THE ENDOGENOUS MINK CELL FOCUS-FORMING (MCF) gp70 LINKED TO THE Rmcf GENE RESTRICTS MCF VIRUS REPLICATION IN VIVO AND PROVIDES PARTIAL RESISTANCE TO ERYTHROLEUKEMIA INDUCED BY FRIEND MURINE LEUKEMIA VIRUS

BY RICHARD S. BULLER,* MARC SITBON,* AND JOHN L. PORTIS*

From the *Laboratory of Persistent Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rocky Mountain Laboratories, Hamilton, Montana 59840; and the †Institut National de la Santé et de la Recherche Médicale Unité 152, Hôpital Cochin, 75104 Paris, France

Inoculation of IRW or BALB/c neonatal mice with Friend murine leukemia virus (F-MuLV) induces an erythroleukemia 1–3 mo after infection (1, 2). Some strains of mice, such as DBA/2 and C57BL/6, exhibit different levels of resistance to disease induction (3–6). In DBA/2 mice the incidence of erythroleukemia is low and for those mice that develop this form of leukemia the onset of disease is relatively late (4–6 mo) (3). C57BL/6 mice on the other hand, are completely resistant (4, 6). In both of these strains, resistance is genetically dominant.

Despite these differences in susceptibility to erythroleukemia, there appears to be no correlation between disease susceptibility and replication of the inoculated ecotropic virus (4, 6). Within several weeks of inoculation of susceptible mice with F-MuLV, however, a new class of viruses called mink cell focus-forming (MCF) viruses appear (4, 7) which result from recombination between input ecotropic and specific endogenous nonecotropic retroviral sequences (8, 9). These viruses are thought to play a role in the induction of erythroleukemia. They are readily isolated from susceptible mice but are recovered only with difficulty (DBA/2) (3) or not at all (C57BL/6) from resistant strains (4, 10). The specific role of MCF viruses in leukemia induction is not known, but their expanded cellular tropism may allow them to infect relevant target cells.

Genetic studies have shown the existence of several host genes that mediate resistance to F-MuLV erythroleukemia, although the mechanisms of action of these genes are not well characterized. Backcross analyses have revealed that DBA/2 mice appear to possess a single dominant gene controlling resistance to F-MuLV–induced erythroleukemia (5, 4), although more recent evidence suggests that this strain possesses more than one resistance gene (11). One of the

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Abbreviations used in this paper: IC, infectious center; IRW, inbred Rocky Mountain white mice; FIA, focal immunofluorescence assay; FMF, flow microfluorometric; F-MuLV, Friend murine leukemia virus; SFFV, spleen focus-forming virus.
genes that may play a role in the resistance of DBA/2 mice is the Rmcf gene on chromosome 5 (12). Embryo cell cultures from mouse strains carrying the resistance allele (Rmcf°), such as DBA/2 and CBA/N, display a resistance to infection by MCF viruses in vitro when compared with mice having the sensitive allele (Rmcf'), such as BALB/c and IRW (12-14). The resistance is a cell surface phenomenon apparently mediated by viral interference due to the expression of endogenous retroviral gp70 capable of competing with MCF viruses for the MCF receptor (12-14).

Although Rowe and Hartley (15) addressed the question of the role of the Rmcf gene in vivo using F1 hybrids between the Rmcf° strain AKR and the Rmcf° strains DBA/2 and CBA/N (15), the best evidence to date that the Rmcf gene has a function in vivo comes from the work of Ruscetti et al. (11). Since the Rmcf gene is linked to several known genetic markers (12, 16), these markers were used to establish partially congenic lines of BALB/c mice carrying the segment of chromosome 5 bearing the Rmcf gene from DBA/2 mice (16). These studies indicate that mice carrying this chromosome segment are resistant to erythroleukemia induced by F-MuLV (11). However, as the Rmcf gene is defined by its effect in vitro, namely the restriction of MCF viral replication in cell culture, and thus, can be analyzed only by cumbersome functional criteria, the role of the Rmcf gene in disease resistance has not been tested in genetic crosses that allow random assortment of genes from Rmcf° and Rmcf° mice.

We recently reported (14) the characterization of two allelic endogenous gp70 molecules that appear to be encoded by the Rmcf locus. Cell cultures from mice carrying the Rmcf° allele (DBA/2 and CBA/N) express an MCF gp70, whereas cultures from Rmcf° mice express either a xenotropic gp70 (C57BL/6, A/WySn, and CBA/J) or they do not express any detectable gp70 (BALB/c, IRW, and NFS/N) (14). Because these gp70 molecules can be readily identified on dermal cell cultures derived from neonatal mice using mAbs, this provided a direct method for examining the relationship between the endogenous MCF env gene from DBA/2 mice and the in vivo resistance of this strain to F-MuLV-induced erythroleukemia. Results of a backcross indicated that progeny heterozygous for this endogenous env gene were partially resistant to erythroleukemia and that this endogenous gp70 was associated with a restriction of MCF virus expression after infection with F-MuLV, as well as the delayed onset of the anemia and splenomegaly associated with erythroleukemia. However, unlike the Rmcf°/° parent, most of the resistant progeny eventually succumbed to erythroleukemia within 6 mo.

**Materials and Methods**

**Mice.** Mice used in this study were bred at the animal facilities of the Rocky Mountain Laboratories, (RML) Hamilton, Montana. DBA/2J, mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Inbred Rocky Mountain White mice (IRW) were derived (3) and maintained at RML. The Rmcf phenotypes of these strains have been reported previously (12, 14).

**Typing of Mice for Expression of Rmcf-linked gp70.** Within 24 h of birth and before infection with virus, mice were marked for future identification by toe clipping; tails of the newborn mice were then clipped and used to start primary tail-skin cultures accord-
ing to the method of Lander et al. (17). After sufficient growth, cell cultures were analyzed for expression of Rmcf-linked env gene by flow microfluorometric (FMF) analysis.

**FMF Analysis.** FMF analysis of fibroblastic cell cultures for the expression of endogeneous Rmcf-linked env gene has been described previously (14). Briefly, after trypsinization, single-cell suspensions of tail-skin cells were centrifuged through a FCS cushion and passed through nylon mesh in order to remove acellular debris. Approximately $5 \times 10^5$ cells were incubated with 0.1 ml of the MCF-specific mAb 514 (18) on ice for 30 min in the wells of a 96-well tray. After washing, cells were incubated an additional 30 min with 0.1 ml of a 1:200 dilution of FITC-conjugated goat anti-mouse Ig (Cappel Laboratories, Cooper Biomedical, Malvern, PA). After washing, 10,000 cells were analyzed with a FACS analyzer (Becton Dickinson & Co., Mountain View, CA). The analyzer was operated in log gain, with full-scale fluorescence being 3.0 log. Controls consisted of cells reacted with the FITC–goat anti-mouse conjugate alone. Backcross progeny whose tail skin cell cultures expressed endogenous MCF gp70 are referred to in this report as gp70+ mice.

**Virus, Viral Assay, and Cells.** The molecularly cloned F-MuLV strain 57 was used in this study (2). Titration of ecotropic F-MuLV and MCF viruses in the spleens of infected mice was accomplished by overlaying *Mus dunni* (19) cells with various dilutions of single-cell suspensions derived from spleen cells of infected mice. Foci of infection were counted by means of a focal immunofluorescence assay (FIA) (20) using the F-MuLV–specific mAb 48 (18) or the MCF-specific mAb 514 (18). *Mus dunni* cell cultures were maintained in RPMI 1640 medium supplemented with 10% 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).

**Leukemogenesis.** Newborn mice <24 h old were inoculated intraperitoneally with $1.25 \times 10^4$ focus-forming units of F-MuLV in tissue culture supernatant from Fisher rat embryo cells chronically infected with F-MuLV 57. Mice surviving the weaning period were followed for splenomegaly by palpation under ether anesthesia. Mice having grossly enlarged spleens (>800 mg) were considered leukemic and were killed for cytological analysis. This analysis consisted of staining single cell preparations of spleen cells with Sudan Black, a myeloid marker, and for fluoride inhibitable α-naphthylbutyrate esterase (erythroid marker) as described previously (3).

**Determination of Anemia.** Mice were followed for development of anemia associated with leukemia every 2 wk starting 1 mo after infection. Earlier time points were not used in order to avoid confusion with the early hemolytic anemia induced by F-MuLV 57 (21). Mice were bled from the retro-orbital sinus with a 70-µl microhematocrit tube, the tubes were centrifuged, and the hematocrits were expressed as percent of total blood volume.

### Results

**The Endogenous MCF gp70 from DBA/2 Mice Segregates with Resistance to Erythroleukemia in Backcross Progeny.** The two parental strains, IRW and (DBA/2 × IRW)F1, differ in susceptibility to erythroleukemia, with IRW being sensitive and the F1 being resistant. The Rmcf allele expressed by each strain can be typed by examining dermal cell cultures for the expression of endogenous MCF gp70 with an MCF-specific mAb. DBA/2 mice are gp70+ whereas IRW cells are gp70− (14). As the expression of the MCF gp70 is dominant, the F1 strain produced by crossing DBA/2 and IRW also expresses MCF gp70. (DBA/2 × IRW) × IRW backcross progeny were tested for gp70 expression as neonates and subsequently inoculated with F-MuLV 57. As expected from previous studies (14), the env gene from DBA/2 mice segregated as a single dominant gene, with 29 mice being gp70+ and 21 mice gp70−. All mice were followed periodically for two parameters of disease: late anemia (21) and splenomegaly.
It was evident that the gp70\(^{-}\) group developed anemia more rapidly than the gp70\(^{+}\) mice (Fig. 1). As in the susceptible IRW parent (21), anemia in the gp70\(^{-}\) group developed between the fourth and eighth week after inoculation. During this same period no change was observed in the hematocrits of the gp70\(^{+}\) mice. By 12 wk, however, anemia was observed among some of the gp70\(^{+}\) mice.

The difference in disease susceptibility was revealed further by direct measurement of splenic weights at 5 wk after inoculation in a second set of (DBA/2 \(\times\) IRW) \(\times\) IRW backcross progeny infected at birth with F-MuLV 57 (Table I). Although there was considerable heterogeneity within the groups of both parental and backcross progeny, similar to the IRW parent, most of the gp70\(^{-}\) backcross mice had enlarged spleens (>400 mg; normal range 80–100 mg), whereas a majority of the gp70\(^{+}\) backcross mice had only slightly enlarged spleens (200–400 mg). However, the resistance to splenomegaly in the gp70\(^{+}\) backcross mice was less dramatic than that observed in the F1 parent.

Many of these spleens were also examined for the frequency of erythroid cells using enzyme histochemistry (Table I). Mice with abnormally high values (>15%...

**Table I**

| Splenomegaly and Erythroblastosis in Parental and Backcross Mice Infected with F-MuLV |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                 | IRW                             | F1                              | gp70\(^{-}\)                     | gp70\(^{+}\)                      |
| Spleen weight (mg)\(^{*}\)     | 430 (270–910)                   | 170 (140–420)                   | 580 (210–1590)                   | 290 (220–810)                    |
| Percent erythroid\(^{2}\)      | 35 (10–80)                      | 7 (2–51)                        | 98 (5–70)                       | 13 (5–35)                       |

Mice were infected at birth with F-MuLV 57. At 5 wk of age, animals were killed and spleen weights and the percent of splenic erythroid cells were determined.

\(^{*}\) Median spleen weight and range in milligrams. Each group contains data from 15–25 mice.

\(^{2}\) Median percentage and range of splenic erythroid cells as determined by histochemical staining as described in Materials and Methods. Each group contains 15–25 mice except gp70\(^{-}\), which contains 9 mice.
erythroid cells) were clearly more common in the gp70− groups (gp70− backcross and IRW parent) than in the gp70+ groups (gp70+ backcross and F1 parent). Despite the obvious differences in the erythroproliferative responses between the two groups of backcross progeny, even at this relatively early time point, some gp70+ backcross mice, as well as the F1 parent, had evidence of abnormal erythroblastosis.

When the overall incidence of leukemia in backcross progeny was plotted over a 6-mo period of observation, the level of resistance observed for gp70+ backcross mice was not as great as that of the F1 parent in which only 15% of the mice had become leukemic by 6 mo (Fig. 2). By 6 mo, >70% of the gp70+ backcross progeny had become leukemic. Thus, DBA/2 mice possess other resistance loci, the segregation of which must be independent of the endogenous MCF gp70 associated with the Rmcf gene.

**The Endogenous MCF gp70 from DBA/2 Mice Segregates in Backcross Progeny with Restriction of MCF Virus Expression.** Fibroblastic cell cultures derived from mice homozygous for the resistance allele of the Rmcf locus exhibit moderate restriction to MCF virus infection (~100–1,000-fold) (12, 14). The interference expressed by cells derived from heterozygous (Rmcf+/−) mice is even less marked (~10–100-fold) (12, 14). In a previous study, the level of restriction appeared to be directly related to the amount of endogenous envelope protein expressed at the cell surface (14).

Because of the relatively weak interference displayed by F1 cells in vitro, we were surprised to find that (DBA/2 X IRW) X IRW progeny heterozygous for the endogenous Rmcf−-linked env gene from DBA/2 mice had a clear-cut resistance to MCF expression in vivo. 5 wk after inoculation, backcross and parental mice were killed and their spleen cells were seeded onto Mus dunni indicator cells which are susceptible to infection by both ecotropic and MCF viruses. Although no difference was observed in the level of ecotropic virus infectious centers (ICs) between the gp70− and gp70+ backcross progeny and parental
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Figure 3. Replication of F-MuLV 57 and MCF viruses in backcross and parental mice 5 wk after neonatal infection with F-MuLV 57. Mice were killed at 5 wk and dilutions of their spleen cells were layered onto Mus dunni cells and foci of infection were counted with the ecotropic-specific monoclonal 48 for detection of F-MuLV, or the MCF-specific monoclonal 514 for the detection of MCF viruses. Each dot represents the splenic titer of a single mouse. Dots on or below the lower line in the MCF panel represent viral titers below the limit of detection (5 \times 10^{-1} ICs/10^6 spleen cells).

strains (Fig. 3), a striking difference in MCF-specific IC's was noted (Fig. 3). MCF viruses were detected in a majority of the gp70^- mice, with most titers ranging between 10^2 and 10^3 IC/10^6 spleen cells. These titers were, however, on average 10-fold lower than the MCF virus titers seen in the susceptible IRW parent. In contrast, only about one-half of the gp70^+ mice expressed detectable levels of MCF-specific ICs with only one mouse having a titer comparable to the majority of the gp70^- progeny. MCF titers of the resistant F1 parent were consistently low or below the level of detection whereas those of the gp70^+ backcross progeny were more variable. These data indicated that, even when present as a single copy, the Rmcf-linked env gene from DBA/2 mice had a pronounced effect on MCF virus expression in vivo.

The correlation between the lack of MCF virus expression and resistance to leukemia seen in the gp70^+ backcross mice indicated, as has been suggested by other studies (4, 7), that MCF viruses may be the primary transforming agent in F-MuLV-induced erythroleukemia. The backcross used in this study provided an inadvertent test of this hypothesis. As the gp70^- backcross progeny were restricted for MCF virus expression but often lacked other DBA/2 genes conferring resistance to leukemia, most of these mice eventually developed leukemia. By enzyme histochemistry all of the leukemias from these mice exhibited a predominance of erythroid cells (>80% of the cells stained positive for fluoridesensitive \(\alpha\)-naphthyl-butyrate esterase). We therefore tested a series of these leu-
TABLE II
Detection of MCF Viruses in the Spleens of Leukemic (DBA/2 × IRW)F1 × IRW Backcross Mice Infected with F-MuLV

| Endogenous MCF gp70* | Age | Spleen weight | MCF Ics/106 spleen cells |
|----------------------|-----|---------------|--------------------------|
| +                    | 70  | 1.0           | < 0.5                    |
| +                    | 115 | 0.8           | < 0.5                    |
| +                    | 129 | 1.6           | 1.0                      |
| -                    | 144 | 2.0           | 2.6 × 10³                |
| -                    | 56  | 2.2           | 2 × 10³                  |
| -                    | 70  | 1.4           | 4 × 10²                  |
| -                    | 85  | 3.0           | 7 × 10⁷                  |
| -                    | 52  | 1.4           | 2 × 10³                  |
| -                    | 66  | 1.8           | 4 × 10²                  |
| -                    | 73  | 1.9           | 0.5                      |
| -                    | 51  | 1.8           | 4 × 10³                  |
| -                    | 65  | 1.8           | 2 × 10³                  |
| -                    | 65  | 1.3           | 1 × 10³                  |
| -                    | 49  | 2.3           | 9 × 10³                  |
| -                    | 70  | 1.6           | 1 × 10³                  |
| -                    | 70  | 1.3           | 1 × 10³                  |

* These mice were the same animals typed and followed for disease in Fig. 2.

† Time, in days, after birth when mice were killed. Mice were killed when judged leukemic by splenic palpation and hematocrit. All leukemias were typed as erythroid by histochemical staining as described in the Materials and Methods.

‡ Splenic infectious centers were assayed on Mus dunni cells with the MCF specific mAb. 514.

Discussion

In the current study we have established the association of the expression of the Rmef-linked endogenous MCF gp70 of DBA/2 mice and the resistance of this strain to the development of erythroleukemia induced by F-MuLV. The segregation of the endogenous MCF gp70 with the restriction of MCF virus expression is the most direct demonstration that the Rmef gene functions in vivo to restrict MCF virus expression by viral interference.

There is precedence for the ability of expressed endogenous murine retroviral env genes to interfere with the replication of retroviruses and provide resistance to disease. For example, the Fv-4 gene has been shown to prevent the replication of ecotropic viruses through the expression of an endogenous ecotropic env.
gene (22, 23). It is thought that Fv-4 resistance is mediated by viral receptor blockade. Since the Rmcf gene appears to operate through a similar mechanism, this raises several interesting questions about the role of MCF viruses in leukemogenesis in this system. The events leading to the generation and spread of MCF viruses in vivo, and their precise role in leukemia induction are presently not well understood. Data indicating that the Rmcf locus restricts the in vitro replication of MCF viruses were obtained from experiments in which MCF viruses were titrated on cells in the absence of the inducing ecotropic viruses (12, 14). In vivo, after infection with F-MuLV, evidence suggests that MCF viruses are extensively pseudotyped with ecotropic viral envelope proteins (24) and, therefore, may not be susceptible to interference mediated by endogenous MCF gp70. Additionally, due to the early replication of ecotropic virus, it is likely that the relevant erythroid target cells are already infected with ecotropic virus at the time when MCF viruses first appear and these cells, thus, should be effectively blocked for superinfection by pseudotyped pathogenic MCF viruses. If this argument is correct, it is not obvious how to explain the association between restriction of MCF virus expression seen in the gp70+ backcross mice and resistance to erythroleukemia.

As first demonstrated in avian leukoses (25–28) and later in murine leukemias (29–32), the presumed mechanism of leukemogenesis for nonacutely transforming retroviruses such as F-MuLV is insertional activation of cellular onc genes. In Moloney MuLV-induced thymomas it is the recombinant MCF viruses that appear to be integrated in proximity to cellular onc genes (31, 32). Although currently there are no reports of preferred integration sites for F-MuLV in erythroleukemia, several preferred integration sites have been found for this virus in myelogenous leukemias (33–36). Thus, it is possible that several rounds of infection by the ecotropic virus may be necessary to cause transformation of an erythroid target cell. After the initial burst of ecotropic viral replication, subsequent rounds of infection could only be accomplished through entry of pseudotyped ecotropic virus via the MCF receptor. Cells that expressed endogenous MCF gp70 would be resistant to transformation. Although such a mechanism is speculative, the existence of F-MuLV pseudotyped with MCF coat proteins has been observed (24). Additionally, Chesebro et al. (37) reported that sequential infection of mice with a pathogenic Friend MCF virus at birth followed by infection with F-MuLV at 5–7 wk causes a marked acceleration in the induction of leukemia, suggesting a synergistic effect between the two viruses. It should be noted that in the present study the restriction of MCF virus expression mediated by the endogenous gp70 appeared to be more potent than its ability to block the induction of erythroleukemia as evidenced by our failure to detect MCF viruses in some of the gp70+ backcross mice with erythroleukemia (Table II). Thus, expression of MCF viruses appeared not to be necessary for maintenance of the leukemic phenotype.

An alternative mechanism of resistance mediated by the Rmcf locus involves the possible function of MCF viruses as mitogenic signals. The gp55 glycoprotein encoded by the env gene of Friend spleen focus-forming virus (SFFV) is leukemogenic (38, 39). It has been proposed (40) that a domain of this protein binds to MCF receptors on erythroid target cells and causes a mitogenic stimu-
lation resulting in erythroblastosis. On the basis of structural similarities between SFFV gp55 and MCF viral gp70, Li et al. (40) suggested that MCF viruses may also bind to these cell surface receptors and cause a premalignant proliferation of erythroid cells. In this model, the \( Rmef \)-linked endogenous MCF gp70 may be related to the gp70 of nonleukomogenic MCF viruses. Such a non-pathogenic endogenous protein could bind to the cell surface receptor, effectively blocking the binding of pathogenic MCF viruses, thus preventing the mitogenic stimulation envisioned in this model.

Whatever the mechanism, the protection afforded by the expression of this \( env \) gene in backcross mice was not as complete as that observed in the (IRW \( \times \) DBA/2)\( F_1 \) parent. Although the onset of erythroleukemia among the gp70\( ^+ \) progeny, as determined by either late anemia or splenomegaly, was delayed by 1–2 mo, the overall incidence of erythroleukemia over a 6-mo period approached that of the gp70\( ^- \) mice. This observation supports the idea proposed by Ruscetti et al. (11) that the resistance of DBA/2 mice to erythroleukemia induced by F-MuLV is a function of more than one gene.

The fact that several gp70\( ^+ \) backcross progeny had measurable titers of MCF infectious centers at 5 wk after inoculation (Fig. 3), could be explained as a stochastic variation in MCF expression by these mice. Because it was possible to detect, albeit at low levels, MCF viruses in both the heterozygous (IRW \( \times \) DBA/2)\( F_1 \) (Fig. 3) and the homozygous DBA/2 (3, Sitbon, M., unpublished observations), the presence of endogenous gp70 does not completely abrogate the expression of MCF viruses. The variation observed among the gp70\( ^+ \) progeny compared with the \( F_1 \) parent, thus, may be the result of the segregation of other DBA/2 genes controlling the replication of MCF viruses. In support of this idea was the finding that MCF virus expression among the gp70\( ^- \) progeny was approximately tenfold lower than the IRW parent (Fig. 3).

It is more difficult to explain the one gp70\( ^- \) backcross mouse in Fig. 3 that failed to generate a detectable level of MCF viruses. It is known that at least two endogenous sequences participate in the generation of MCF viruses after infection of NFS mice with F-MuLV (8, 9). Perhaps more than one endogenous retroviral sequence must be inherited from the IRW parent in this backcross in order to generate detectable levels of MCF viruses. Thus, the one MCF virus-negative mouse may have represented a backcross offspring that, although negative for the expression of endogenous gp70, did not receive the number of proviruses required to display the IRW phenotype for MCF virus generation. In an attempt to better understand the mechanism of the resistance to erythroleukemia afforded by the expression of the endogenous MCF gp70, we are currently studying hematopoietic tissues in order to identify the cell lineage and differentiation state of the cells expressing the endogenous retroviral gene.

**Summary**

The \( Rmef \) locus restricts the in vitro replication of mink cell focus-forming (MCF) viruses in cell cultures derived from mice carrying the resistance allele. Previously we reported (14) that in cell cultures from first backcross progeny, this \( Rmef \)-linked restriction segregates with the expression of an endogenous
retroviral gp70 is serologically related to that of MCF viruses. The current report details the results of genetic studies designed to examine the possible association of this endogenous gp70 with resistance of mice to Friend murine leukemia virus (F-MuLV)-induced erythroleukemia. This *env* gene segregates as a single dominant trait in (DBA/2 × IRW) × IRW progeny, in which the expression of the gene can be detected by serological techniques. Results indicated that the gp70− progeny developed leukemia at the same rate as the susceptible IRW parent, whereas the tempo of disease among the gp70+ progeny was significantly slower. However, the resistance mediated by this gene was only partial, since most of the gp70+ offspring eventually developed erythroleukemia when followed for 6 mo. This endogenous gp70 also segregated with a restriction to the expression of recombinant MCF viruses after infection with F-MuLV. Since in this study all unlinked genes segregated independently, this is direct evidence that MCF viruses participate in the induction of erythroleukemia.

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