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Identification of Two Forms of an Endogenous Murine Retroviral *env* Gene Linked to the *Rmcf* Locus

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The *Rmcf* gene restricts the replication of recombinant murine mink cell focus-inducing (MCF) viruses in cell cultures derived from mice carrying the resistance allele (*Rmcf*^{r}) and may play a role in resistance to retrovirus-induced leukemias in vivo. We have characterized the endogenous gp70 expressed by *Rmcf*^{r} and *Rmcf*^{s} mice with a panel of type-specific monoclonal antibodies which discriminate xenotropic and MCF gp70. Embryo and tail skin cultures derived from *Rmcf*^{r} mice (DBA/2 and CBA/N) expressed gp70 bearing a determinant unique to MCF viruses, whereas cultures from *Rmcf*^{s} mice expressed either no detectable gp70 (NFS/N and IRW) or a gp70 serologically related to a subgroup of xenotropic viruses (C57BL/6, CBA/J, and A/WySn). Studies of progeny embryos derived from a (C57BL/6 × DBA/2) × C57BL/6 backcross established that the *Rmcf* resistance allele was linked to the expression of the MCF gp70 and that the gene encoding the xenotropic gp70 expressed by C57BL/6 *Rmcf*^{s} mice was allelic with the MCF gp70 from *Rmcf*^{r} mice. These data indicate that the *Rmcf* locus contains an endogenous gp70 gene having two allelic forms, one of which inhibits exogenous MCF infection in vitro by a mechanism of viral interference.

The chromosomal DNA of a number of vertebrate species has been found to contain integrated retroviral sequences (9). The murine genome is particularly rich in these endogenous sequences, which have been estimated to constitute up to 0.05% of mouse chromosomal DNA (3). Some of these retroviral sequences exist as complete proviruses, which are expressed as infectious virus either spontaneously or after chemical induction. However, the majority of these endogenous sequences consist of incomplete proviruses, most of which remain transcriptionally silent.

Expression of retroviral genes during embryogenesis and the tissue-specific expression of endogenous viral envelope genes have suggested that these sequences may have some function in cellular differentiation (13, 18). Although Astrin et al. (1) reported that chickens lacking endogenous sequences develop normally, even these chickens, and other birds previously thought to be free of endogenous viruses, have been found to harbor at least *pol*-related sequences (4, 10). Chickens which express certain endogenous *env* genes were found, however, to be protected from infections by subgroup E avian leukosis viruses (24). Data suggesting that endogenous murine retroviruses may confer resistance to infection have been reported for mice carrying the *Fv-4* gene, which encodes an ecotropic retroviral envelope protein (14, 15). These mice exhibit resistance to replication of both endogenous and exogenously inoculated ecotropic viruses. This resistance is thought to operate via viral interference, by which the expression of endogenous gp70 molecules at the plasma membrane of potential target cells prevents infection by viruses bearing related envelope proteins by competitive inhibition of viral receptors (14).

Another mouse gene, *Rmcf*, located on chromosome 5, restricts the replication of recombinant mink cell focus-inducing (MCF) retroviruses in cultures derived from mice carrying the resistance allele (12). This gene may participate in the resistance of some mouse strains to erythroleukemia induced by Friend ecotropic virus (26), although genetic studies with backcross analysis have not been reported. Embryo cells from the *Rmcf*^{r} strain DBA/2 express a nonecotropic gp70, and it has been suggested that this gp70 might interfere with MCF infection (2). In support of this hypothesis is the finding that *Rmcf*^{r}-associated restriction could be overcome by phenotypic mixing of MCF viruses with an ecotropic envelope (12). However, embryo cells from mice carrying the sensitive allele (*Rmcf*^{s}), although fully susceptible to MCF virus infection, were also found to constitutively express endogenous nonecotropic gp70 (25). This inconsistency may be the result of the fact that the heterologous antisera used in these studies (25) lacked the specificity to distinguish between qualitatively different gp70 molecules. More precise characterization of these proteins should provide a clearer picture of the mechanism of restriction conferred by the *Rmcf* gene.

Using type-specific monoclonal antibodies, we have characterized the endogenous gp70 molecules expressed by embryo cells from *Rmcf*^{r} and *Rmcf*^{s} mice. In backcross analysis, two genes encoding qualitatively distinct gp70 molecules were found to be linked to the *Rmcf* locus. On the basis of results from genetic experiments, these two gp70 molecules appeared to act as alleles. One of these genes encoded a gp70 bearing a unique determinant of MCF viruses, and this gene product was linked to the resistance allele of the *Rmcf* gene. The second gp70, expressed only in *Rmcf*^{s} mice, was xenotropic and appeared not to interfere with MCF virus replication in vitro.

MATERIALS AND METHODS

Mice. All mice used in this study were bred at the animal facilities of the Rocky Mountain Laboratories (RML). DBA/2J, C57BL/6J, CBA/J, A/WySnJ, and (C57BL/6 × DBA/2)F_1J [(B6 × D2)F_1] parental stocks were purchased from the Jackson Laboratory, Bar Harbor, Maine. NFS/N and CBA/N parental stocks were acquired from the Small Animal Production Center, National Institutes of Health.

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The *Rmcf* phenotypes of these mice have been reported previously (12). Inbred Rocky Mountain white (6) mice were derived and maintained at RML and were typed as $Rmcf^{s/s}$ in our laboratory.

Virus and cells. As $Fv\text{-}1^n$ and $Fv\text{-}1^b$ mice were used in this study, the assay for infectivity of MCF virus was carried out with the NB-tropic HIX Moloney MCF virus, obtained from B. Chesebro of RML. Stocks were prepared from chronically infected mink lung cells as described previously (20). Primary murine embryo cell cultures were prepared from day-14 embryos resulting from timed matings. Embryos were disaggregated by the cold trypsin method of Freshney (11). Primary tail skin cultures were prepared by the method of Lander et al. (16). The mink lung cell line Mv1Lu (ATCC CCL64) and embryo cell cultures were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), sodium bicarbonate (2.0 g/liter), and penicillin (200 U/ml). Tail skin cultures were maintained in similar medium with the addition of gentamicin (5 μg/ml) and amphotericin B (2.5 μg/ml).

Virus assay. Mice were typed for the *Rmcf* phenotype by infectivity of HIX MCF virus on embryo cell cultures by a modified UV mink cell procedure (12). Embryo cells were seeded onto 60-mm dishes at a concentration of 3×10^5 cells per plate. The following day, the cells were infected with various dilutions of virus in the presence of 16 μg of Polybrene (Abbott Laboratories, North Chicago, Ill.) per ml. Four days later the embryo cells were UV irradiated (2.4×10^5 ergs/cm^2) and overlaid with 2×10^5 mink cells per plate. Five days after addition of mink cells, a focal immunofluorescence assay (28) was performed with monoclonal antibody 83A25 to detect foci of infection. Antibody 83A25, an antiretroviral rat monoclonal with broad specificity which produced bright foci in the focal immunofluorescence assay, was supplied by L. Evans of RML.

Antibodies. Three monoclonal antibodies were used to characterize retroviral gp70. The derivation and description of these antibodies have been reported previously. Briefly, antibody 18-1 has broad specificity, reacting with the gp70 of all exogenous and endogenous viruses tested, except Friend ecotropic virus (21). Antibody 514 (5) is specific for MCF gp70, reacting with all the MCF viruses tested so far and not reacting with any known xenotropic, ecotropic, or amphotropic viruses. Antibody 18-6 (21) reacts with the gp70 of a subset of xenotropic viruses.

FMF analysis. For flow microfluorometric (FMF) analysis, embryo and tail skin cells were removed from flasks by trypsinization. To remove acellular debris, cells were initially centrifuged through a 2-ml FCS cushion at $400 \times g$ for 7 min, followed by passage through nylon mesh (74-μm pore diameter; Small Parts, Miami, Fla.). From 2×10^5 to 7×10^5 embryo or tail skin cells were incubated with 0.1 ml of the appropriate monoclonal antibody tissue culture supernatant (18-6 and 514) or a 1:100 dilution of ascites fluid (18-1) for 30 min on ice in a 96-well tray, washed with Dulbecco phosphate-buffered saline containing 2% FCS, and incubated on ice for 30 min with 0.1 ml of a 1:200 dilution of fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse immunoglobulin (Cappel Laboratories, Cooper Biomedical, Malvern, Pa.). The dilutions of primary and secondary reagents used were determined to be in the optimal range. After being washed, 10,000 cells were analyzed with a fluorescence-activated cell sorter (FACS) analyzer (Becton Dickinson, Mountain View, Calif.). The analyzer was operated in the log gain mode, with full-scale fluorescence being 3.0 log. Volume gates were set so that no cells were excluded. Controls consisted of cells reacted with the FITC goat anti-mouse immunoglobulin alone. All experiments were performed a minimum of three times.

Immunoprecipitation and SDS-PAGE. ^{125}I surface labeling of cells was carried out with Iodogen (Pierce Chemical, Rockford, Ill.) as described previously (19). Cells were lysed with 0.5% Nonidet P-40 in 0.01 M Tris hydrochloride–0.15 N NaCl–0.001 M EDTA, pH 7.2, and the lysates were precleared with normal goat serum and Formalin-fixed *Staphylococcus aureus* Cowan I strain cells as reported earlier (8). Volumes of lysate containing 2.5×10^6 to 5.0×10^6 cpm were incubated with monoclonal antibodies, followed, for antibody 514, with rabbit anti-mouse immunoglobulin. The immune complexes were precipitated with Formalin-fixed *S. aureus* Cowan I cells, and precipitates were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Dried gels were exposed on Kodak X-Omat AR film with an intensifying screen as reported earlier (19).

RESULTS

Reactivity of anti-retroviral gp70 antibodies with mouse embryo cells. To characterize the gp70 molecules expressed by embryo cell cultures from strains of mice differing at the *Rmcf* locus, we used a group of type-specific anti-gp70 monoclonal antibodies. The broadly reactive antibody 18-1 detected a gp70 on embryo cells of both DBA/2 ($Rmcf^r$) and C57BL/6 ($Rmcf^s$) mice (Fig. 1). However, the gp70 molecules from these strains were serologically distinct. That from DBA/2 was related to the gp70 of MCF viruses (antibody 514-positive and 18-6-negative), whereas that expressed by C57BL/6 was related to a subgroup of xenotropic viruses (514-negative and 18-6-positive). Embryo cells from

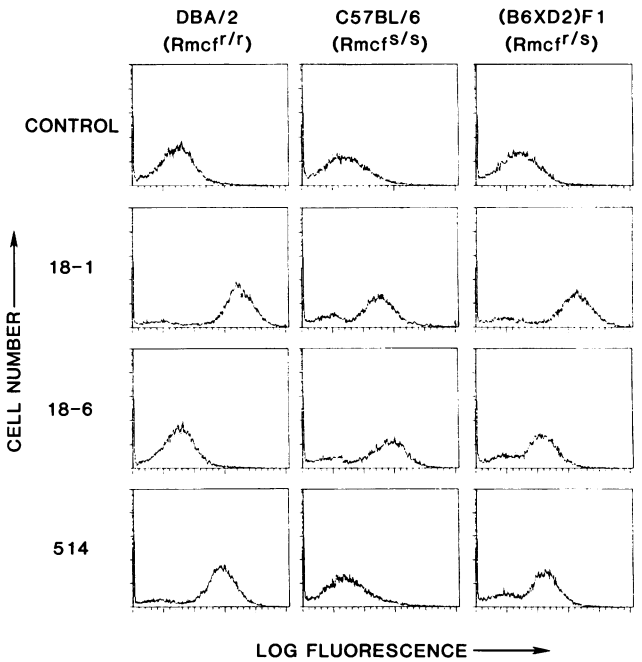

FIG. 1. FMF analysis of gp70 expressed by embryo cell cultures derived from mice differing at the *Rmcf* locus. Cells were incubated with antibody 18-1 (broadly reactive), 18-6 (specific for xenotropic gp70), or 514 (specific for MCF gp70), followed by FITC goat anti-mouse immunoglobulin. Controls consisted of cells exposed to the FITC conjugate alone.

(B6 × D2)F_1 mice (*Rmcf*$^{r/s}$) consistently expressed both gp70 molecules, although each was expressed at a lower mean density than in the parental cells, as evidenced by lower relative fluorescence (Fig. 1). (See also Fig. 5, where the same magnitude of difference in the level of expression of gp70 between the F_1 and parental mice was also seen in the backcross data.) The resistance of DBA/2 embryo cells to infection by MCF viruses (12) thus correlated with the expression of an endogenous MCF gp70. This correlation was further supported by examination of embryo cultures derived from CBA sublines differing at the *Rmcf* locus (12) (Fig. 2). Embryo cells from CBA/J (*Rmcf*$^{s/s}$) mice, like those from C57BL/6 mice, expressed a xenotropic-like gp70 (514-negative and 18-6-positive), whereas embryo cells of CBA/N (*Rmcf*$^{r/r}$) expressed MCF-like gp70, i.e., similar to DBA/2 (514-positive and 18-6-negative). An endogenous xenotropic gp70 reacting with antibody 18-6 was also found to be expressed by embryo cells derived from the *Rmcf*$^{s/s}$ strain A/WySn mice (data not shown). Two other *Rmcf*$^{s/s}$ strains, NFS/N (Fig. 2) and IRW (not shown), did not express any detectable endogenous gp70 (18-1-negative). Additionally, tail skin fibroblast cultures derived from both neonatal and adult mice of the above strains expressed gp70 serologically identical to that expressed by embryo cell cultures (data not shown).

To confirm that the monoclonal antibodies were indeed reacting with cell surface gp70, we used these antibodies to immunoprecipitate lysates of ^{125}I surface-labeled DBA/2 and C57BL/6 embryo cells. Antibody 18-1 precipitated a 70-kilodalton protein present on cells of both strains, the gp70 expressed by DBA/2 being precipitated only by antibody 514 and that expressed by C57BL/6 cells being precipitated only by antibody 18-6 (Fig. 3). Thus, a gp70 bearing a unique determinant of MCF viruses was expressed only by embryo cells from *Rmcf*$^{r/r}$ and *Rmcf*$^{r/s}$ mice. *Rmcf*$^{s/s}$ mouse strains were either gp70 negative or expressed a gp70 related to xenotropic viruses.

Endogenous MCF gp70 of DBA/2 mice segregates as a single gene with the resistance allele of the *Rmcf* locus. As shown in Fig. 1, on the basis of intensity of fluorescence, 514-positive gp70 was expressed by (B6 × D2)F_1 embryo cells at lower

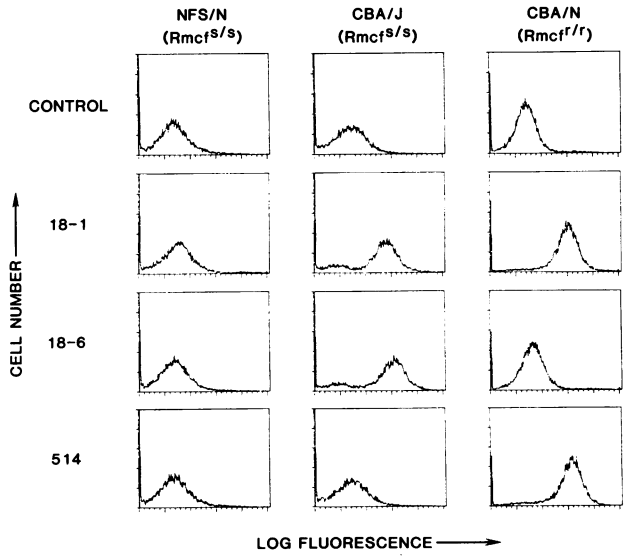

FIG. 2. FMF analysis of gp70 expressed by embryo cell cultures derived from NFS/N, CBA/J, and CBA/N mice. Cells were analyzed as described in the legend to Fig. 1.

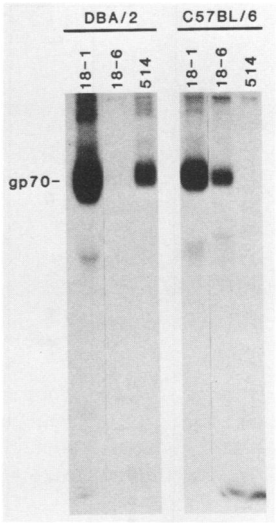

FIG. 3. Immunoprecipitation of ^{125}I surface-labeled embryo cells by anti-gp70 monoclonal antibodies. Precipitates were resolved by SDS-PAGE analysis. Note that both cell types expressed endogenous gp70, which was precipitated by the broadly reactive antibody 18-1. However, the gp70 molecules expressed by the two strains were clearly differentiated by reactivity with MCF- (antibody 514) and xenotropic virus-specific (antibody 18-6) monoclonal antibodies.

levels than by DBA/2 cells. However, by FMF analysis the difference between F_1 (514-positive) and C57BL/6 (514-negative) parental cells was consistent and readily apparent. Therefore, individual embryo cultures derived from (B6 × D2)F_1 × C57BL/6 backcross progeny were examined for the expression of 514-positive gp70. Of 51 embryos derived from eight different litters, all expressed 18-1-positive gp70; 41% of the embryos were 514 negative, and 59% were 514 positive. Chi-square analysis indicated that these data were statistically consistent with the segregation of the 514-positive gp70 as a single gene. Additionally, when 25 of these same backcross embryo cultures were typed for the *Rmcf* phenotype, the results indicated that expression of this endogenous MCF gp70 segregated with resistance to MCF virus infection (Fig. 4), establishing linkage between this gp70 and the resistance allele of the *Rmcf* locus on chromosome 5.

Endogenous gp70 molecules expressed by DBA/2 and C57BL/6 embryo and tailskin cells appear to be allelic. During the course of characterizing the gp70 expressed by (B6 × D2)F_1 × C57BL/6 backcross embryos, an interesting correlation was noted between the level of expression of 514- and 18-6-positive gp70 molecules. Without exception, among 51 progeny the level of expression of 18-6-positive gp70 was higher by a factor of ca. 3 in 514-negative than in 514-positive embryo cultures (Fig. 5). This observation suggested that these two gp70 molecules were allelic, i.e., embryo cells expressing high levels of 18-6-positive gp70 were homozygous for the 18-6-positive gp70 gene and those expressing low levels were heterozygous. To test this hypothesis, (B6 × D2)F_1 mice, expressing both 514-positive and 18-6-positive gp70 molecules, were crossed with IRW mice, a strain which did not express either gp70. FMF analysis of tail skin cultures derived from 50 progeny revealed that 514- and 18-6-positive gp70 molecules were expressed in a mutually exclusive fashion, with 24 progeny being 514 positive and 18-6 negative and 26 progeny being 514 negative and 18-6

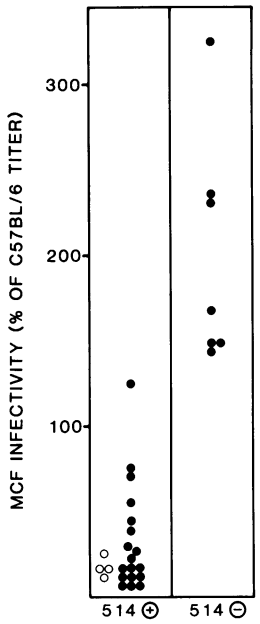

FIG. 4. Segregation of the *Rmcf*^{r} allele with the expression of endogenous MCF gp70 by (B6 × D2)F_1 × C57BL/6 backcross embryo cells. Expression of MCF (514-positive) gp70 was assessed by FMF analysis of embryo cells. MCF virus infectivity was determined by a modified UV mink cell procedure with a broadly reactive monoclonal antibody, 83A25 (see Materials and Methods). Infectivity is expressed as the percentage of the virus titer on C57BL/6 (*Rmcf*$^{s/s}$) embryo cells (solid circles). Open circles represent the relative infectivity of (B6 × D2)F_1 (*Rmcf*$^{r/s}$) controls. Each point represents the mean of at least two assays.

positive. These results, together with the backcross data, indicated that of the 101 mice which we examined for the segregation of these two gp70 molecules, no recombinants were observed, indicating a map distance of ≤1 centimorgan. Thus, the genes coding for these gp70 molecules acted as alleles, and the level of expression of the gene products was dependent on gene dose.

DISCUSSION

Using monoclonal antibodies which discriminate between MCF and xenotropic gp70, we have shown that while both C57BL/6 (*Rmcf*$^{s/s}$) and DBA/2 (*Rmcf*$^{r/r}$) mice expressed an endogenous gp70 in embryo and tail skin cultures, only the DBA/2 gp70 was serologically related to that of MCF. The endogenous gp70 expressed by C57BL/6 embryo cell cultures was characterized as xenotropic-like. Furthermore, these two gp70 molecules were allelic, based on the reciprocal relationship between the level of expression of MCF and xenotropic gp70 in (B6 × D2)F_1 × C57BL/6 backcross embryos and the mutually exclusive expression of these two molecules in progeny of (B6 × D2)F_1 × IRW mice. Analysis of MCF replication in embryo cell cultures derived from (B6 × D2)F_1 × C57BL/6 backcross progeny revealed that the expression of endogenous MCF gp70 segregated with the restriction of MCF virus replication, i.e., was linked to the *Rmcf*^{r} allele. Thus, the Rmcf^{r} phenotype, expressed by DBA/2 and CBA/N mice, was linked to the expression of endogenous MCF gp70, while the Rmcf^{s} phenotype was associated with either no detectable expression of gp70 (NFS/N and IRW) or expression of the endogenous xenotropic gp70 (CBA/J, C57BL/6, and A/WySn). These data suggest that the *Rmcf* gene is a structural gene which encodes these gp70 molecules. However, the existence of a gene controlling the expression of multiple murine endogenous proviral loci has been reported (17). It therefore remains possible that the genes we have described represent segregating control elements which specifically activate one or another envelope sequence. Final resolution of this point must await molecular analysis.

The restriction of MCF virus replication imparted by the *Rmcf*^{r} allele was found by Hartley et al. (12) to be overcome by pseudotypic mixing of MCF with ecotropic virus envelope. This suggested that the restriction was expressed at an early stage of the virus replication cycle. The results of the current study support this hypothesis and indicate that the restriction was probably a result of viral interference imposed by the expression of endogenous MCF gp70. It is of interest that the xenotropic gp70 expressed by embryo cells of some *Rmcf*$^{s/s}$ strains did not interfere with MCF virus replication. It is known, however, that the ability of xenotropic gp70 to interfere with infection by MCF viruses depends on the nature of the interfering xenotropic gp70 and the type of cell (7, 23). Therefore, although embryo cells expressing endogenous xenotropic gp70 detected by anti-

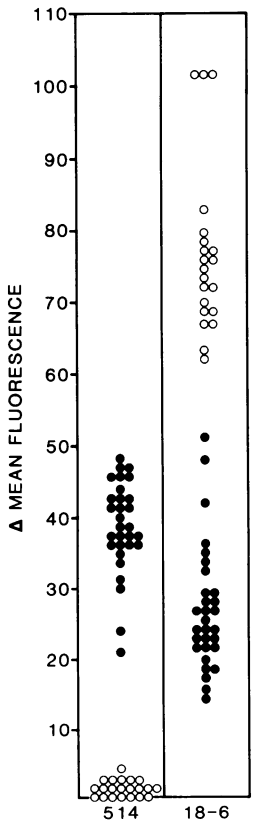

FIG. 5. Relative levels of expression of endogenous MCF and xenotropic gp70 by embryo cells derived from (B6 × D2)F_1 × C57BL/6 backcross progeny. The level of gp70 expression was determined by relative fluorescence as assayed by FMF analysis. Symbols: ○, embryo cells with no detectable MCF (514-positive) gp70; ●, embryo cells which expressed both MCF (514-positive) and xenotropic (18-6-positive) gp70. The vertical axis represents the mean fluorescence of cells reacted with monoclonal antibody 514 or 18-6 and FITC goat anti-mouse immunoglobulin minus the mean fluorescence of cells reacted with FITC conjugate alone.

body 18-6 were susceptible to MCF infection in vitro, it is possible that the expression of this gp70 by different cell types in vivo could interfere with the replication of MCF viruses in mice. We are currently studying the possible relationship between the expression of this gp70 and the known restriction of MCF virus replication in C57BL mice (27).

The effect of the *Rmcf* gene on MCF virus replication is weak (30- to 100-fold) (12) compared with the restriction (10^3- to 10^4-fold) exhibited in other retroviral interference systems in which homogeneous cell lines have been used (7, 22, 23). This difference can be partly explained by the heterogeneity of the secondary embryo cultures used to study the *Rmcf* gene. In our hands, the level of expression of endogenous gp70 by secondary embryo cells from DBA/2 and C57BL/6 mice was found to be consistently lower than that by embryo cell lines derived from the same mouse strains. Accordingly, MCF virus replication has been found to be restricted by 10^3- to 10^4-fold in a DBA/2 embryo cell line (2), approximately 100-fold greater restriction than that observed in secondary DBA/2 embryo cultures (12; this study). Thus, the derivation of cell lines from these embryo cultures appears to select for cells expressing higher levels of endogenous gp70. A similar relationship between the level of MCF gp70 expression and restriction of virus replication is observed in *Rmcf*$^{r/s}$ mice, which expressed lower levels of MCF gp70 than *Rmcf*$^{r/r}$ mice (Fig. 1) and exhibit accordingly lower restriction of virus replication than the homozygous strain (12).

The finding that the endogenous MCF and xenotropic *env* genes described here acted as alleles is intriguing. We believe this to be the first demonstration of the expression of alternate alleles for endogenous murine retroviral *env* genes. A common ancestral gene may have undergone mutation or recombination, resulting in the appearance of two alleles. In this case the mouse strains which were negative for both alleles (e.g., NFS/N and IRW) may still contain the structural gene, the expression of which has been lost. Alternatively, these two envelope sequences could reflect independent germ line integrations, in which case they may occupy tightly linked but not identical sites in the genome. Molecular analysis will be required to clarify this point.

The possible function of these endogenous gp70 molecules is still an open question. The linkage between the MCF gp70 allele and the resistance allele of the *Rmcf* gene suggests that this gp70 may function in preventing the spread of MCF viruses in vivo. However, it is not clear that these gp70 molecules are expressed by cells other than fibroblasts. In preliminary experiments, these gp70 molecules appear not to be expressed by lymphoid cells in either the spleen or bone marrow. We are currently examining different cell populations in both fetal and adult tissues and carrying out linkage studies relating parameters of disease resistance to expression of these gp70 molecules.

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