4 RNA level in the spleen, in which there was no significant difference in dot blot results in any of the groups and no detectable Fv-4 RNA in homozygous mice as determined by Northern blot analysis. The Fv-4 RNA in the thymus was thus correlated with resistance to FrMLV, while in the spleen it was not. Considering that the target cell for the virus is the erythroblast (found in the spleen but not thymus), this provides further support for soluble Fv-4 antigen binding to cells resulting in resistance far from its site of production (Kitagawa *et al.*, 1995, 1996). Fv-4 RNA is formed in the spleens of crosses between wild Fv-4 and BALB/c mice (Ikeda *et al.*, 1985), indicating that genetic elements not included in our construct are important for expression of the native gene in the spleen. Antibodies for FACS analysis were not available to us for study of protein localization. Therefore, we cannot make any conclusions about Fv-4 protein formation in our mice. However, since Fv-4 protein is a circulating antigen that binds to cells not expressing the *Fv-4* gene (Kitagawa *et al.*, 1995), the study of Fv-4 RNA is a better indicator of tissue-specific gene expression. Fv-4 RNA and the resistance to FrMLV were observed only in our homozygous transgenic mice, demonstrating that at the transcriptional and phenotypic levels, the native gene is not as effective as recombinant constructs.

Previous work has shown that Fv-4 envelope protein is released into the serum and that only 30% of cells must express the *Fv-4* gene for interference to be observed (Kitigawa *et al.*, 1995). Another study showed that interference to superinfection with FrMLV occurred when only 10% of splenocytes formed envelope glycoprotein on their surfaces, and only 1% of splenocytes appeared to be producing infectious virus (Mitchell and Risser, 1992). Although not specifically examined in this study, this raises the possibility that envelope glycoprotein shed by infected cells was also binding to uninfected cells, thus resulting in the dramatic (more than 100-fold) decrease in susceptibility of mice to superinfecting FrMLV.

Some nontransgenic mice had small spleens or undetectable virus, indicating that the immune response to the virus may contribute to resistance associated with *Fv-4* gene, as suggested previously (Higo *et al.*, 1997). Wild *Fv-4* mice are resistant to FrMLV as heterozygotes, but similarly to our transgenic mice, cells cultured from these wild mice are resistant only as homozygotes (Yoshikura and Odaka, 1978). Nude mice homozygous for *Fv-4* gene are resistant to FrMLV infection, but heterozygous nude mice are not (Higo *et al.*, 1997). Resistance to spleen focus-forming virus has been accomplished in mice by infecting with FrMLV before challenge. This has been done in normal mice as well as mice incapable of producing neutralizing antibodies or genetically deficient for T cells (Mitchell and Risser, 1992). These studies indicate that components of the immune system present *in vivo* that are not directly related to *Fv-4* gene play an important role in resistance to ecotropic viruses. However, the immunity is not necessary for interference to produce a significant effect if there is sufficient expression of the interfering virus gene.

Although surface receptors appear to be blocked by Fv-4 protein, other sites may contribute to retroviral interference. A chimeric feline leukemia provirus containing all but 40 C-terminal amino acids of the surface glycoprotein of an endogenous feline leukemia provirus element, is not transported beyond the endoplasmic reticulum, yet causes viral interference without blocking viral entry (Bechtel *et al.*, 1994).

The study of interference of viral infection in mice expressing viral envelope glycoprotein genes may provide the basis for a more far-reaching application in agricultural animals. This would involve expression of genuine viral envelope glycoprotein genes in transgenic animals to produce disease resistance. Transgenic sheep expressing the Visna virus envelope glycoprotein gene are currently under study to determine their resistance characteristic (Clements *et al.*, 1994, 1996). Study of retroviral and nonretroviral gene expression in mice should continue to be important in studying mechanisms of viral interference and in determining if this approach is feasible on a broader scale.

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