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Activation of erythropoietin receptors by Friend viral gp55 and by erythropoietin and down-modulation by the murine *Fv-2*^{r} resistance gene

(Friend virus/leukemia/cancer genes)

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ABSTRACT **The leukemogenic membrane glycoprotein (gp55) encoded by Friend spleen focus-forming virus appears to bind to erythropoietin receptors (EpoR) to stimulate erythroblastosis [Li, J.-P., D'Andrea, A. D., Lodish, H. F. & Baltimore, D. (1990) *Nature (London)* 343, 762–764]. To directly compare the effects of gp55 with erythropoietin (Epo), we produced retrovirions that encode either gp55, Epo, or EpoR. After infection with EpoR virus, interleukin 3-dependent DA-3 cells bound ^{125}I-labeled Epo and grew without interleukin 3 in the presence of Epo. These latter cells, but not parental DA-3 cells, became factor-independent after superinfection either with Epo virus or with Friend spleen focus-forming virus. In addition, Epo virus caused a disease in mice that mimicked Friend erythroleukemia. Although *Fv-2*^{r} homozygotes are susceptible to all other retroviral diseases, they are resistant to both Epo viral and Friend viral erythroleukemias. These results indicate that both gp55 and Epo stimulate EpoR and that the *Fv-2* gene encodes a protein that controls response to these ligands. However, the Fv-2 protein is not EpoR because the corresponding genes map to opposite ends of mouse chromosome 9. These results have important implications for understanding signal transduction by EpoR and the role of host genetic variation in controlling susceptibility to an oncogenic protein.**

Recent studies suggested that the leukemogenic membrane glycoprotein (gp55) encoded by Friend spleen focus-forming virus (SFFV) interacts specifically with erythropoietin receptors (EpoR) to cause erythroblastosis (1, 2). After interleukin 3 (IL-3)-dependent hematopoietic cells were coinfected with SFFV and with a helper-free retrovirus that encodes EpoR, the cells proliferated without any growth factor (1). In contrast, cells infected only with SFFV or with EpoR virus remained growth factor-dependent. In addition, these workers obtained evidence that small proportions of gp55 and of EpoR form unexpectedly stable complexes in the rough endoplasmic reticulum (1). The functional significance of these latter results is somewhat uncertain because the proportions of gp55 and EpoR in the complexes were very low (1) and because gp55 folds heterogeneously in the rough endoplasmic reticulum, where it promiscuously forms disulfide bonded complexes with other cellular proteins (3). Moreover, other results suggest that the cell-surface form of gp55 may be the mitogenically active component (4, 5). Although details of gp55 function, therefore, remain uncertain, these recent results support a model that can explain the mitogenic mechanism and target-cell specificity of Friend viral erythroleukemia.

To directly compare gp55 with erythropoietin (Epo), we produced helper-free retrovirions that encode either Epo, EpoR, or gp55. The effects of these viruses were studied in an IL-3-dependent line of hematopoietic cells that lacks any endogenous EpoR. The virus that encodes Epo was also analyzed in mice that were either susceptible or genetically resistant to Friend viral erythroleukemia. The murine *Fv-2* gene controls susceptibility to both Epo viral and Friend viral erythroleukemias but not to other retroviral diseases.

MATERIALS AND METHODS

Cells. The ψ-2 and PA-12 cell lines, which package retroviruses with ecotropic and amphotropic host-range envelopes, respectively (6, 7), were maintained as described (8, 9). DA-3, an IL-3-dependent murine cell line that lacks EpoR (10), was maintained in RPMI 1640 medium/10% fetal bovine serum. Recombinant Epo (1 unit/ml) and IL-3 (40 units/ml) were added to cells as noted.

Plasmids and Vectors. The human *EPO* gene was isolated from a Charon 3A λ phage genomic library. The library was screened by standard methods (11) with two ^{32}P-labeled oligonucleotide probes—5′-CGGGGAAAGCTGAAGCTGTACACAGGGGAGGCCTGCAGGA-3′ and 5′-CGAGTCCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGCCG-3′—that occur in different coding regions of the published *EPO* sequence (12). One positive clone was identified among 5×10^5 phages. The 2425-base-pair (bp) *Apa* I fragment that contains the coding exons but not the poly(A)-addition signal was cut from the purified λ DNA, blunted, ligated to *Xho* I linkers (New England Biolabs), digested with *Xho* I, and ligated into the *Xho* I site of the retroviral vector pSFF (8) to form pSFF-Epo. The SFFV colinear molecular clone pL2-6K that encodes gp55 (4), the vector pSFF (8), and the pSFF-EpoR construct that encodes EpoR (1) have been described. Fig. 1 shows all these retroviral vectors. Ping-pong vector amplifications were done by transfecting calcium phosphate-precipitated DNAs into cocultures that contained 1:1 mixtures of ψ-2 and PA-12 retroviral packaging cells according to protocol B (9). RNA from cocultures and from infected cells was analyzed for vector transcripts by slot blotting (8).

Virus Infections. Viruses were obtained from cell culture media (8, 9). Helper-free Epo virus was from the medium of a ψ-2/PA-12 coculture that had been transfected with pSFF-

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Abbreviations: IL-3, interleukin 3; SFFV, Friend spleen focus-forming virus; gp55, envelope glycoprotein encoded by SFFV; Epo, erythropoietin; EpoR, erythropoietin receptor(s); BFU-E, burst-forming erythroblasts; D2.R, congenic mouse strain D2.B6-Fv-2^r; RI, recombinant inbred.

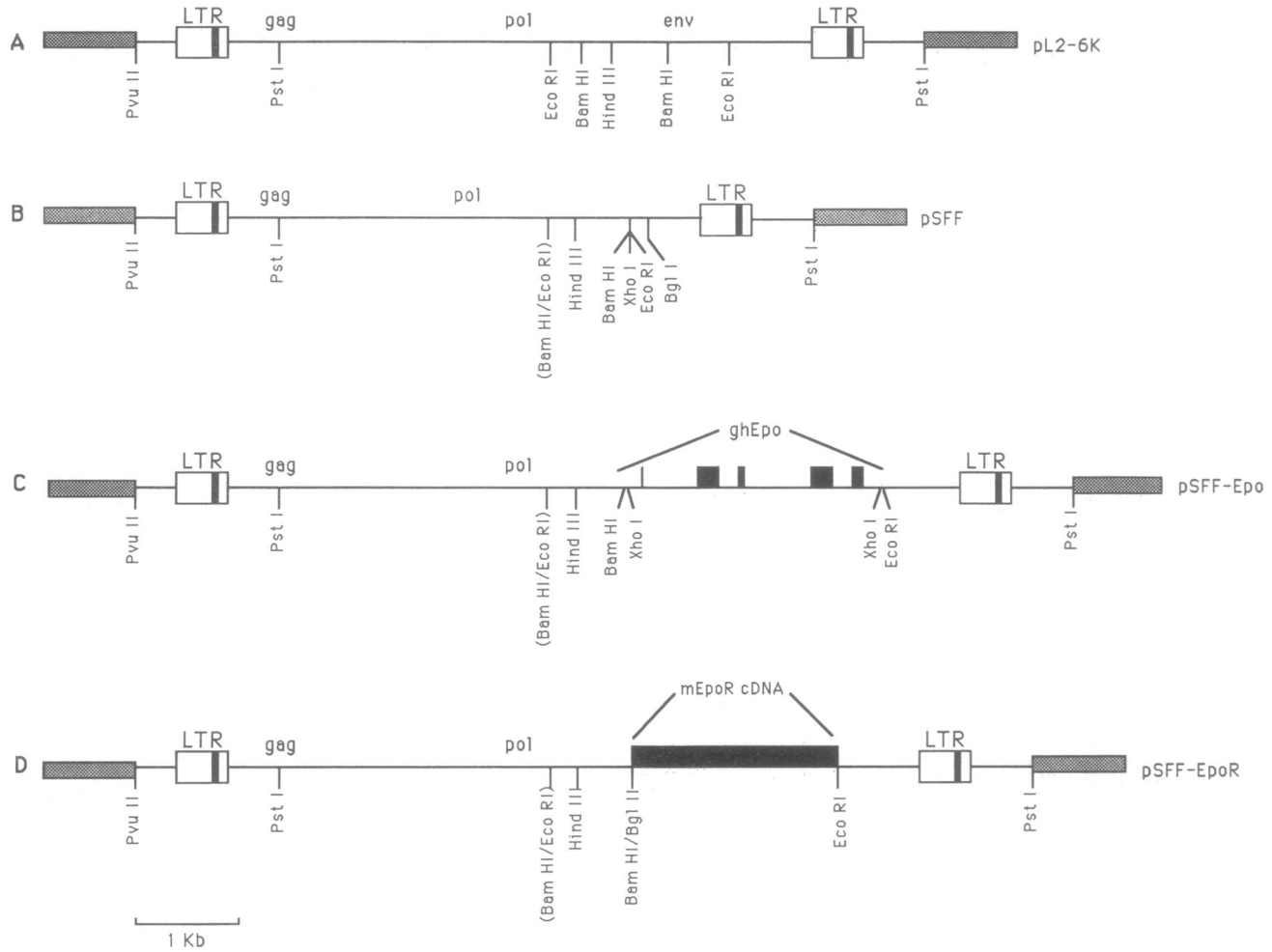

FIG. 1. Maps of retroviral constructs. The maps show relative sizes and major genetic features and restriction sites in the vectors. Hatched areas represent pSP64 vector sequences. (*A*) pL2-6K is the colinear SFFV molecular clone that encodes gp55 (4). (*B*) The retroviral vector pSFF was derived from pL2-6K by eliminating the *Bam*HI and *Eco*RI sites in the *pol* region, deleting the *Bam*HI–*Eco*RI *env* fragment, and creating a *Bam*HI-*Xho* I-*Eco*RI multicloning expression site (8). (*C*) pSFF-Epo encodes human Epo. The black areas in the region that contains the gene for human Epo (ghEpo) are the exons. (*D*) pSFF-EpoR encodes mouse EpoR (1).

Epo and that produced Epo at ≈1 mg/liter. Epo virus was mixed in a ratio of 7:3 with a biologically cloned nonpathogenic Rauscher murine leukemia virus helper (13). The virus mixture (0.7 ml), injected i.v. into 4- to 6-week-old female NIH/Swiss mice, caused massive splenomegaly by 10 days. Serially passaged Epo virus (obtained by homogenizing infected spleens in 10 vol of 0.02 M Tris·HCl, pH 7.4/0.10 M NaCl/0.001 M EDTA and centrifuging twice at 10,000 × *g* for 15 min) was stored at −70°C. Passaged Friend virus (Lilly–Steeves polycythemia strain) was also from spleen homogenates (14). Mouse strains included NFS/N, DBA/2J (both *Fv-2*^{s}), and the *Fv-2*^{r} congenic strain D2.B6-Fv-2^r (D2.R). The latter strain contains the *Fv-2*^{r} gene from C57BL/6 in a DBA/2J genetic background (15).

Gene Mapping. The mouse gene for EpoR was mapped by Southern blot analysis of DNAs from Chinese hamster × mouse somatic-cell hybrids (16) and from SWR/J × C57L/J recombinant inbred (RI) strains using a 1.5-kilobase-pair (kbp) *Kpn* I–*Sty* I fragment of EpoR from pXMN 190 (17) as the hybridization probe. DNAs from different mouse strains and from RI strains were from The Jackson Laboratory.

Protein Detection. Described methods were used for Epo bioassay (18), radioimmunoassay (19), cell immunofluorescence (9), gp55 detection by immunoblotting (4), and for identifying EpoR on cells by binding ^{125}I-labeled Epo (3000–4000 Ci/mmol; 1 Ci = 37 GBq) (Amersham-Searle, Arlington Heights, IL) for 2.5 hr at 37°C (17).

RESULTS

Epo and gp55 Both Specifically Stimulate EpoR. Retroviral vectors that encode gp55 (pL2-6K), Epo (pSFF-Epo), and EpoR (pSFF-EpoR) (see Fig. 1) were efficiently amplified after transfection into cocultures of cells that package retrovirions with ecotropic (ψ-2 cells) and amphotropic (PA-12 cells) host-range envelopes. As described (8, 9) for gp55 and for human growth hormone, the cocultures produced substantial quantities of protein and high titers of the corresponding helper-free virions. After transfection with pSFF-Epo, the cells secreted biologically active Epo and Epo virions (see below). Cocultures that had amplified pSFF-EpoR synthesized EpoR RNA and bound ^{125}I-labeled Epo. For example, in one study three amplified cocultures with ≈5 × 10^5 cells bound 2480, 5230, and 2350 cpm of ^{125}I-labeled Epo, whereas control ψ-2 and PA-12 cultures bound 27 and 12 cpm, respectively.

IL-3-dependent DA-3 cells were first infected with EpoR virus to obtain derivative cells (DA3-EpoR) that grew in the presence of either IL-3 or Epo. As expected, DA3-EpoR cells also bound ^{125}I-labeled Epo, whereas uninfected DA-3 cells did not bind this hormone. Table 1 shows that superinfection of DA3-EpoR cells either with the gp55-encoding SFFV virus or with Epo virus enabled the cells to grow without any factor, whereas DA-3 cells infected only with the latter viruses remained IL-3 dependent.

Table 1. Properties of DA-3 cells and of derivatives that contain EpoR, Epo, and/or SFFV viruses

Cells*	Factor required for growth
DA3	IL-3
DA3-EpoR	Epo or IL-3
(DA3-EpoR)Epo	Neither
(DA3-EpoR)SFFV	Neither
(DA3)Epo	IL-3
(DA3)SFFV	IL-3

*The nomenclature for cells indicates the helper-free viruses that were used to infect the IL-3-dependent DA3 cell line. DA3 cells were first infected with EpoR virus, and the resulting DA3-EpoR cells were selected by growth in Epo without IL-3 (see *Results*). The DA3 and DA3-EpoR cell populations were then infected with Epo virus or with SFFV, and the resulting cells were tested for abilities to grow with Epo or IL-3 or without any growth factor.

Pathogenesis by the Epo-Encoding Retrovirus. The hypothesis that gp55 acts as an Epo agonist implies that an Epo-encoding retrovirus would cause a disease similar to Friend erythroleukemia. Accordingly, NIH/Swiss mice infected with Epo virus in the presence of a nonpathogenic Rauscher murine leukemia virus helper rapidly developed splenomegaly and polycythemia. This development was not caused solely by Epo in the innoculum because the disease could be serially passaged indefinitely using cell-free virus preparations from infected animals. Table 2 shows results of a typical experiment in which passaged Epo virus was used to infect NIH/Swiss mice. Blood smears of infected mice contained 10–25% reticulocytes and elevated numbers of nucleated erythroid progenitor cells. In contrast to the predominately lymphoid cells in normal spleens, enlarged spleens of infected mice contained almost exclusively erythroblasts and their differentiating progeny (see Fig. 2). Thus, Epo virus mimics the disease caused by Friend virus (polycythemia strain). A similar pathogenesis has been reported for transgenic mice that contain the human *EPO* gene (20).

Mice of the congenic D2.R strain (DBA/2J homozygous for the *Fv-2*^{r} allele) and mice of *Fv-2*^{s} strains NFS/N and DBA/2J were studied for their relative responses to both Friend virus and Epo virus. Titration of an Epo virus preparation by the spleen focus method (21) yielded a titer of 7.1 ($\pm$ 2.8) $\times$ 10^3 focus-forming units/ml in the *Fv-2*^{s} strains, but among 10 D2.R mice receiving 1 ml of a 1:10 or 1:50 dilution of this preparation (700 or 140 focus-forming units, respectively) only a single spleen focus in one mouse was seen, a response typical of that seen with high-titer preparations of Friend virus in these mice (15). In long-term experiments,

Table 2. Splenomegaly and polycythemia caused by passaged Epo virus in NIH/Swiss mice

Mice*	Spleen weight, g	Hematocrit, %
Uninfected	0.09–0.12	39–43
11d	1.3	70
14d	2.7, 0.64, 0.2	64, 52, 56
19d†	0.07, 0.22	46, 38
25d	1.0	78
32d	4.5	80
40d	3.1	82

*Mice from this *Fv-2*^{s} strain either were uninfected or they were sacrificed for analysis on the day (d) indicated after infection. Spleen weights of uninfected mice were determined at the end of study. Each number in a series corresponds to a different mouse; for example, the 14-day (14d) spleen that weighed 0.64 g was from a mouse with an hematocrit of 52%.

†The mice killed in this experiment at 19d did not have substantial splenomegaly or polycythemia. In other experiments, mice analyzed at that time had extensive disease.

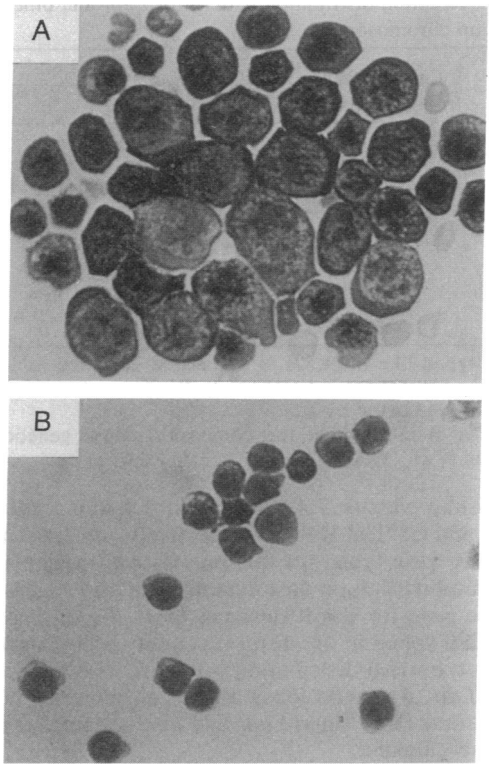

FIG. 2. Typical cells from the spleens of mice. Spleen fragments were gently shaken in culture medium, large fragments were removed, and cells were then sedimented onto slides in a cytocentrifuge. The cells were then stained with Wright's stain. (*A*) Cells from enlarged spleen of a mouse infected with passaged Epo virus. The cells consist primarily of erythroblasts and their smaller more differentiated progeny, suggesting that the spleen had become a site of substantial and effective erythropoiesis. This conclusion was confirmed by benzidine staining for hemoglobin. The enlarged spleens also entrap many circulating blood cells. (*B*) Cells from a normal spleen. The major cells are normal lymphocytes. The splenic red pulp also contains many mature erythrocytes that are underrepresented in this field. (×1200.)

both viruses rapidly induced grossly indistinguishable disease syndromes in *Fv-2*^{s} hosts, including massive splenomegaly within 10 days after a high virus dose, elevated hematocrits, and 100% mortality within 3–5 weeks. In contrast, D2.R mice injected with these same virus preparations developed only an indolent form of the disease. These mice showed a markedly prolonged latent period (≥4 weeks) for the development of moderate splenomegaly, moderately elevated hematocrits, and extremely prolonged survival (10 to >15 weeks). We conclude that homozygosity for *Fv-2*^{r} causes very similar resistance to both Friend and Epo viral diseases.

Genetic Mapping of the EpoR Gene. To determine whether the *Fv-2* and EpoR genes might be identical, we mapped the EpoR gene using DNAs from panels of somatic-cell hybrids and RI mice. Southern blot analysis using the 1.5-kbp *Kpn* I–*Sty* I fragment of EpoR as the probe identified *Pst* I fragments of 1.7, 1.3, and 1.0 kbp in Chinese hamster DNA and 1.3 and 0.85 kbp in mouse DNA. Six of 18 somatic-cell hybrids contained the mouse-specific EpoR fragment, and the presence or absence of this fragment correlated perfectly with mouse chromosome 9. There were at least two discrepancies for all other mouse chromosomes.

Because chromosome 9 also contains *Fv-2*, an effort was made to position the EpoR gene in RI strains that had been typed for *Fv-2*. Because the *Fv-2*^{r} allele occurs in C57BL/J and related strains, RI strains derived from C57BL crosses were typed for polymorphisms of EpoR. *Apa* I produced 9.3-

Table 3. RI strain-distribution pattern for *Epor* and other markers on chromosome 9

Locus	Centimorgans from centromere†	SWXL strain*						
		4	7	12	14	15	16	17
Epor		L	S	S	S	S	L	S
Ldlr	4	L	S	S	S	S	L	S
			X	X			X	
Apoa-1	25	L	L	L	S	S	S	S
		X		X				
Mod-1	44	S	L	S	S	S	S	S
							X	
Fv-2	55	S	L	S	S	S	L	S

*Strains typed like the C57L/J parent are indicated as L. Strains typed like SWR/J are indicated as S. Positions of crossovers in RI strains are indicated by X.

†Distances obtained from the composite mouse genetic map of Davisson *et al.* (1990) (22).

and 3.1-kbp versus 7.4-, 3.1-, and 1.1-kbp fragments in SWR/J and C57L/J DNAs, respectively. The seven SWXL RI strains were typed for this polymorphism (see Table 3). The strain-distribution data demonstrate that *Fv-2* is distinct from the gene for EpoR (termed *Epor*). Furthermore, *Fv-2* has been mapped in the distal region of chromosome 9 (23), whereas the strain-distribution pattern for *Epor* is identical to that of *Ldlr*, a genetic locus at the centromeric end of this chromosome (24). Thus, *Epor* and *Fv-2* are neither identical nor closely linked.

DISCUSSION

Stimulation of EpoR by gp55 and by Epo. These results support and extend previous evidence (1, 2) that the SFFV-encoded gp55 glycoprotein acts as an Epo agonist to stimulate EpoR. After infection with EpoR virus, IL-3-dependent DA-3 hematopoietic cells bound ^{125}I-labeled Epo and grew without IL-3 in the presence of Epo. Superinfection of these DA3-EpoR cells with either gp55- or Epo-encoding viruses converted them to factor-independent proliferation. Moreover, gp55 and Epo were only mitogenic for cells that contained EpoR (see Table 1). These results establish that our gp55, Epo, and EpoR viruses encode biologically active proteins and that EpoR can interact with either gp55 or Epo to generate a mitogenic signal.

Resistance to Epo Viral and to Friend Viral Erythroleukemias Caused by Homozygosity for *Fv-2^r*. Additional strong evidence for an overlap of gp55 and Epo mechanisms of action was suggested by the resistance of *Fv-2^r* homozygous mice to both Epo and Friend viral erythroleukemias. Although homozygosity for the *Fv-2^r* allele confers almost absolute resistance to the induction of 9-day spleen foci by Friend virus (15, 25), high doses of virus can lead to an indolent version of disease in D2.R mice, with less pronounced splenomegaly and elevated hematocrits developing after latency periods markedly longer than those in *Fv-2^s* mice. This same pattern of relative resistance occurs in *Fv-2^r* homozygotes after administration of Epo virus. All other retroviral diseases, including erythroleukemias caused by infection of newborn mice with the pure Friend murine leukemia virus helper, are uninhibited by homozygosity for *Fv-2^r* (26–29).

Several lines of evidence suggest that resistance of homozygous *Fv-2^r* mice to Friend and to Epo viral erythroleukemias may be caused by down-modulation of the EpoR signal-transduction pathway in burst-forming erythroblasts (BFU-E) rather than by a block in retroviral infection of these cells. (*i*) Erythroblasts and other cells from *Fv-2^r* strains can be productively infected by SFFV and by other retroviruses (25, 30, 31). Our results also indicate that Epo and SFFV viruses cause attenuated erythroproliferative diseases in the *Fv-2^r* D2.R mice. (*ii*) Mutations of the gp55 gene can yield SFFV variants capable of causing erythroleukemia in *Fv-2^r* as well as in *Fv-2^s* strains (31, 32). Because gp55 is not a component of virions (5), it is unlikely that these mutations could alter the efficiency of cell infection. (*iii*) BFU-E in uninfected congenic *Fv-2^r* strain mice are less actively engaged in mitotic cycling than BFU-E in *Fv-2^s* mice (33–35). Because Epo is the prime regulator of mitosis in these uninfected erythroblasts, these results are consistent with our suggestion that the Epo response pathway is down-modulated in the resistant mice. Other evidence using chimeric mice suggests that BFU-E from *Fv-2^r* strains are inherently less responsive to SFFV-induced mitogenesis than *Fv-2^s* BFU-E (32, 36), perhaps due to a mitotic inhibitor on the cell surfaces (34, 35).

Although one explanation for our pathogenesis results would be that the *Fv-2* gene encodes EpoR and that the *Fv-2^r* allele is relatively sluggish in mitogenic signaling, our gene mapping studies indicate that *Fv-2* and *Epor* genes are distinct. Therefore, we propose that *Fv-2* encodes another protein that controls EpoR mitogenic signaling. This protein could possibly be another EpoR subunit, a transducin that intermittently associates with ligand–EpoR complexes, or a protein that controls the metabolism or cell-surface shedding of EpoR.

Our results provide important evidence concerning the mechanism by which host genetic variation can modulate pathogenesis caused by an oncogenic retrovirus. In this case, gp55 acts as an agonist to constitutively activate EpoR of erythroblasts. In *Fv-2^r* strains, the EpoR signal-transduction pathway in BFU-E appears relatively down-modulated, thereby causing a substantially reduced mitogenic response to either gp55 or to Epo. Because productive infection and release of progeny retrovirions both require cell proliferation (37–39), viral replication and consequent pathogenesis would amplify much more quickly in *Fv-2^s* than in *Fv-2^r* strains. Because mitosis would amplify virus production exponentially, even a small decrease in proliferative response would substantially reduce pathogenesis. It is interesting that the down-modulation of BFU-E mitogenesis in *Fv-2^r* strains (33) does not cause erythrocyte insufficiency in uninfected mice maintained in normal conditions.

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