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Fv-4 Resistance Gene: a Truncated Endogenous Murine Leukemia Virus with Ecotropic Interference Properties

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***Fv-4* is a mouse gene which controls susceptibility to infection by ecotropic murine leukemia virus (MuLV). We previously cloned part of an endogenous MuLV associated with the resistance allele of the *Fv-4* gene (*Fv-4*^{r}). In this report, we describe an extended clone of the *Fv-4*^{r} allele consisting of a 17-kilobase DNA fragment containing the retroviral sequence and its 5′-flanking sequence. The new DNA clone contains a truncated MuLV with Δ*pol-env*-long terminal repeat sequences but no other MuLV-reactive sequence within 13 kilobases upstream of the truncated MuLV. Transfection of this clone into mouse cells led to transcription of *Fv-4 env* mRNA, expression of the *Fv-4*^{r}-specific MuLV envelope protein, and resistance to infection with ecotropic MuLV but not amphotropic and dualtropic MuLVs. Restriction of ecotropic viruses appears to occur at or before viral cDNA synthesis. This result is consistent with a model of receptor interference for *Fv-4* restriction. Our data also suggest that the 5′ non-MuLV sequence is important for biological function, since a DNA clone which lacks most of the 5′-flanking sequence did not efficiently confer the resistance phenotype.**

There are many copies of murine leukemia virus (MuLV)-related sequences in the mouse genome (8, 48). These sequences were probably generated by MuLV infection of germ cells or early embryonic cells. Endogenous MuLVs may affect biological functions of host animals in several ways. Insertional mutations by MuLVs have been found as heritable mutations at several loci (9, 13, 25, 46, 49). Expression of infectious viruses derived from endogenous proviral loci and amplification of the viruses or their recombinant progeny are key events in spontaneous viral leukemogenesis (52). Viral leukemogenesis appears to be modified by expression of endogenous retroviral proteins. For example, alleles at *Fv-4* (27, 50) and *Rmcf* (3, 16, 43) are associated with expression of MuLV envelope (env) glycoproteins and resistance to retroviral leukemogenesis. It is also known that in chickens a few avian endogenous retrovirus loci have similar resistance effects (2, 17, 40).

Mouse gene *Fv-4* (27, 50) and an apparently identical gene, *Akvr-1* (10, 11, 34), are located on chromosome 12 (24, 36). *Fv-4* controls susceptibility to retrovirus infection and therefore to virus-induced tumors. The resistance allele at the *Fv-4* locus (*Fv-4*^{r}) is dominant over the susceptibility allele (*Fv-4*^{s}) (50). Two phenomena are associated with the *Fv-4*^{r} allele: constitutive expression in a variety of cells of a unique MuLV env-related antigen (22, 23, 54) and inheritance of a MuLV *env* sequence (21, 30). These markers were found in three of three *Fv-4*^{r} congenic mouse strains (21; H. Ikeda; unpublished data) which were established from independent wild or laboratory *Fv-4*^{r} mice. On the basis of clear genetic concordance of these markers with *Fv-4*^{r}, we have proposed a receptor interference model for *Fv-4* restriction (22, 23). Virus interference refers to the phenomenon in which cells chronically infected with one virus are resistant to superinfection with other viruses bearing the same virus envelope specificity. Interference is probably due to blockage of the virus receptors by the envelope glycoprotein of the initially infecting virus.

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We have previously cloned a 5.2-kilobase (kb) DNA fragment which contains an *Fv-4*^{r}-associated, MuLV-related sequence (21). The cloned DNA consists of the 3′ half of an ordinary endogenous MuLV, including the 3′ portion of the *pol* region, the entire *env* gene, and the 3′ long terminal repeat (LTR). Sequence analysis indicated that *Fv-4 env* is distinct from but most related to the *env* gene of exogenous ecotropic MuLV isolates and of the AKV virus, which is the only strain of endogenous ecotropic MuLV found in laboratory mice (8). *Fv-4 env* is probably related to endogenous MuLVs carried by wild mice (6, 7, 31), which is consistent with the finding that many wild mice of Japan, Southeast Asia, and North America have an *Fv-4*^{r}-like resistance gene(s) (11, 35, 36, 51). 5′ of the *pol* sequence in the 5.2-kb DNA clone was about 250 base pairs of a non-MuLV sequence, suggesting that the *Fv-4*^{r} provirus is truncated.

This report describes another genomic DNA clone containing the *Fv-4*^{r}-associated, MuLV-like structure and its 5′-flanking region. We tested the biological activity of this cloned DNA by transfection. The results showed that this DNA, although lacking a 5′ LTR, induces efficient expression of the *Fv-4*-specific env protein and makes transfected *Fv-4* env$^+$ cells resistant specifically to ecotropic MuLVs. These functional activities suggest that the *Fv-4*^{r} gene is a combination of the non-MuLV sequence and the truncated MuLV.

MATERIALS AND METHODS

DNA cloning from a genomic library. A DNA library was prepared by ligation of *Bam*HI arms of bacteriophage λ J1, provided by J. Mullins (Harvard University), and 10- to 20-kb fragments of partially *Mbo*I-digested DNA from livers of BALB/c *Fv-4w*^{r} mice (36). The library was screened with a ^{32}P-labeled *Fv-4 env*-specific probe pFv4env (21). One clone (λ Fv4-17) was isolated from approximately 3×10^5 recombinant phage. The insert of λ Fv4-17 was 17 kb long and contained the *Fv-4*-associated MuLV structure and its 5′-flanking sequence.

Plasmids. pFv4 is a plasmid with a 5.2-kb *Eco*RI insert

containing the *Fv-4*^{r}-associated MuLV (21) (see Fig. 1). pcDFv4env is a pcDX-derived vector (38) in which the *Fv-4 env* region was inserted between the simian virus 40 (SV40) early promoter and the SV40 polyadenylation signal (see Fig. 3). pcDFv4env was constructed by ligation of the following three DNA fragments: (i) a 240-base-pair fragment containing the SV40 promoter and splice donor and acceptor sites which was isolated from a 1.3-kb *Pst*I fragment from pcDX DNA which was blunt ended with *Escherichia coli* DNA polymerase I and then restricted with *Hin*dIII; (ii) a 2.4-kb *Acc*I-*Kpn*I fragment containing the *Fv-4 env* region from pFv4, the ends of which were blunt ended with *E. coli* DNA polymerase I; and (iii) a 2.6-kb fragment containing the SV40 polyadenylation signal and a plasmid sequence isolated from a 3.0-kb *Bam*HI fragment of pcDX, which was blunt ended and restricted with *Hin*dIII. These three fragments were ligated and propagated in *E. coli* HB101 cells. Two DNA constructs with *Fv-4 env* in opposite orientations, termed pcDFv4env and pcDFv4env(R), were obtained. pSV2neo is a vector containing the SV40 early promoter and the neomycin resistance gene (45). p0.8 is a plasmid containing a 0.8-kb *Bam*HI fragment from Friend MuLV *env* which was used to detect Friend MuLV cDNA (44).

DNA transfection into mouse cells. NIH 3T3 cells were transfected with pSV2neo and *Fv-4 env*-containing plasmid (or phage) DNAs by the calcium phosphate method (38). Selection of Neo^r cells began 2 days after transfection with addition of G418 (GIBCO Laboratories) to culture medium at a concentration of 400 μg/ml.

IF staining. For immunofluorescence (IF) staining, normal and transfected NIH 3T3 cells were trypsinized, washed with 199 medium containing 1% fetal calf serum, incubated for 30 min at 37°C with *Fv-4* env-specific antiserum (BALB/c anti-BALB/c *Fv-4w*^{r}) (22), washed, and reacted with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin. Fluorescence-positive cells were counted under a fluorescence microscope or with a cytofluorograph (Ortho Spectrum III).

Virus assay. NIH 3T3 cells and transfected NIH 3T3 cells were infected with a mixture of nondefective helper MuLV and mouse sarcoma virus (MSV) pseudotyped with helper MuLV [MSV (MuLV)]. The helper MuLVs used were amphotropic (4060A strain), dualtropic (AKR13 strain), and ecotropic (Friend, Moloney, and AKR623 strains) MuLVs which were kindly provided by Janet Hartley (National Institutes of Health). Sarcoma virus was rescued from C-182 cells (4) by ecotropic MuLVs and from S^+ L^- mink cells (provided by J. Hartley) by amphotropic and dualtropic MuLVs. Before infection, cells were treated with Polybrene at a concentration of 20 μg/ml for 6 to 18 h.

Analysis of DNA and RNA. Genomic and cytoplasmic DNAs were isolated as previously described (20, 21, 32). RNA was prepared by the guanidine thiocyanate procedure (32). Southern and Northern (RNA) blot analyses were performed with nick-translated probes. Hybridization was performed as previously described (21), except that the hybridization temperature was 50°C rather than 45°C.

RESULTS

Cloning of DNA associated with *Fv-4* resistance. We previously cloned a 5.2-kb *Eco*RI fragment which is associated with the resistance allele of the *Fv-4* locus (*Fv-4*^{r}) and contains an MuLV segment (pFv4 in Fig. 1) (21). To understand this chromosomal region better, we cloned a 17-kb DNA fragment (λ Fv4-17) which contains the *Fv-4*^{r}-associated retroviral sequence (about 4 kb long) plus its upstream region (about 13 kb) (Fig. 1). The cloned DNA was digested with restriction enzymes and hybridized with several MuLV segments to identify sequences homologous to MuLV. No MuLV-reactive sequence was found in the upstream region from the insert of pFv4 (data not shown). It was necessary to determine whether the newly cloned DNA was derived from an intact genomic DNA or was a cloning artifact, because the phage library of genomic DNA was prepared from partially *Mbo*I-digested DNA from *Fv-4*^{r}-carrying mice. Our previous Southern blot analysis showed that the *Fv-4 env*-specific probe (probe A in Fig. 1) hybridized to a 14-kb *Kpn*I fragment of *Fv-4*^{r} mouse DNA. The insert of λ Fv4-17 also had a 14-kb *Kpn*I fragment, and both the A probe and a *Kpn*I-*Hin*dIII fragment (probe B in Fig. 1) located about 14 kb upstream from the *Kpn*I site of the *Fv-4* 3′ LTR hybridized to the same 14-kb *Kpn*I fragment of *Fv-4*^{r} mouse DNA, indicating that the insert was derived from intact genomic DNA.

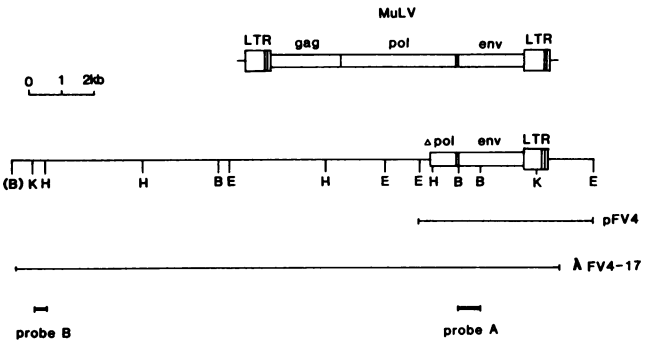

FIG. 1. Structure of *Fv-4*^{r}-associated MuLV and its flanking sequences. The general structure of an integrated MuLV is shown at the top. The restriction map was developed with two DNA clones, pFv4 and λ Fv4-17, from BALB/c *Fv-4w*^{r} mouse DNA. The enzyme sites indicated are *Hin*dIII (H), *Pst*I (P), *Eco*RI (E), *Kpn*I (K), and *Bam*HI (B). The retroviral sequence is indicated by the boxed area. Probes A and B indicate a *Bam*HI fragment of *Fv-4 env* and a *Kpn*I-*Hin*dIII fragment of the 5′-flanking sequence.

Introduction of *Fv-4* MuLV DNAs into tissue culture cells. Despite close genetic linkage of *Fv-4* resistance, the presence of the endogenous MuLV-like sequence (21, 30), and expression of the unique MuLV env protein in *Fv-4*^{r} mice (22, 23), there had been no direct evidence that these are functions of the same *Fv-4*^{r} gene. To resolve this question, we performed DNA transfection experiment with newly cloned *Fv-4* DNA. These experiments were particularly interesting because the *Fv-4*-associated MuLV-like structure lacks a 5′ LTR, the regulatory element for transcription of retroviral genes. Hence, it was unclear whether the cloned DNA could have biological activity.

The cloned DNAs or DNA constructs derived from them were cotransfected with a selectable marker (pSV2neo) into tissue culture mouse cells. Cell clones growing in medium containing G418 were isolated, analyzed for expression of *Fv-4* env protein, and tested for susceptibility to infection with MuLVs. NIH 3T3 cells were used for these experiments, because (i) they are susceptible to infection by MuLVs of three interference groups (amphotropic, dualtropic, and ecotropic MuLVs) (12, 15, 29, 33, 37, 42) and (ii) they do not carry endogenous ecotropic MuLVs which cross-hybridize with the *Fv-4 env* probe.

Expression of the *Fv-4* env glycoprotein was detected with *Fv-4* env-specific antiserum (23) by membrane IF (by light

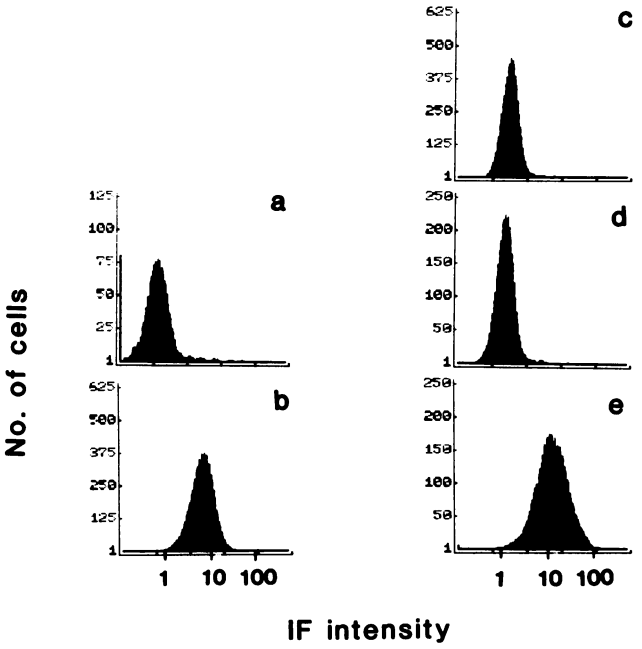

FIG. 2. IF flow cytometry analysis of *Fv-4* env expression with anti-*Fv-4*^{r} alloantiserum. Single-cell suspensions were incubated with BALB/c anti-BALB/c *Fv-4w*^{r} alloantiserum (37°C, 30 min), followed by fluorescein-conjugated rabbit anti-mouse immunoglobulin G (37°C, 30 min). Panels: a, embryo fibroblast cells from BALB/c mice; b, embryo fibroblast cells from BALB/c *Fv-4w*^{r} mice; c, NIH 3T3 cells; d, G418-resistant NIH 3T3 cells containing pSV2neo; e, G418-resistant, *Fv-4* env$^+$ NIH 3T3 cells containing pSV2neo and pcDFv4env.

microscopy in early experiments and by flow cytometry later). *Fv-4*^{r} antiserum reacted with embryo fibroblasts from BALB/c *Fv-4w*^{r} (*Fv-4*^{rr}) congenic mice but not with those from BALB/c (*Fv-4*^{ss}) mice (Fig. 2). The *Fv-4* env antigen was undetectable on NIH 3T3 cells or pSV2neo-transfected NIH 3T3 cells (Fig. 2). Furthermore, no significant fluorescence was found on NIH 3T3 cells persistently infected with various ecotropic, dualtropic, and amphotropic MuLVs (data not shown), indicating that the reactivity of the antiserum was highly specific for the *Fv-4* env protein under our assay conditions.

Transfected DNAs were pFv4, λ Fv4-17, pcDFv4env, and pcDFv4env(R) (Table 1). The latter two DNAs were made by inserting the coding region of *Fv-4 env* (*Acc*I-to-*Kpn*I fragment) between the SV40 early promoter and the polyadenylation signal from the pcDX vector (38) (see Fig. 3). On the basis of alignment of *Fv-4 env* with other MuLV *env* sequences, the *Acc*I site in *Fv-4* MuLV lies between the splice acceptor and the initiation codon of the *env* region, whereas the *Kpn*I site is located 13 bp upstream of the polyadenylation signal in the LTR (21).

TABLE 1. Generation of *Fv-4* env$^+$ cell clones by transfection with various DNAs[a]

DNA(s) transfected	No. of *Fv-4* env$^+$ clones/no. of Neo^r clones (%)
pSV2neo	0/8 (0)
pSV2neo–λ Fv4-17	25/41 (61)
pSV2neo–pFv4	6/36 (17)
pSV2neo–pcDFv4env	30/48 (63)
pSV2neo–pcDFv4env(R)[b]	0/12 (0)

[a] NIH 3T3 cells were transfected with pSV2neo (1 μg) alone or with the indicated DNAs (10 μg) (Fig. 1 and 3). Neo^r cell clones were analyzed for expression of *Fv-4* env protein by indirect IF with BALB/c anti-BALB/c *Fv-4w*^{r} serum (23).

[b] The *Fv-4 env* region was inserted in the reverse orientation with respect to the SV40 promoter.

After transfection of λ Fv4-17 and pSV2neo DNAs, 61% (25 of 41) of Neo^r clones expressed detectable levels of the *Fv-4* env antigen (Table 1). In contrast, pFv4 DNA yielded only 17% (6 of 36) *Fv-4* env$^+$ clones. The low frequency of *Fv-4* env$^+$ cell clones was probably not due to a defect in the coding region of *Fv-4 env*, because NIH 3T3 cells expressed the *Fv-4* env antigen at a high frequency (63%; 30 of 48) when they were transfected with pcDFv4env DNA (Table 1). No *Fv-4* env$^+$ clones were obtained following transfection with pSV2neo or pSV2neo plus pcDFv4env(R).

The relative amount of antigen on each *Fv-4* env$^+$ clone was estimated on the basis of the mean fluorescence intensity (Fig. 3). The *Fv-4* env antigen was highly expressed on cell clones transfected with λ Fv4-17 DNA (in most clones, 11 to 100 times over the background level of NIH 3T3 cells). Both pcDFv4env- and pFv4-transfected cells expressed lower levels of the antigen (5 to 50 and 3 to 11 times the background, respectively).

The copy number of transfected DNA was estimated by Southern blot analysis for several independent cell clones from each group of transfectants. All cell clones tested appeared to carry one to several copies of the transfected DNAs. There was no correlation between the copy number and level of *Fv-4* env expression (data not shown).

By Northern blot analysis, the *Fv-4 env* RNA in transformed *Fv-4* env$^+$ cells was compared with the transcript in *Fv-4*^{r}-carrying mice. *Fv-4 env* mRNA in vivo is about 3.0 kb long (Fig. 4, lane 10). This size is indistinguishable from the MuLV *env* mRNA in MuLV-infected cells (21) (Fig. 4, lane 11). Cell clones carrying λ Fv4-17 expressed a 3.0-kb *Fv-4 env* RNA (Fig. 4, lanes 5 and 6). pcDFv4env-transfected cells expressed a few different species of *Fv-4 env* RNA (Fig. 4, lanes 3 and 4), but the major transcript had the expected size, namely, about 250 base pairs shorter than in vivo *Fv-4 env* RNA.

Three pFv4-transfected cell clones which produced low amounts of *Fv-4* env antigen expressed two major species of *Fv-4 env* RNA, both of which were slightly longer than the normal *Fv-4 env* RNA in *Fv-4*^{r}-carrying mice (Fig. 4, lanes 7 to 9). These abnormal-size RNAs probably derive from a transfected DNA construct rather than peculiar integration sites in chromosomal DNA, because all of three cell clones expressed the same two RNAs. Our preliminary S1 mapping analysis using a probe derived from the 5′ end of the *Fv-4 env* region suggests that in these RNAs the same splicing acceptor site of *env* is used as in cells transfected with λ Fv4-17 DNA (data not shown). Thus, the larger RNAs could be readthrough products or could contain a unique 5′-untranslated exon. Although we did not characterize these RNAs further, the available evidence suggests that pFv4 DNA does not contain the normal Fv-4^r promoter which leads to a 3.0-kb *Fv-4 env* mRNA.

Resistance to ecotropic MuLVs conferred by λ Fv4-17. We examined the susceptibility of the transformed cells to infection by various MuLVs. Preliminary experiments showed that primary clones of *Fv-4* env$^+$ cells—although they expressed large amounts of *Fv-4*^{r} antigen—were variably susceptible to ecotropic MuLVs. For instance, of 13

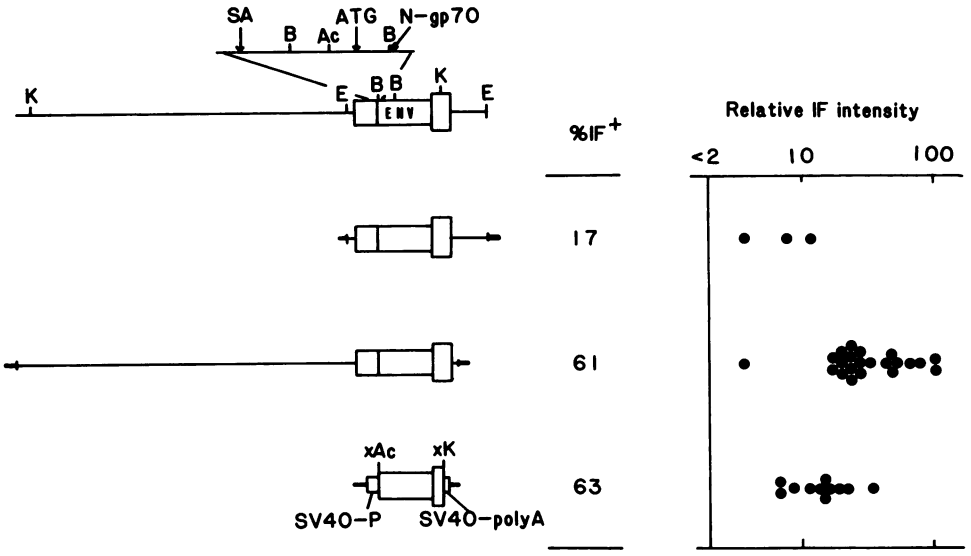

FIG. 3. Expression of the *Fv-4* env antigen on NIH 3T3 cells transfected with various DNAs. The DNAs indicated were cotransfected with pSV2neo, and individual G418-resistant cell clones were analyzed for *Fv-4* env expression on the cell surface by IF flow cytometry (Fig. 2). %IF$^+$, Percentage of *Fv-4* env$^+$ cell clones among the G418-resistant clones tested. The structure of *Fv-4^r*-associated MuLV and its flanking sequences is indicated at the top. The three DNAs used for transfection are shown below the top map. The restriction enzymes shown are *Acc*I (Ac), *Bam*HI (B), and *Eco*RI (E), and *Kpn*I (K). Abbreviations: SA, splice acceptor site; ATG, initiation codon of *env*; N-gp70, N terminus of gp70; SV40-P, promoter of SV40. In the graph of relative IF intensity, the mean fluorescence intensity of each cell clone is indicated as a point. Background fluorescence (IF intensity, <2) indicates the mean fluorescences of NIH 3T3 cells and pSV2neo-transfected NIH 3T3 cells. Data on cell clones with a mean IF intensity of <2 are not shown.

Fv-4 env$^+$ cell lines which were transfected with λ Fv4-17 DNA, 8 clones were resistant, whereas 5 were intermediately or fully susceptible to infection by ecotropic MuLV. Since minor contamination (<2%) with *Fv-4* env$^-$ cells could lead to susceptibility to MuLV, we isolated two or three secondary clones by a limiting dilution from three resistant and three susceptible cell lines. All of the secondary clones expressed *Fv-4* env antigen in amounts comparable to those of the parental cells, and all were resistant to infection by ecotropic MuLVs. For further analyses, we used the secondary clones carrying pFv4, λ Fv4-17, and pcDFv4env. The mean *Fv-4* env IF intensities of the secondary cell lines were 11, 23, and 13, respectively.

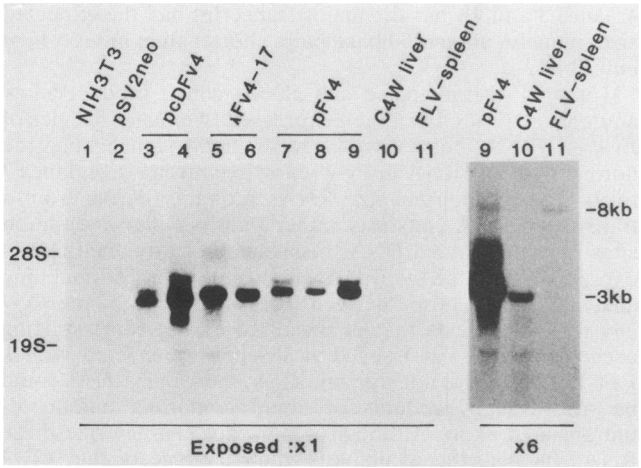

FIG. 4. Expression of *Fv-4 env* RNA in cells transfected with various DNAs. Five micrograms of total cellular RNA was electrophoresed and transferred to a nitrocellulose filter. The filter was hybridized with a ^{32}P-labeled *Bam*HI fragment from *Fv-4 env* (probe A in Fig. 1). The rightmost panel, showing lanes 9 to 11 (indicated as exposed ×6), was exposed six times longer than the film on the left. Note that in lane 11, 8 and 3 kb indicate full-length and spliced *env* mRNAs, respectively, of Friend MuLV (FLV) which cross-hybridized with probe A.

The secondary cell lines were infected by MSV pseudotyped with ecotropic (Moloney, Friend, and AKV623 strains), amphotropic (4060A), or dualtropic (AKR13) MuLVs. Parental and pSV2neo-transfected NIH 3T3 cells were susceptible to all of these viruses (Fig. 5). λ Fv4-17- and pcDFv4env-transfected NIH 3T3 cells were susceptible to amphotropic and dualtropic MuLVs but strongly resistant to the three strains of ecotropic MuLVs, being at least 10^3-fold less susceptible than NIH 3T3 cells. pFv4-transfected cells were also susceptible to amphotropic and dualtropic MuLVs but intermediately resistant to ecotropic MuLVs. These results indicated that the cloned *Fv-4^r*-associated MuLV-like structure and its flanking sequence are biologically able to induce resistance specific to ecotropic MuLV infection.

However, the following results suggested that the restriction to ecotropic MuLVs in *Fv-4* env$^+$ cells is not absolute. Normal and transfected *Fv-4* env$^+$ NIH 3T3 cells were infected by ecotropic MuLVs or mixtures of MSV pseudotyped with ecotropic MuLV and ecotropic MuLV, and then the virus replication of ecotropic MuLV was assayed by the XC plaque test. XC plaques were detectable in *Fv-4* env$^+$ cells, but the number of plaques was about 10^2-fold less than in control NIH 3T3 cells (data not shown). Furthermore, XC plaques generated in *Fv-4* env$^+$ cells were smaller than those in NIH 3T3 cells. In such experiments, it was not known whether these *Fv-4* env$^+$ cells had generally higher sensitivity to various groups of MuLVs, since the XC plaque test cannot be used to detect nonecotropic MuLVs. Probably,

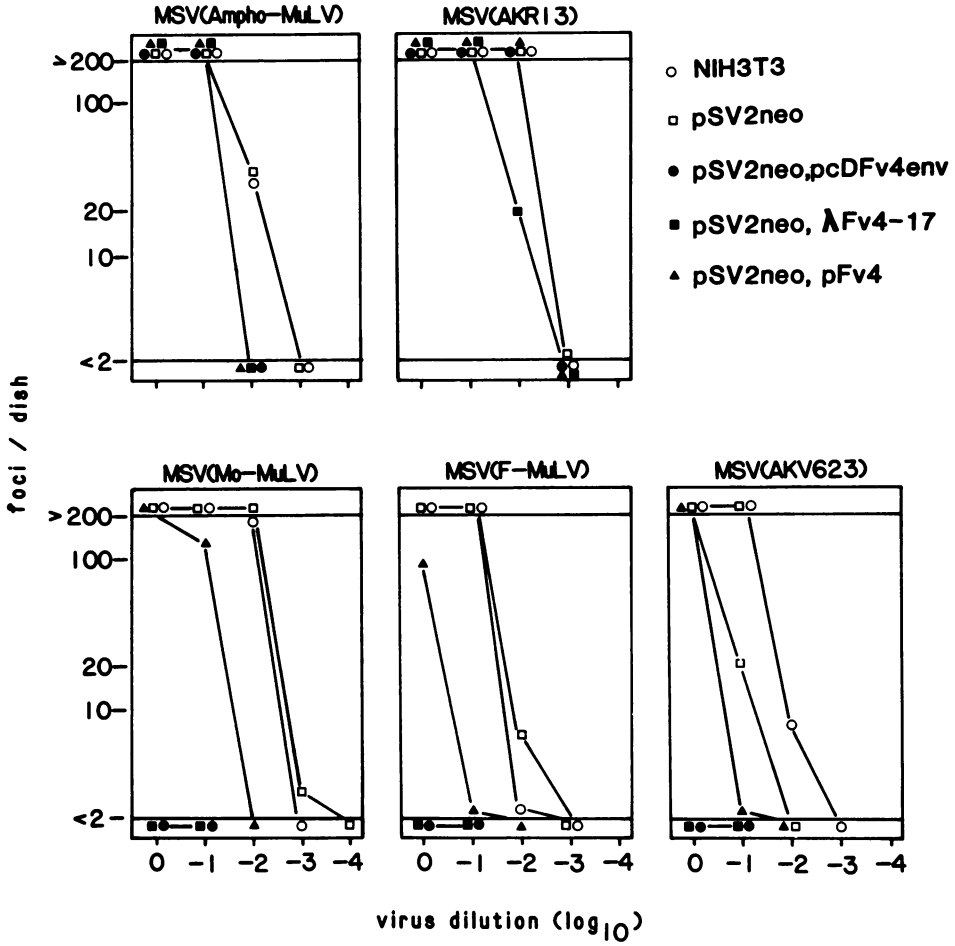

FIG. 5. Susceptibility of transformed NIH 3T3 cells to MuLVs. MSV pseudotyped with amphotropic (Ampho), dualtropic (AKV13), or ecotropic (Moloney [Mo], Friend [F], and AKV623 strains) MuLVs were used to infect NIH 3T3 cells and transfected NIH 3T3 cells. MSV foci were scored 5 days postinfection.

the more sensitive virus assay system, i.e., the XC test, allowed detection of smaller amounts of virus replication.

Possible occurrence of *Fv-4* restriction before or at proviral synthesis. In the previous experiment, virus susceptibility of cells was tested by an MSV focus assay (Fig. 5), which involves many cycles of viral replication. We therefore wished to examine the susceptibility of these cells to the initial steps of viral replication. After retroviruses attach to and enter cells, viral RNA is reverse transcribed into cDNA and linear and closed circular forms of viral DNAs are detectable in the cytoplasm of infected cells. NIH 3T3 cells and *Fv-4* env$^+$ NIH 3T3 cells carrying pcDFv4env were infected by a high titer of Friend MuLV, and Hirt supernatant from each cell type was isolated 6, 12, 24 and 48 h postinfection. In infected NIH 3T3 cells, both linear (form III) and closed circular (form I) viral cDNAs were found 6 h postinfection, and the amount of viral cDNA increased until 24 h postinfection. In contrast, in infected pcDFv4env-transfected cells, viral DNAs were not detectable until 12 h postinfection, and the amount of viral cDNA was much less (Fig. 6). This leakiness of the restriction of viral cDNA synthesis in *Fv-4* env$^+$ cells appeared to be consistent with the incomplete resistance of these cells to ecotropic MuLVs when virus replication was assayed by the XC plaque test (see above). Thus, *Fv-4* restriction appeared to function at some step before or at viral cDNA synthesis, such as virion adsorption, penetration, and uncoating.

DISCUSSION

We cloned and characterized an *Fv-4^r*-associated DNA about 17 kb long which contains a truncated endogenous MuLV and about 13 kb of 5′ nonretroviral sequences. DNA transfection experiments demonstrated that the DNA is transcriptionally active, encodes the *Fv-4* env-specific antigenic determinants, and confers resistance to retrovirus infection on transfected cells, all of which are phenomena previously shown to be associated with the *Fv-4* resistance allele. Therefore, we conclude that the cloned 17-kb fragment contains the functional *Fv-4^r* gene. Furthermore, the *env* region within the 17-kb DNA must be directly related to virus resistance, since the same resistance phenotype was shown with pcDFv4env, which contains only the *env* region of *Fv-4^r* in an SV40 expression vector.

One of the unexpected structural features of the MuLV-like sequence of the *Fv-4^r* gene is the absence of a 5′ LTR within 13 kb of the env-coding sequence. The previous sequence analysis indicated that the 3′ LTR of *Fv-4* MuLV has the characteristics of a complete 3′ LTR of infectious MuLV (21). We therefore suspect that the truncated *Fv-4*

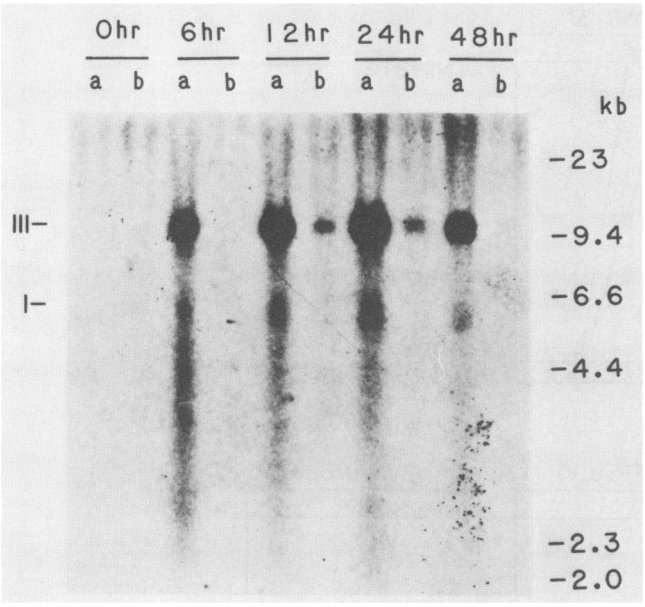

FIG. 6. Restricted synthesis of viral cDNA in *Fv-4* env$^+$ NIH 3T3 cells postinfection by ecotropic MuLV. NIH 3T3 (a) and pcDFv4env-transfected NIH 3T3 (b) cells were infected by ecotropic Friend MuLV, and Hirt supernatants were isolated 6, 12, 24, and 48 h postinfection. Cytoplasmic DNA was blotted and hybridized to a Friend MuLV *env* probe (44). I and III indicate sizes of closed circular and linear viral cDNAs, respectively.

MuLV was originally part of an endogenous MuLV and that either a deletion of more than 5 kb in the 5′ proviral sequence or an insertion of more than 12 kb in the 5′ half of the putative endogenous MuLV occurred. Furthermore, the demonstrated interference specificity of *Fv-4 env* (Fig. 5) and the characteristics of the *Fv-4 env* sequence (21) suggest that the prototype MuLV of *Fv-4^r* would be a member of the ecotropic MuLV class.

In contrast to λ Fv4-17, pFv4, which contains the truncated *Fv-4* MuLV and its short 5′ and 3′ cellular sequences (Fig. 1), showed weak or atypical biological activity when transfected into NIH 3T3 cells. (i) *Fv-4 env* mRNA expressed in pFv4-transfected cells was larger than the natural mRNA in *Fv-4^r* mice (Fig. 4), (ii) Fv-4 env$^+$ cell clones were generated at a relatively low frequency (Table 1) and expressed a lower concentration of the env antigen (Fig. 3), and (iii) these *Fv-4* env$^+$ cells showed weaker resistance to infection by ecotropic MuLVs (Fig. 5). pFv4 structurally differs from λ Fv4-17 in the absence of a 13-kb 5′-flanking cellular sequence and the presence of a 1.5-kb 3′-flanking cellular sequence and the bacterial vector DNA. Although there are many possibilities, it seems most likely that the difference between pFv4 and λ Fv4-17 is due to the presence of the 5′-flanking region of λ Fv4-17, which contains transcriptional regulatory (enhancer and promoter) elements, splice donor sites, and/or an untranslated exon(s). We are currently searching for promoter activity in the upstream region which may lead to high expression of *Fv-4 env* mRNA in a variety of tissues in mice with *Fv-4^r* (F. Laigret and H. Ikeda, unpublished data).

We previously hypothesized that *Fv-4* restriction is mediated via receptor interference (22, 23). Mouse cells have at least three virus receptor types (15), *Rec-1* for ecotropic MuLVs (12, 33, 37, 42), *Ram-1* for amphotropic MuLVs (12), and *Rmcf-1* for dualtropic MuLVs (29). Recently, a cDNA clone that may encode the receptor for ecotropic MuLV has been identified (1), but the biochemical nature of these receptors is still unclear (see reference 26). In view of the resistance specificity of *Fv-4* env$^+$ cells, the *Fv-4* env protein may saturate the *Rec-1* receptor. The receptor interference model is also consistent with the finding that *Fv-4* env$^+$-transformed cells restricted viral cDNA synthesis after ecotropic MuLV infection (Fig. 6). Similar restricted cDNA synthesis was reported by Dandekar et al. (10), who characterized the virus susceptibility of embryo fibroblast cells with *Akvr-1^{rr}* and *Akvr-1^{ss}* genotypes, which are identical to the *Fv-4^{rr}* and *Fv-4^{ss}* genotypes (34).

Several mouse strains, such as AKR and SL/Ni, express *env* glycoprotein of endogenous ecotropic MuLV on the cell surface (19, 39, 53), but these mice are not as strongly resistant to exogenous infection by ecotropic MuLVs as are *Fv-4^r* mice. There are two major differences between ecotropic AKV proviral loci in these laboratory mice and the *Fv-4^r* gene. One is in the *env* sequences. The amino-terminal (variable) region of *Fv-4 env* shares about 70 to 75% of the nucleotides and deduced amino acid homology with AKV *env* (21). The difference in env polypeptides might be crucial for interference activity in vivo. The other difference lies in the structures outside of the *env* region. Most of the AKV proviral loci in AKR mice appear to be structurally competent for full expression of infectious virus (5, 41), while the *Fv-4^r* gene contains only a truncated provirus. This may result in either quantitative or qualitative differences between the env glycoproteins, leading to different resistance activities. *Fv-4* env might accumulate on the cell surface more than AKV env, since AKV env could be released from the cell surface as virions, whereas *Fv-4* env would not be expected to. Alternatively, unidentified transcriptional regulatory sequences of the *Fv-4^r* gene might provide tissue-specific expression different from that of AKV loci, whose expression should be controlled by the 5′ LTR. Cell-specific expression of endogenous *env*, especially by target cells for MuLV, must be extremely important for in vivo resistance. It is also possible, but less likely, that lack of viral protease sequences (encoded in the *gag-pol* region) in *Fv-4^r* results in different processing of the env protein precursor, Pr80^{env}, because while Pr80^{env} is thought to be cleaved into gp70 and p15(E) by a host protease, p15(E) is further cleaved to p12(E) by the viral protease (28). However, the second cleavage is thought to occur in virions (18). Thus, it remains unknown why *Fv-4^r*-endogenous *env* has such strong in vivo activity.

Most endogenous viruses are thought to have no biological effect on host animals (8), but in several cases, deleterious or beneficial effects have been found. Deleterious effects include leukemogenesis caused by infectious MuLVs which have arisen from endogenous virus loci (such as AKV loci) (52) and insertional mutation at essential genes of host animals (9, 13, 25, 46, 49). Beneficial effects were proposed for the avian endogenous virus locus (*ev-3*), which reduces the immunological response of a host to viral proteins post-infection with exogenous viruses, leading to reduction of a wasting that seems to be induced by a strong immune reaction to infected lymphoid cells (14). The other beneficial effect—interference with exogenous infection—is probably a consequence of expression of retrovirus envelope glycoproteins on the cell surface. The *Fv-4^r* gene is one such example. Analogous genes exist in the avian system and include endogenous virus loci *ev-3*, *ev-6*, and *ev-9*. These endogenous viruses have various deletions or mutations in the viral genomes but are able to express env glycoprotein on the host

cell surface and thereby interfere with exogenous infection by the same group of viruses (2, 17, 40). An additional example is the mouse *Rmcf* gene, which seems control both expression of an *env* glycoprotein related to dualtropic MuLV and resistance to infection by dualtropic MuLV (3, 16, 43). However, it has not been elucidated whether *Rmcf* is an endogenous virus locus or a regulatory gene for expression of other endogenous virus loci.

It is well documented that endogenous virus loci responsible for viral interference restrict the spread of viruses in somatic cells, but it is not known whether they affect infection of germ cells in vivo. If the latter occurs, endogenous retroviruses could restrict the evolutionary amplification of endogenous viruses in host species and thus might provide a selective advantage by minimizing insertional mutations and reducing retrovirus-induced diseases. Studies of endogenous retroviruses in mice (8, 48) and humans (47) indicated that certain endogenous retroviruses exist in multiple copies, all of which appear to be inactive. In evolutionary terms, these might reflect ancient, repeated acquisition of infectious virus. Reasons for the disappearance of such active viruses from each species are not known, but they could involve generation of interfering *env* loci, such as the *Fv-4*^{r} gene.

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LITERATURE CITED

1. **Albritton, L. M., L. Tseng, D. Scadden, and J. M. Cunningham.** 1989. A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell **57**:659–666.
2. **Baker, B., H. Robinson, H. E. Varmus, and J. M. Bishop.** 1981. Analysis of endogenous avian retrovirus DNA and RNA: viral and cellular determinants of retrovirus gene expression. Virology **144**:8–22.
3. **Bassin, R. H., S. Ruscetti, I. Ali, D. K. Haapala, and A. Rein.** 1982. Normal DBA/2 mouse cells synthesize a glycoprotein which interferes with MCF virus infection. Virology **123**:139–151.
4. **Bassin, R. H., N. Tuttle, and P. J. Fischinger.** 1971. Rapid cell culture assay technique for murine leukemia viruses. Nature (London) **229**:564–566.
5. **Buckler, C. E., S. P. Staal, W. P. Rowe, and M. A. Martin.** 1982. Variation in the number of copies and in the genomic organization of ecotropic murine leukemia virus proviral sequences in sublines of AKR mice. J. Virol. **43**:629–640.
6. **Chattopadhyay, S. K., M. W. Cloyd, D. L. Linemeyer, M. R. Lander, E. Rands, and D. R. Lowy.** 1982. Cellular origin and role of mink focus-forming viruses in murine thymic lymphomas. Nature (London) **295**:25–31.
7. **Chattopadhyay, S. K., A. I. Oliff, D. L. Linemeyer, M. R. Lander, and D. R. Lowy.** 1981. Genomes of murine leukemia viruses isolated from wild mice. J. Virol **39**:777–791.
8. **Coffin, J.** 1982. Endogenous viruses, p. 1109–1203. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Copeland, N. G., N. A. Jenkins, and B. K. Lee.** 1983. Association of the lethal yellow (A^y) coat color mutation with an ecotropic murine leukemia virus genome. Proc. Natl. Acad. Sci. USA **80**:247–249.
10. **Dandekar, S., P. Rossitto, S. Pickett, G. Mockli, H. Bradshaw, R. Cardiff, and M. Gardner.** 1987. Molecular characterization of the *Akvr-1* restriction gene: a defective endogenous retrovirus-borne gene identical to *Fv-4*^{r}. J. Virol. **61**:308–314.
11. **Gardner, M. B., S. Rasheed, B. K. Pal, J. D. Estes, and S. J. O'Brien.** 1980. *Akvr-1*, a dominant murine leukemia virus restriction gene, is polymorphic in leukemia-prone wild mice. Proc. Natl. Acad. Sci. USA **77**:531–535.
12. **Gazda, A. F., H. Oie, P. Lalley, W. W. Moss, and J. D. Minna.** 1977. Identification of mouse chromosomes required for murine leukemia virus replication. Cell **11**:949–956.
13. **Habbers, K., M. Kuehn, H. Delius, and R. Jaenisch.** 1984. Insertion of retrovirus into the first intron of 1(I) collagen gene leads to embryonic lethal mutation in mice. Proc. Natl. Acad. Sci. USA **81**:1504–1508.
14. **Halpern, M. S., D. L. Ewert, L. J. Flores, and L. B. Crittenden.** 1983. The influence of the *ev 3* locus on the inducibility of serum antibody reactivity for envelope glycoprotein group-specific determinants. Virology **128**:502–504.
15. **Hartley, J. W., N. K. Wolford, L. L. Old, and W. P. Rowe.** 1977. A new class murine leukemia virus associated with development of spontaneous lymphomas. Proc. Natl. Acad. Sci. USA **74**:789–792.
16. **Hartley, J. W., R. A. Yetter, and H. C. Morse III.** 1983. A mouse gene on chromosome 5 that restricts infectivity of mink cell focus-forming recombinant murine leukemia viruses. J. Exp. Med. **158**:16–24.
17. **Hayward, W. S., S. B. Braverman, and S. M. Astrin.** 1980. Transcriptional products and DNA structure of endogenous avian proviruses. Cold Spring Harbor Symp. Quant. Biol. **44**:1111–1122.
18. **Henderson, L. E., R. Sowder, T. D. Copeland, G. Smythers, and S. Oroszlan.** 1984. Quantitative separation of murine leukemia virus proteins by reversed-phase high-pressure liquid chromatography reveals newly described *gag* and *env* cleavage products. J. Virol. **52**:492–500.
19. **Hiai, H., Y. O. Buma, H. Ikeda, K. Moriwaki, and Y. Nishizuka.** 1987. Epigenetic control of endogenous ecotropic virus expression in SL/Ni mice. JNCI **79**:781–787.
20. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
21. **Ikeda, H., F. Laigret, M. A. Martin, and R. Repaske.** 1985. Characterization of a molecularly cloned retroviral sequence associated with *Fv-4* resistance. J. Virol. **55**:768–777.
22. **Ikeda, H., and T. Odaka.** 1983. Cellular expression of murine leukemia virus gp70-related antigen on thymocytes of uninfected mice correlates with *Fv-4* gene-controlled resistance to Friend leukemia virus infection. Virology **128**:127–139.
23. **Ikeda, H., and T. Odaka.** 1984. A cell membrane "gp70" associated with *Fv-4* gene: immunological characterization, and tissue and strain distribution. Virology **133**:65–76.
24. **Ikeda, H., H. Sato, and T. Odaka.** 1981. Mapping of the *Fv-4* gene controlling resistance to murine leukemia viruses. Int. J. Cancer **28**:237–240.
25. **Jenkins, N. A., N. G. Copeland, B. A. Taylor, and B. K. Lee.** 1981. Dilute (*d*) coat colour mutation of DBA/2J mice is associated with the site of an ecotropic MuLV genome. Nature (London) **293**:370–374.
26. **Johnson, P. A., and M. R. Rosner.** 1986. Characterization of murine-specific leukemia virus receptor from L cells. J. Virol. **58**:900–908.
27. **Kai, K., H. Ikeda, Y. Yuasa, S. Suzuki, and T. Odaka.** 1976. Mouse strain resistant to N-, B-, and NB-tropic murine leukemia viruses. J. Virol. **20**:436–440.
28. **Katoh, I., Y. Yoshinaka, A. Rein, M. Shibuya, T. Odaka, and S. Oroszlan.** 1985. Murine leukemia virus maturation: protease region required for conversion from "immature" to "mature" core form and for virus infectivity. Virology **145**:280–292.
29. **Kozak, C. A.** Genetic mapping of a mouse chromosomal locus required for mink cell focus-forming virus replication. J. Virol. **48**:300–303.

30. **Kozak, C. A., N. J. Gromet, H. Ikeda, and C. E. Buckler.** 1984. A unique sequence related to the ecotropic murine leukemia virus is associated with the *Fv-4* resistance gene. Proc. Natl. Acad. Sci. USA **81**:834–837.
31. **Kozak, C. A., and R. R. O'Neill.** 1987. Diverse wild mouse origins of xenotropic, mink cell focus-forming, and two types of ecotropic proviral genes. J. Virol. **61**:3082–3088.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Marshall, T. H., and U. R. Rupp.** 1979. Gene controlling receptor for ecotropic and xenotropic type C virus in *Mus cervicolor* and *Mus musculus*. J. Virol. **29**:501–506.
34. **O'Brien, S. J., E. J. Berman, J. D. Estes, and M. B. Gardner.** 1983. Murine retroviral restriction genes *Fv-4* and *Akvr-1* are alleles of a single locus. J. Virol. **47**:649–651.
35. **Odaka, T., H. Ikeda, K. Moriwaki, A. Matsuzawa, M. Mizuno, and K. Kondo.** 1978. Genetic resistance in Japanese wild mice (*Mus musculus molossinus*) to an NB-tropic Friend murine leukemia virus. JNCI **67**:1123–1127.
36. **Odaka, T., H. Ikeda, H. Yoshikura, K. Moriwaki, and S. Suzuki.** 1981. *Fv-4*: gene controlling resistance to NB-tropic Friend murine leukemia virus. Distribution in wild mice, introduction into genetic background of BALB/c mice, and mapping of chromosomes. JNCI **67**:1123–1127.
37. **Oie, H. K., A. F. Gazdar, P. A. Lalley, E. K. Russel, J. D. Minna, J. DeLarco, G. J. Todaro, and U. Francke.** 1978. Mouse chromosome 5 codes for ecotropic murine leukemia virus cell-surface receptor. Nature (London) **274**:60–63.
38. **Okayama, H., and P. Berg.** 1983. A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. Mol. Cell. Biol. **3**:280–289.
39. **Old, L. J., and E. Stockert.** 1977. Immunogenetics of cell surface antigens of mouse leukemia. Annu. Rev. Genet. **11**: 127–160.
40. **Robinson, H. L., S. M. Astrin, A. M. Senior, and F. H. Salazar.** 1981. Host susceptibility to endogenous viruses: defective, glycoprotein-expressing proviruses interfere with infections. J. Virol. **40**:745–751.
41. **Rowe, W. P.** 1972. Studies of genetic transmission of murine leukemia virus by AKR mice. I. Crosses with *Fv-1*^{n} strains of mice. J. Exp. Med. **136**:1272–1285.
42. **Ruddle, N. H., B. S. Conta, L. Leinward, C. Kozak, F. Ruddle, P. Besmer, and D. Baltimore.** 1978. Assignment of the receptor for ecotropic murine leukemia virus to mouse chromosome 5. J. Exp. Med. **148**:451–465.
43. **Ruscetti, S., R. Matthai, and M. Potter.** 1985. Susceptibility of BALB/c mice carrying various DBA/2 genes to development of Friend murine leukemia virus-induced erythroleukemia. J. Exp. Med. **162**:1579–1587.
44. **Silver, J., and C. Kozak.** 1986. Common proviral integration region on mouse chromosome 7 in lymphomas and myelogenous leukemia induced by Friend murine leukemia virus. J. Virol. **57**:526–533.
45. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
46. **Stavenhagen, J. B., and D. M. Robins.** 1988. An ancient provirus has imposed androgen regulation on the adjacent mouse sex-limited protein gene. Cell **55**:247–254.
47. **Steele, P. M., A. Martin, A. B. Rabson, T. Bryan, and S. J. O'Brien.** 1986. Amplification and chromosomal dispersion of human endogenous retroviral sequences. J. Virol. **59**:545–550.
48. **Stoye, J. P., and J. M. Coffin.** 1988. Polymorphism of murine endogenous proviruses revealed by using virus class-specific oligonucleotide probes. J. Virol. **62**:168–175.
49. **Stoye, J. P., S. Fenner, G. E. Greenoak, C. Moran, and J. M. Coffin.** 1988. Role of endogenous retroviruses as mutagens: the hairless mutation of mice. Cell **54**:383–391.
50. **Suzuki, S.** 1975. *Fv-4*: a new gene affecting the splenomegaly induction by Friend leukemia virus. Jpn. J. Exp. Med. **45**: 473–478.
51. **Suzuki, S., K. Tsuji, and K. Moriwaki.** 1981. Friend murine leukemia virus resistance in Japanese wild mice: possible allelism with *Fv-4* in FRG mice. JNCI **66**:729–731.
52. **Teich, N., J. Wyke, T. Mak, A. Bernstein, and W. Hardy.** 1982. Pathogenesis of retrovirus-induced disease, p. 785–998. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
53. **Tung, J. S., P. V. O'Donnell, E. Fleissner, and E. A. Boyse.** 1978. Relationship of gp70 of MuLV envelopes to gp70 components of mouse lymphocyte plasma membranes. J. Exp. Med. **147**: 1280–1284.
54. **Yoshikura, H., and T. Odaka.** 1982. Surface antigen expressed in hematopoietic cells derived from *Fv-4*^{r} mouse strain. JNCI **68**:1005–1009.