# THE ROLE OF THE ERYTHROPOIETIN RECEPTOR IN RETROVIRAL *ENV*-MEDIATED PATHOGENESIS

by

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A DISSERTATION

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## Abstract

Friend virus causes a rapid-onset disease in susceptible mice characterized by a fulminant splenomegaly driven by a massive erythroid hyperplasia. The molecular basis for the erythroblast proliferation has been shown to be the mitogenic stimulation of the erythropoietin receptor (EpoR) by the *env* glycoprotein (gp55) encoded by the defective spleen focus-forming component (SFFV) of the Friend viral complex. The studies described in this dissertation have focused on the interaction of the EpoR with SFFV-related *env* glycoproteins.

A retrovirus encoding Epo caused a disease indistinguishable from Friend erythroleukemia. Both diseases were restricted in *Fv-2*$^{rr}$ homozygous mice, which are sensitive to all other retroviral diseases. Gene mapping studies showed that *Fv-2* and EpoR genes are unlinked, eliminating the possibility that the *Fv-2*$^{r}$ and *Fv-2*$^{s}$ alleles are an EpoR polymorphism. These results imply that *Fv-2*-encoded proteins may interact closely with EpoR to control its activation by Epo or by gp55. This hypothesis was supported by two observations. First, SFFV mutations that alter gp55 structure can overcome *Fv-2*$^{rr}$ restriction. Second, human EpoR was not activated by gp55, but it was easily activated by a SFFV mutant that overcomes *Fv-2*$^{rr}$ restriction.

I observed the genesis of a new SFFV and was able to isolate the stages of progenitor evolution. The earliest progenitor appeared to be a replication competent retrovirus of the mink cell focus-inducing class (MCF) that was able to activate EpoR. The results support the hypothesis that MCFs form from endogeneously inherited retroviral genes and that the MCFs can then rapidly evolve in stages to form increasingly pathogenic derivatives including SFFVs. The idea that SFFVs can rapidly form *de novo* is a surprising result, with major implications for understanding the role of endogenously inherited retroviruses in

the spontaneous leukemias of mice. A novel SFFV mutant encoding the smallest *env* glycoprotein (gp41) known to activate EpoR was also isolated. This mutant was found to have a large deletion in the ecotropic-specific domain of the encoded glycoprotein. These results suggest that the ecotropic-specific domain of SFFV *env* glycoproteins is not required for EpoR activation or to cause erythroblastosis *in vivo*.

Finally, to investigate suggestions in the literature that EpoR might function as an MCF cell surface receptor, EpoR was expressed on cells lacking MCF receptors. We found that these cells remained resistant to two well-characterized MCF viruses. These results suggest that MCFs do not use EpoR as a cell surface receptor, despite published accounts of mitogenic and physical interactions.

# Introduction

## I. Retroviral *env* glycoproteins

Murine leukemia viruses (MuLVs) that are replication competent encode *gag, pol* and *env* proteins. The *env* gene encodes a glycoprotein that is cleaved by partial proteolysis to form the products gp70 surface (SU) glycoprotein plus a p15E transmembrane (TM) protein (70, 87, 117). The combination of the SU and TM proteins form structures on the outer coat of the virus that bind to specific corresponding receptors on the surfaces of susceptible cells. *Env* is the most variable retroviral gene (88), apparently the result of selective forces.

Many retroviral *env* genes (in addition to the spleen focus-forming virus *env* gene that will be discussed in detail) have been implicated pathogenesis, including *env* genes of HIV-1 (22), HTLV-1 (37), feline leukemia and immunodeficiency viruses (80, 83), avian subgroup F leukosis viruses (109), MuLVs (54) and myeloproliferative leukemia virus (110). Consequently, it is very important to understand the structures and functions of retroviral *env* glycoproteins.

The MuLVs have been grouped according to their host ranges. The gp70s of ecotropic MuLVs bind to receptors that occur only on cells of mice or rats, while xenotropic gp70s bind to receptors that occur on most mammalian cells but are absent from most strains of laboratory mice (40, 61). Amphotropic and dualtropic MuLVs both have a broad host-range although they bind to different receptors (17, 59, 96).

II. Friend disease

A. Discovery

Friend erythroleukemia virus (FV) is a complex of a replication competent murine leukemia virus (MuLV) and a replication-defective spleen focus-forming virus (SFFV) (87, 99, 117). The prototype virus reported in 1957 by Charlotte Friend was isolated from mice that developed splenomegaly 14 months after injection with a cell-free filtrate derived from Erlich ascites carcinoma cells (electron microscopic examination of these cells had revealed virus-like particles) (34). It was initially unknown if FV was associated with the tumor cells, or if it emerged in the mice independently (35). This original FV isolate (FV-A) caused a syndrome in mice associated with splenomegaly and anemia. Subsequently, the similar Rauscher erythroleukemia and Cas virus complexes were isolated (3, 66, 95). After distribution of the original FV-A to other laboratories, FV derivatives formed that were associated with splenomegaly and polycythemia (termed FV-P) (87, 99, 117).

B. Pathogenic Characteristics

The course of Friend disease can be divided into two stages. In the initial 4-6 weeks a fulminant splenomegaly occurs that is driven by erythroid hyperplasia. During this stage of the disease the infected erythroblasts retain their commitment to terminally differentiate. In contrast, the second stage of the disease is distinguished by erythroblast immortalization, demonstrated by the ability of the cells to be transplanted to secondary recipients (127). Dimethylsulfoxide treatment of cell lines derived from the tumors that form in secondary recipients induces expression of hemoglobin and other proteins associated with erythroid differentiation (36, 86). Experiments with helper-free preparations of

SFFV have shown that SFFV can cause the initial polyclonal proliferation of erythroblasts as well as the subsequent erythroblast immortalization (111, 128). Recent work has shown that SFFV integrates near the *ets*-related transcription factor PU.1 (79, 89, 111) and that expression of PU.1 immortalizes erythroblasts (105).

C. Target Cells

The primary target cells of SFFV are specific erythroid progenitors, called burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E). Hematopoiesis, the process by which all formed elements of the blood are produced, is strictly controlled by a series of interacting factors present in the bone marrow environment and expressed by the hematopoietic cells (reviewed in (87)). The pluripotential hematopoietic stem cell forms progenitor cells committed to the main marrow cell lines through cell proliferation and differentiation. The BFU-E and CFU-E are the late erythroid intermediates in this hierarchical cascade. The CFU-E are a class of rapidly dividing cells that are highly responsive to the presence of erythropoietin, a hormone produced in the kidney that regulates the erythroid pathway (64). The BFU-E are more immature cells that divide less frequently and, unlike CFU-E, require a "burst promoting activity" (such as interleukin-3) as well as Epo to develop into erythroblasts (51). The presence of EpoR on the cell surfaces corresponds well to Epo response (104). As expected from *in vivo* manifestations, FV-P infection of murine bone marrow erythroblasts in culture results in their Epo-independent proliferation and differentiation (41).

D. Origin and pathogenic mechanism of SFFV

The structure of SFFV suggests that it could have derived from a dualtropic mink cell focus-forming virus (MCF) by a 585 base pair deletion and by a single base pair addition in the ecotropic-specific portion of the *env* gene (14) (see Figure 1). Evidence suggests that both the 585 base pair deletion and the frameshift mutation probably contribute to SFFV pathogenesis (2, 125). The pathogenic differences between FV-A and FV-P strains of virus have also been ascribed to amino acid sequence differences in the ecotropic-specific portion of the *env* glycoproteins (12, 100).

SFFV encodes an *env* glycoprotein (gp55) that is inefficiently processed from the rough endoplasmic reticulum to form a larger cell surface derivative ($gp55^P$) (38). Recent evidence has shown that SFFV-encoded gp55 binds to erythropoietin receptors (EpoR) to cause erythroblast proliferation and splenomegaly in susceptible mice (31, 67, 69, 102, 130). Cross-linking studies with [$^{125}$I] Epo have suggested that the critical mitogenic interaction between gp55 and EpoR probably occurs exclusively on cell surfaces (8, 31). This is in agreement with earlier evidence that SFFV mutants lacking the $gp55^P$ cell surface component were nonpathogenic (68).

E. Interaction of gp55 and EpoR

The site(s) on gp55 necessary for EpoR binding and activation remain uncertain. Different studies have suggested that the dualtropic domain, the ecotropic domain, or the carboxyl terminal hydrophobic membrane anchor region may be critical (2, 12, 63, 67, 68, 73, 112, 125, 126, 133). However, a gp42 glycoprotein encoded by the BB6 *env* mutant of SFFV activates EpoR, yet it contains a large deletion in its ecotropic-specific region (63, 75) (see Figure 1). An EpoR-gp55 fusion protein that lacks

dualtropic *env* sequences can activate wild-type EpoR, perhaps by a mechanism that involves the remaining *env* sequences (107).

F. Host Resistance to Friend disease

It is generally believed that Friend disease is restricted to certain strains of mice and that humans and rats are resistant (23). By crossing inbred strains of resistant mice with susceptible strains, specific resistance genes have been identified. Of the genes identified in this way, the *Fv-2* and *Fv-5* genes are unique because they specifically control SFFV-induced disease and have no effect on the replication of helper virus or on immune responses (10, 60, 91, 106). Consequently, they might possibly control the interaction of gp55 with EpoR or the subsequent signal transduction pathway.

Mice homozygous for the *Fv-2* $^{r}$ allele are resistant to SFFV-induced erythroblastosis, but they are susceptible to all other retroviral diseases (101, 108), Resistance of *Fv-2* $^{rr}$ homozygotes is not due to a block in SFFV infection or in gp55 expression (25, 28, 48, 122). In *Fv-2* $^{rr}$ / *Fv-2* $^{ss}$ chimeric mice (susceptible mice are termed *Fv-2*$^{s}$), infection with Friend virus causes selective proliferation of the *Fv-2* $^{ss}$ erythroblasts (5, 108).

It is not understood how *Fv-2* $^{rr}$ homozygotes resist gp55-induced erythroblastosis, however *Fv-2* and *Epor* genes are unlinked, ruling out the possibility that the *Fv-2*$^{r}$ and *Fv-2*$^{s}$ alleles are an EpoR polymorphism (7, 48, 70). Interestingly, the truncated SFFV mutant (BB6) obtained by forced passage of SFFV in *Fv-2*$^{rr}$ congenic mice is able to overcome *Fv-2* resistance (a congenic mouse is *Fv-2*$^{rr}$ in an otherwise susceptible DBA/2 genetic background) (75). BB6 encodes a gp42 *env* glycoprotein with a large deletion in its membrane-proximal ecotropic-specific domain (75) .

A cell culture assay similar to that used to establish SFFV activation of EpoR was used to study BB6 activation of EpoR. In this assay, interleukin-3 dependent hematopoietic cells (BaF3) are converted to Epo dependence after infection by an EpoR-encoding virus (these cells are termed BaF3/EpoR). When factor -dependent BaF3/EpoR cells were infected with SFFV (termed BaF3/EpoR/SFFV) they became factor-independent. Similarly, factor-dependent BaF3/EpoR cells infected with BB6 were converted to factor-independence. The growth factor requirements of BaF3 cells not expressing EpoR were unchanged by BB6 and SFFV infection. These results suggested that, like gp55, the smaller BB6-encoded *env* glycoprotein, gp42, specifically activates EpoR (63) .

The proximity of EpoR to other cell surface proteins has been examined by crosslinking [$^{125}$ I] Epo to cell surface EpoR (8, 31, 63) . Casadevall and others found that certain proteins associated with the EpoR complex were displaced by the cell surface component of gp55 ($gp55^{P}$) in Friend virus-infected cells (8). When cell lysates from factor-independent BaF3/EpoR/BB6 and BaF3/EpoR/SFFV cells were examined by crosslinking [$^{125}$ I] Epo to surface EpoR, proteins were detected with immunological properties and sizes consistent with [$^{125}$ I] Epo-gp42 and [$^{125}$ I] Epo-gp55 complexes, respectively. Comparison of the pattern of the other [$^{125}$ I] Epo-containing complexes in these lysates revealed that certain EpoR accessory proteins were differentially displaced by the BB6 and SFFV-encoded *env* glycoproteins. One model for understanding *Fv-2*$^{r}$ restriction that is consistent with these results is that *Fv-2*-encoded protein (*Fv-2*$^{r}$ or Fv-2$^{s}$) directly associates with EpoR to control its activation, and that $gp55^{P}$ is unable to activate EpoR in *Fv-2*$^{rr}$ mice because of steric interference due to the presence of the ecotropic-specific domain. The

absence of a substantial portion of the ecotropic-specific domain in the BB6-encoded *env* glycoprotein might relieve this interference and allow it to activate EpoR in Fv-$2^s$ and *Fv*-$2^{rr}$ mice (63) .

III. Mink Cell focus-forming viruses

A. Origin

Mink cell focus-inducing viruses (MCFs) (1, 6, 14, 129) are a class of replication competent MuLVs that have a broad host-range called dualtropic (or polytropic) (30, 44). MCFs have been implicated in murine retroviral pathogenesis (11, 16, 27, 39, 44, 52, 101, 123, 124) and specifically occur in the affected tissues in the cases of thymic lymphomas, B cell leukemias, erythroleukemias and central nervous system degeneration (26, 30, 101, 113, 118). Mice that fail to form MCFs appear to be resistant to these diseases (98, 101). MCF isolates appear to be relatively stringent in the tissue-specificities of their replication *in vivo* compared with ecotropic MuLVs (15, 29, 82, 97).

Although MCFs are not inherited as replication-competent intact proviruses, the mouse genome contains numerous dispersed dualtropic *env* gene sequences (9, 32, 62). MCFs apparently readily form *de novo* by recombination when ecotropic host-range MuLVs replicate in mice (27, 30, 58, 101). MCF *env* genes typically are hybrid recombinants that contain a 5' dualtropic-specific region and a 3' ecotropic-specific portion (58) (see Figure 1). In addition, MCFs often differ from ecotropic MuLVs in their long terminal repeat sequences (9, 33, 49, 54, 56, 93).

B. Interaction with gp55

Recent studies suggest that MCF envelope glycoproteins may be mitogens that bind to erythropoietin receptors and to other members of

this hemopoietin receptor superfamily (63, 67, 119). This is an interesting and important issue because mitogenic activation of cells susceptible to infection provides a powerful enhancement to retroviral replication, thus driving selection of oncogenic retroviruses (55).

Previous evidence (63, 67, 119) does not establish whether cell surface receptors for MCF mitogenesis can also function as receptors for infection. Although the cell surface receptors that mediate MCF infection of mouse fibroblasts appear to be encoded by a genetic locus distinct from that of EpoR (7, 48, 59, 61), it is conceivable that MCFs could use different receptors to infect different tissues. HIV-1 appears to use different receptors on different cells (13, 42, 43).

## IV. Erythropoietin Receptor

### A. Structure and function

Murine EpoR is a 507 amino acid protein with a single 23 amino acid transmembrane domain between amino acids 250 and 272 (19). The human homologue contains 508 amino acids and is 82% similar to the murine receptor (53). The EpoR extracellular domain has several structural features in common with other members of the recently identified and expanding hemopoietin receptor superfamily: four cysteine residues in conserved positions in the N-terminal extracellular region and a WSXWS motif in the extracellular region adjacent to the membrane (4). There is no known kinase domain in the intracellular region. However, closely related members of this family (EpoR, IL-2Rβ, IL-3R, IL-4R and the gp130 signal transducer for IL-6R) share two highly homologous cytoplasmic regions in the first 60 amino acids after the end of the transmembrane domain (81). It has been proposed that these highly

conserved segments interact with structurally similar signaling molecule(s) (81).

The existence of EpoR subunits has been proposed based on crosslinking studies of [$^{125}$ I] labeled Epo (8, 19, 31, 53, 63, 76), experiments with an Epo-encoding retrovirus (48) and by analogy to the large family of cytokine receptors to which EpoR belongs. For example, the IL-2Rβ chain cytoplasmic region interacts with the *src*- family kinase p56$^{lck}$ to transduce a signal (46). Association of EpoR with phosphorylated proteins, including a soluble cytoplasmic tyrosine kinase, has been demonstrated (71, 131). EpoR can form high affinity and low affinity binding sites (for review see(21)), however the nature of the differences between the high and low affinity forms is not yet understood. It is likely that EpoR associates with other proteins to form receptor complexes of high and low affinity similar to other hemopoietin receptors in the cytokine receptor family. A third IL-2R subunit, IL-2Rγ, was recently discovered that participates in the formation of high and low affinity complexes with the IL-2Rβ (115). A common β subunit is shared between the GM-CSF, IL-3, and IL-5 receptors in the mouse to form the corresponding high affinity receptors (57, 116).

Deletion mapping has revealed several non-overlapping regulatory domains in the mEpoR C-terminal domain (20, 78, 133). Additionally, EpoR expression in FDC-P1 cells has been shown to down-modulate responsiveness of the cells to GM-CSF. This down modulation was relieved by C-terminal deletions in EpoR, suggesting that GM-CSFR and the C-terminal region of EpoR may compete for limiting regulatory factor(s) (92).

B. Interaction with gp55

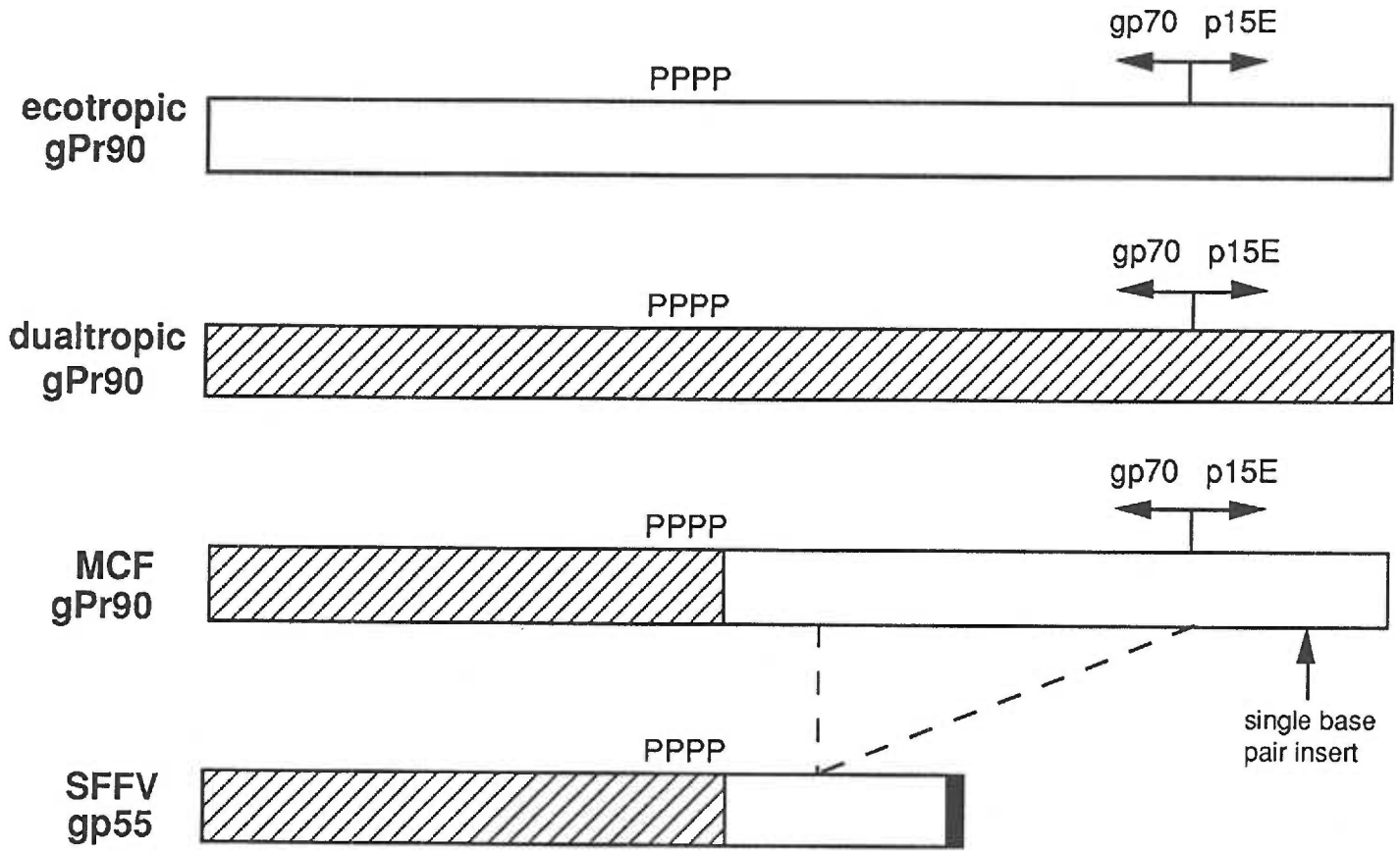
The region(s) of EpoR required for gp55 activation is not known, although the EpoR transmembrane region has been proposed to be essential but not sufficient for activation based on EpoR truncations, interleukin-3 receptor/EpoR chimeras, and gp55/EpoR chimeras (20, 107). Cross linking studies with [$^{125}$] I Epo have suggested that EpoR, Epo and gp55$^{P}$ might occur in a ternary complex at the cell surface. This implies that Epo and gp55 interact with nonoverlapping sites on EpoR (8, 31, 63).

V. Thesis Rationale

As I began the experiments described in this thesis EpoR had just been cloned. Shortly thereafter, the mitogenic activation of EpoR by the *env* glycoprotein encoded by SFFV was reported. That gp55 could act as an erythropoietin agonist explained SFFV target cell specificity and the striking erythroid proliferation that followed infection of susceptible animals. We obtained evidence supporting this important discovery using a retrovirus encoding Epo. We also found that while the Epo virus was able to cause a Friend-like disease in susceptible mice, it was restricted in *Fv-2*$^{rr}$ homozygotes, the same mice that are specifically resistant to Friend virus. We mapped *Epor* to chromosome 9 in a region unlinked to *Fv-2* and thereby ruled out the possibility that *Fv-2*$^{r}$ encodes a defective allele of the EpoR (see Manuscript #1). A second Epo virus was constructed using an LTR from a mink cell focus-forming virus in place of the SFFV LTR described in the first manuscript. We found that despite the strict thymotropic nature of this MCF, a Friend-like disease resulted following infection of susceptible mice. This result has important implications for the design of retroviral vectors, particularly for expression

of growth factors (see Manuscript #2). Several reports had suggested that EpoR might possibly be a cell surface receptor for MCFs based on 1) mitogenic activation of EpoR and other members of the hematopoietin receptor superfamily by MCFs, and 2) chemical crosslinking of MCF-encoded *env* glycoproteins to EpoR. EpoR was expressed on cells lacking MCF receptors. We found that these EpoR-expressing cells were resistant to two well-characterized MCF viruses. These results suggest that MCFs do not use EpoR as a cell surface receptor (see Manuscript #3). We hoped to exploit retroviral genetic drift to isolate a potent, constitutively activated EpoR mutant. What we discovered instead was a series of SFFV evolutionary intermediates, including a replication competent MCF, that were able to activate the EpoR (see Manuscript #4). A SFFV mutant with a significant deletion in the ecotropic-specific domain was analyzed. Sequence and pathologic results suggest that the ecotropic-specific domain of SFFV *env* glycoproteins is not required for EpoR activation (see Manuscript #5). Finally, SFFV activation of human EpoR and murine EpoR were compared. We found that human EpoR was resistant to activation by SFFV, but was easily activated by a SFFV mutant that overcomes *Fv-2*$^{r}$ restriction. Our results suggest that the EpoR functions as a multisubunit assemblage and that the resistance of non-murine species to SFFV may be controlled in part by *Fv-2*-encoded homologues (see Manuscript #6).

Figure 1. Comparison of the structures of SFFV-related *env* glycoproteins. PPPP refers to a flexible proline-rich linker region of approximately 50 amino acids (54, 74). Endogenously inherited dualtropic *env* genes recombine with replicating ecotropic MuLVs to form MCFs (27, 30, 58, 101). MCFs generally have an amino terminal region derived from the dualtropic *env* gene that extends into the proline-rich region. Formation of SFFV is believed to have involved a 585 base pair deletion (which eliminated the gp70-p15E cleavage site in the gPr90 protein precursor) and a single frame-shift mutation that led to a short novel hydrophobic sequence (shown in black) and premature termination of the protein (14). The BB6 *env* gene has an additional 159 base deletion resulting in a 53 amino acid deletion from the ecotropic-specific domain (75).


gp70 p15E
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MCF
gPr90
single base
pair insert
PPPP
SFFV
gp55

Manuscript #1

# Activation of Erythropoietin Receptors by Friend Viral gp55 and by Erythropoietin and Down-modulation by the Murine *Fv*-2$^r$ Resistance Gene

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(Friend virus/leukemia/cancer genes)

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 $5 \times 10^5$ 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 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-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 x *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 x mouse somatic-cell hybrids (16) and from SWR/J x 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 x $10^5$ 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.

**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 inoculum 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 (± 2.8) x $10^3$ 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, 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-kpb *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- and 3.1-kbp versus 7.4-, 3.1-, and 1.1-kbp fragments in SWR/J and C57L/J DNAs, respectively. The seven SWXLRI 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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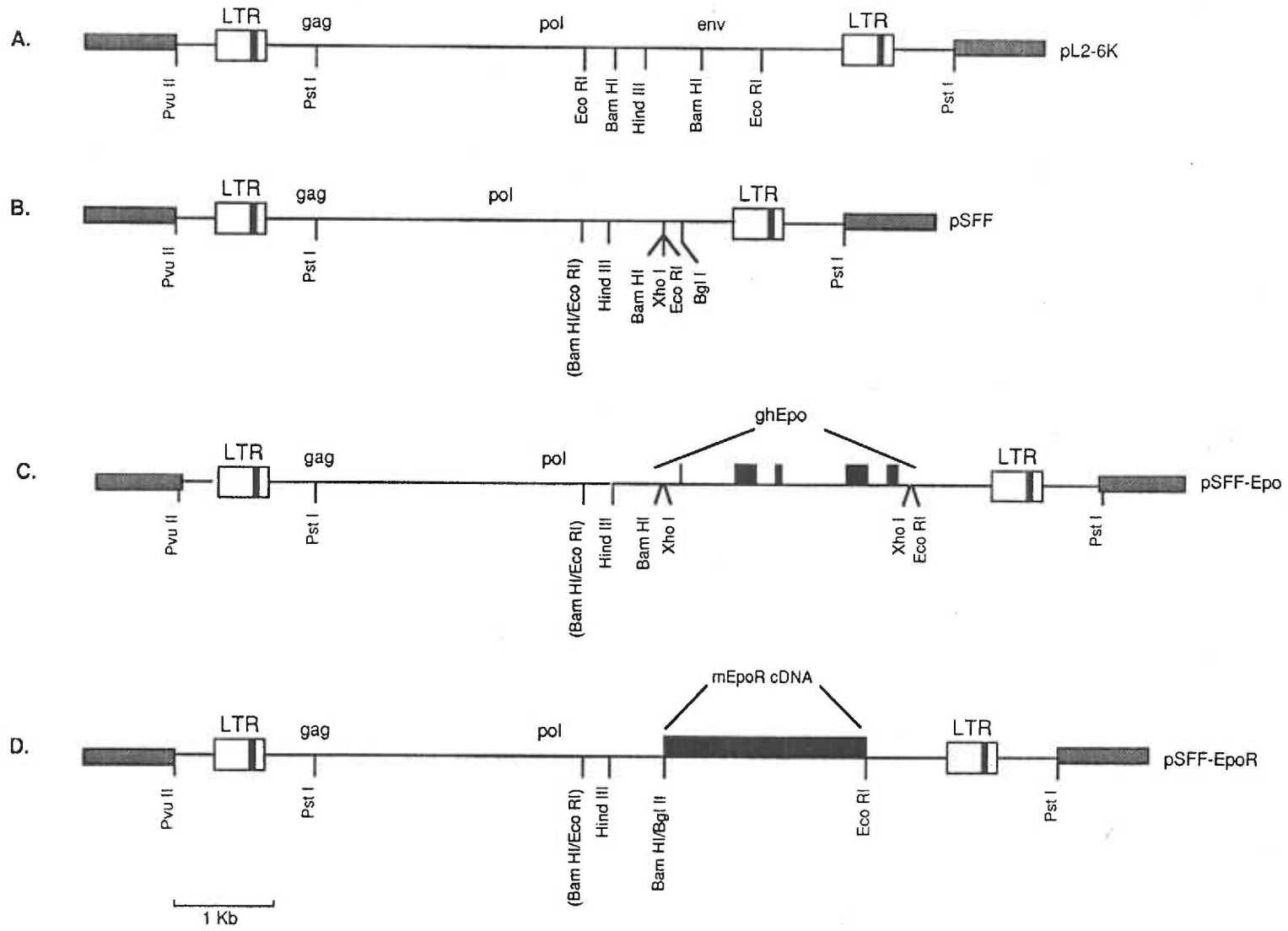
Figure 1. Maps of retroviral constructs The maps show relative sizes and major genetic features and restriction sites in the vectors. Hatched areas represent pSP64 vector sequences. (A) pL2-6K is the colinear SFFV molecular clone that encodes gp55 (4). (B) The retroviral vector pSFF was derived from 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* 1-*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).


A.
LTR
gag
pol
env
LTR
pL2-6K
Pvu II
Pst I
Eco RI
Bam HI
Hind III
Bam HI
Eco RI
Pst I
B.
LTR
gag
pol
LTR
pSFF
Pvu II
Pst I
(Bam HI/Eco RI)
Hind III
Bam HI
Xho I
Eco RI
Bgl I
Pst I
C.
ghEpo
LTR
gag
pol
LTR
pSFF-Epo
Pvu II
Pst I
(Bam HI/Eco RI)
Hind III
Bam HI
Xho I
Xho I
Eco RI
Pst I
D.
mEpoR cDNA
LTR
gag
pol
LTR
pSFF-EpoR
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Eco RI
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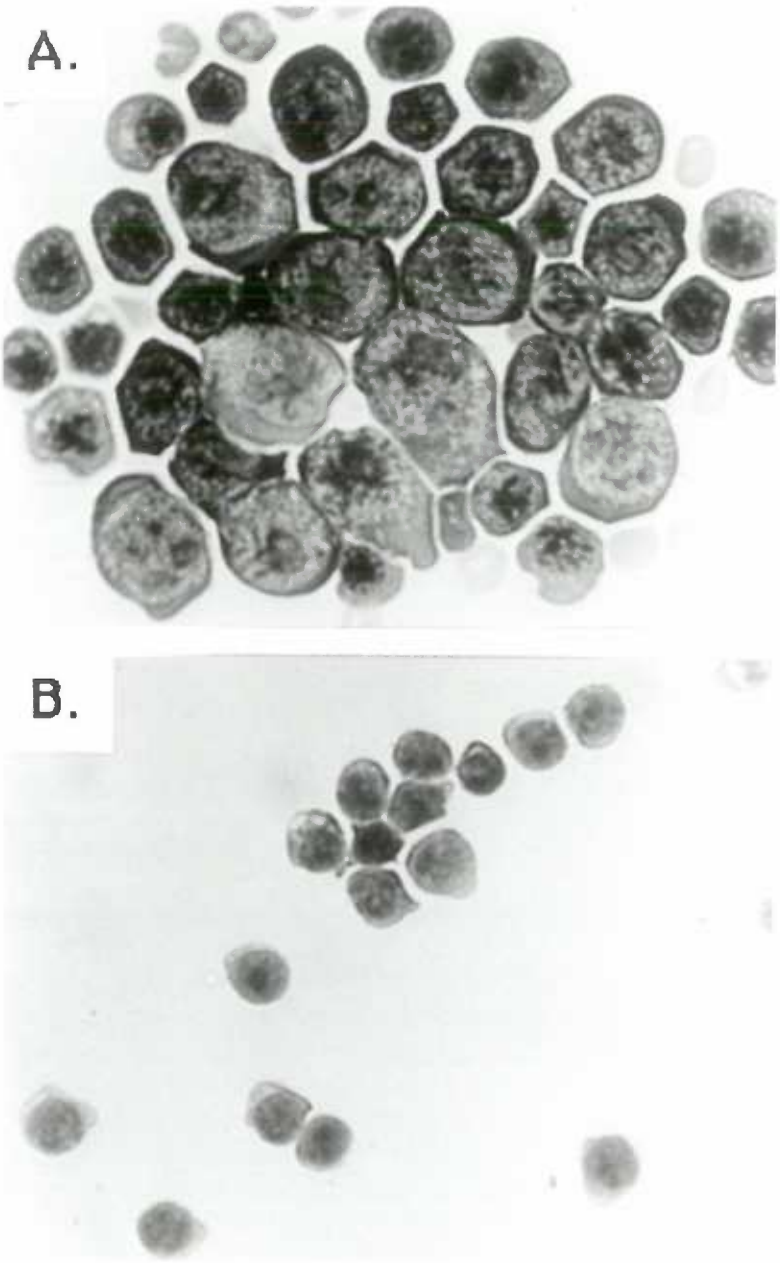
A.
B.

Figure 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. (x 2900)

**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.

**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 |
| lld | 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 l9d did not have substantial splenomegaly or polycythemia. In other experiments, mice analyzed at that time had extensive disease.

**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<br>X | S<br>X | S | S | L<br>X | S |
| *Apoa -1* | 25 | L<br>X | L | L<br>X | S | S | S | S |
| *Mod-1* | 44 | S | L | S | S | S | S<br>X | S |
| *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).

transiently transfected and chromosomally integrated proviruses. Moreover, tissue specificities of retroviral replication are adaptable and can be substantially influenced by the gene products they encode. The results have important implications for design of retroviral vectors for targeting to specific tissues.

INTRODUCTION

Retroviral vectors have been useful for expressing proteins including hemopoietins and their receptors in cell cultures and *in vivo* (Bowtell *et al.*, 1988; Hoatlin *et al.*, 1990; Li *et al.*, 1990; Wong *et al.*, 1989) and they are being investigated for gene therapy applications (Bodine *et al.*, 1990; Miller *et al.*, 1988; Miller and Rosman, 1989; Moore *et al.*, 1989). Unfortunately, retroviral expression can be unstable (Emerman and Temin, 1984; Xu *et al.*, 1989). Moreover, retroviral vectors have not been developed for targeting to specific tissues, despite the fact that such targeting occurs with natural retroviruses (Celander and Hazeltine, 1984; Chatis *et al.*, 1983; Cloyd, 1983; Evans and Morrey, 1987; Ishimoto *et al.*, 1987; Rosen *et al.*, 1985). Members of the mink cell focus-inducing class of murine retroviruses (MCFs) are exceptionally fastidious in their tissue tropisms. For example, thymotropic MCFs can be recovered only from thymic epithelium of infected mice (Cloyd, 1983).

Recently, we developed a relatively efficient and simple method for amplifying retroviral vectors (Bestwick *et al.*, 1988; Kozak and Kabat, 1990). The vector DNA is transfected into a coculture that contains cells (e.g., ψ2 and PA12 fibroblasts) that package retroviruses into distinct host-range envelopes. The ψ2 and PA12 cells release ecotropic and amphotropic virions, respectively (Mann *et al.*, 1983; Miller *et al.*, 1985). Since the virions from either cell type are cross-infections (due to absence of interference) (Weiss *et al.*, 1989), a back-and-forth ("ping-pong") process of infectious amplification ensues until a large proportion of cells express the recombinant protein. Because this process is efficient, dominant selectable marker genes are unnecessary and the vectors can be simplified and optimized for high-level expression of only the recombinant protein. Additionally, helper-free virions of broad host-range

are released in high titers. This method has been adapted for production of high titer retroviruses for gene therapy (Bodine *et al.*, 1990).

Several factors were previously shown to limit ping-pong amplifications. For example, only certain retroviral vectors are efficiently amplified when the vector DNA is transfected directly into the packaging cell cocultures (Bestwick *et al.*, 1988; Kozak and Kabat, 1990). Although the amplifications could theoretically proceed indefinitely until all cells contain multiple proviruses, it ceases prematurely in some cases due to instability of proviral expression and due to recombination of the vector with retroviral envelope genes in the packaging cells (Kozak and Kabat, 1990). These problems were not alleviated by using other retroviral packaging cell lines (Bestwick *et al.*, 1988; Kozak and Kabat, 1990). Presumably, a more transcriptionally active vector would result in higher yields of recombinant protein and perhaps in more rapid and efficient amplification. The only previously developed ping-pong vector (pSFF) that has been shown to amplify directly from transfected DNA was derived from Friend spleen focus-forming virus (SFFV); it has a moderately active enhancer-promotor in its long-terminal repeat (LTR) that has a slight preference for expression in erythroblasts and a low activity in fibroblasts (Spiro *et al.*, 1988). Other retroviral vectors have been constructed using a Moloney murine retrovirus. The Moloney LTR is similarly weak, although it has some preference for T-lymphocytes (Celander and Hazeltine, 1984; Chatis *et al.*, 1983; Evans and Morrey, 1987; Ishimoto *et al.*, 1987; Rosen *et al.*, 1985; Spiro *et al.*, 1988). In this project we used a transient transfection assay (Celander and Hazeltine, 1984; Gorman *et al.*, 1982; Rosen *et al.*, 1985; Short *et al.*, 1987; Spiro *et al.*, 1988; Yoshimura, *et al.*, 1985) to compare transcription activities of different retroviral LTRs for potential use in retroviral vectors. We found that the

LTR from MCF-13 virus was exceptionally active in all cells examined. This LTR was then substituted into pSFF to form a new vector pSFY. Using the human erythropoietin (Epo) gene as a model, we unexpectedly found that pSFF-Epo and pSFY-Epo were amplified and expressed to the same extent in fibroblasts. The resulting viruses both caused massive erythroblastosis and erythrocytosis in infected mice. Moreover, although MCF-13 virus is stringently thymotropic and causes only thymic lymphoma (Cloyd, 1983; Cloyd *et al.*, 1980; Hollen and Yoshimura, 1989), the SFY-Epo virus replicated extensively in erythroblasts.

## MATERIALS AND METHODS

ψ2 and PA12 fibroblasts were used as previously described (Bestwick, *et al.*, 1988; Hoatlin *et al.*, 1990; Kozak and Kabat, 1990). For ping-pong amplifications, cocultures were seeded 24 h before transfection at a 1:1 ratio according to protocols A or B as described (Kozak and Kabat, 1990). The cloning of the gene for human erythropoietin, its insertion into the pSFF retroviral expression vector, and the radioimmunoassay for Epo were also described earlier (Hoatlin *et al.*, 1990), as were methods for DNA and RNA isolation and blotting (Bestwick *et al.*, 1988; Li *et al.*, 1987; Southern, 1975; Thomas, 1983), for immunofluorescence microscopy (Kozak and Kabat, 1990), and for analysis of pathogenesis in infected mice (Hoatlin *et al.*, 1990).

For transient transfection analyses of retroviral LTR transcription activities, the LTRs were linked to the bacterial chloramphenicol acetyl transferase (CAT) gene as described previously (Spiro *et al.*, 1988). The constructs used included pSV2 CAT (with the SV40 viral enhancer-promotor) (Gorman *et al.*, 1982); pp1 CAT (with the SFFV LTR) (Li *et al.*, 1987), pC4 CAT (with a variant SFFV LTR) (Spiro *et al.*, 1988), pM1 CAT (with the U3 and R regions of the Moloney MuLV LTR) (Linney *et al.*, 1984; Overhauser and Fan, 1985), and pMM1 CAT (with the U3 and R regions of the MCF-13 virus LTR) (Hollen and Yoshimura, 1989). These plasmids were transfected in equimolar concentrations into murine NIH/3T3 fibroblasts, FVT/A erythroleukemia cells (Anand *et al.*, 1981), and S49 thymic lymphoma cells (Horbita and Harris, 1970). Cell extracts made 48 h later were analyzed for CAT activity as described previously (Gorman *et al.*, 1982; Spiro *et al.*, 1988). To control for differences in transfection efficiencies in different experiments and in different recipient cells, the CAT activities were normalized to the

activities obtained in parallel transfections with pSV2 CAT (Gorman *et al.*, 1982).

The structures of the expression plasmids pSFF-Epo and pSFY-Epo are diagrammed in Fig. 1. The pSFY vector was constructed by replacing the 600 base pair EcoR V-Kpn I fragment from the 3' LTR of pSFF with a 900 base pair Xba I-Kpn I fragment from MCF-13. The latter fragment was first ligated into the corresponding sites in the polylinker of pUC19 (Yanisch-Perron *et al.*, 1985) and was then recovered as a 900 base pair Hinc II-Kpn I fragment. pSFY was then constructed from pSFF fragments Kpn I-BamH I, BamH I-EcoR V and the Hinc II-Kpn I fragment just described in a three way ligation.

RESULTS

*Analysis of retroviral LTRs and construction of a new vector pSFY.* For comparing transcription activities of different retroviral LTRs, we used a standard transient transfection assay (Celander and Hazeltine, 1984; Gorman, *et al.*, 1982; Rosen *et al.*, 1985; Short *et al.*, 1987; Spiro *et al.*, 1988; Yoshimura *et al.*, 1985). The LTR sequences were constructed in linkage with the bacterial CAT gene and the CAT enzyme activities were measured in cell extracts made 48 h post-transfection (Gorman *et al.*, 1982). These activities were normalized to the activities obtained in parallel transfections of the same cells with pSV2 CAT, a plasmid that contains the promotor-enhancer sequences of the SV40 virus (Gorman *et al.*, 1982; Spiro *et al.*, 1988).

As shown in Fig. 2, the MCF-13 LTR (pMMI-CAT) was substantially more active than other retroviral LTRs in all of the cells examined. The MCF-13 LTR is most active in S49 lymphoma cells, in agreement with the induction of thymic lymphoma by this virus (Cloyd *et al.*, 1980). Similarly, the standard SFFV LTR (pp1 CAT) is relatively erythrotropic; the Moloney LTR (pM1 CAT) is relatively thymotropic; and the variant SFFV LTR (pC4 CAT) is most active in fibroblasts (Spiro *et al.*, 1988). Similar results were obtained in independent experiments.

These transient transfection analyses implied that the MCF-13 LTR is highly active in all cells analyzed, and that it is approximately 10 times more active than the SFFV LTR in NIH/3T3 fibroblasts (Fig. 2). For this reason, and because thymotropic expression vectors would be potentially important for studies of AIDS, we substituted the enhancer-promotor sequences of the 3' LTR of pSFF with the corresponding sequences from MCF-13 to obtain a new retroviral vector pSFY (see Fig. 1 and Materials and Methods). When this vector is transcribed to make RNA and then the RNA is replicated by the

reverse transcription mechanism, the LTR substitution from MCF-13 will be replicated into both LTRs of the resulting proviral DNA (Even *et al.*, 1983; Gilboa *et al.*, 1979; Temin, 1982). Based on the transient transfection LTR-CAT assays (Fig. 2), this would be expected to result in relatively abundant gene expression.

*Ping-pong amplification of pSFF-Epo and pSFY-Epo.* The Epo gene including introns was inserted into these vectors for ping-pong amplification. As illustrated by the results in Fig. 3, synthesis of Epo was detected by radioimmunoassay within 2-3 weeks post-transfection and reached maximum levels by 4-6 weeks. Expression in the cocultures that had amplified pSFY-Epo seemed to be usually somewhat lower than in cocultures transfected with pSFF-Epo. However, expression in replicate cocultures can also vary due to random factors (Kozak and Kabat, 1990). Consequently, our principle conclusion is that the pSFY vector is successfully amplified in these cocultures.

During the amplifications shown in Fig. 3, cells from the cocultures were plated onto coverslips for analysis of Epo-specific immunofluorescence. The Epo was clearly visible in a proportion of the cells as a strong perinuclear fluorescence, consistent with synthesis of a secretory glycoprotein (see Fig. 4). As shown in Table 1, at 30 days post-transfection cocultures with pSFF-Epo had 33-50% Epo-containing cells whereas those with pSFY-Epo were 45-73% positive for Epo. Using counting statistics and assuming the binomial distribution for numbers of proviruses in the cells, these results were used to calculate the multiplicities of infection (i.e., average numbers of proviruses per cell) in the cultures (see Table 1) (Kozak and Kabat, 1990). The calculated multiplicities were 0.39-0.68 proviruses/cell and 0.6-1.29 proviruses/cell for the cocultures that had been transfected with pSFF-Epo and pSFY-Epo

respectively. Dividing the yields of Epo at 30 days in these same cocultures (see Fig. 3) by these multiplicities of infection provides an estimate of the relative levels of Epo synthesis per integrated provirus. This calculation suggested that the SFF-Epo proviruses were on the average approximately 1.2 times more active than the SFY-Epo proviruses.

Thus, the expressions per provirus were very similar for the chromosomally integrated SFF-Epo and SFY-Epo proviruses, in striking contrast to the transient transfection estimates of promotor-enhancer activities (see Fig. 2). We repeated the transfection of these vectors in 5 independent amplification experiments and never observed a greater activity of pSFY-Epo than of pSFF-Epo.

*Characteristics of the ping-pong amplifications.* Cells were cloned from the cocultures undergoing amplification. Cell clones and cocultures were then grown and analyzed for production of Epo by radioimmunoassay and bioassay (Krystal, 1983). Data from four clones are shown in Table 2. Yields from these fibroblast clones ranged from approximately 0.05 mg/ml to 1 mg/l of culture medium. These estimates are based on measurements of total Epo produced over a 96 h period from culture dishes that contained approximately $2 \times 10^6$ cells at harvest in a total volume of 5 ml. Human erythropoietin (70U/μg) was used as a standard (Krystal *et al.*, 1986). The Epo produced was biologically active based on a standard Epo bioassay (Krystal, 1983).

Southern blot analysis of the clone U4 DNA is shown hybridized with a [$^{32}$P]-labeled Epo probe at high stringency in Fig. 5. When the cellular DNA was cleaved using a restriction endonuclease (EcoR I) that cuts only once in the provirus (see Fig. 1), two fragments were seen in the clone U4 digest (lane 6) but not in the digest of control mouse fibroblast DNA (lane 5), suggesting

that the clone U4 cells contain two proviruses. When cut with Xho I, an enzyme that cuts on both sides of the Epo insert (see Fig. 1), a single fragment of approximately 0.6 kilobase pairs was observed in the clone U4 digest (lane 4), and this was substantially smaller than the 2.4 kilobase pair Xho I fragment with Epo sequences from the pSFF-Epo plasmid [lanes 1 (20 pg) and 2 (1 pg)]. The 0.6 kilobase pair size of the fragment from clone U4 cells is compatible with the size expected (582 bp) following removal of introns from the 2.4 kilobase pair genomic Epo insert that was ligated into the vector (Lin *et al.*, 1985). This strongly suggests that amplification of the vector caused intron loss, as expected according to the retroviral transmission mechanism for ping-pong amplification.

Northern blot analysis of RNAs isolated from Ψ2 and PA12 cocultures that had been transfected with either pSFF-Epo or pSFY-Epo is shown in Fig. 6. The blot was hybridized with a [$^{32}$P]-labeled Epo probe. Both cocultures contained similar amounts of Epo-specific RNAs (lanes 3 and 4). These RNAs included full-length viral transcripts of approximately 6 kilobases plus smaller processed RNAs that include the mRNAs that encode Epo. The sizes of the Epo-encoding subgenomic mRNA components [2.2 kb and 2.5 kb in the RNAs from cells transfected with pSFF-Epo and pSFY-Epo respectively], are in agreement with the different sizes of the 3' untranslated regions expected in these RNAs (see vector structures in Fig. 1). In addition, there does not seem to be any significant difference in the abundance of these Epo-specific transcripts in the cocultures or in the ratio of their subgenomic processed mRNAs compared with intact genomic viral RNAs. Consequently, SFF-Epo and SFY-Epo viral transcripts are processed with similar efficiencies to form subgenomic Epo mRNAs.

*Pathogenesis by Epo-encoding retroviruses in mice.* To compare the *in vivo* effects of the Epo-encoding viruses, the helper-free virions from the coculture supernatants were mixed with a Friend MuLV helper virus for injection into NIH/Swiss mice. Mice injected with both SFF-Epo and SFY-Epo viruses had very large hematocrits and enlarged spleens at 26 d post-infection. To determine whether the SFY-Epo and SFF-Epo viruses were both replicating within the proliferating erythroblasts, and to ensure that the neoplasms were not caused only by Epo in the original inocula, passaged virus stocks were prepared by standard methods (Hoatlin *et al.*, 1990) from these enlarged spleens. As shown in Table 3, the viruses recovered from these spleens were equally able to cause erythroblast proliferation and erythrocytosis in secondary recipients.

## DISCUSSION

We have analyzed and attempted to optimize expression of Epo using the ping-pong method (Bestwick *et al.*, 1988; Bodine *et al.*, 1990; Hoatlin *et al.*, 1990; Kozak and Kabat, 1990; Li *et al.*, 1990) to amplify different retroviral vectors. Our data suggests that a high yield of Epo can result from this process of retrovirus-mediated infectious amplification. The pSFF and pSFY vectors that we have developed differ from other retroviral vectors in that they become amplified reproducibly when they are simply added as calcium phosphate precipitates to the cocultures (Bestwick *et al.*, 1988; Kozak and Kabat, 1990). Moreover, the process occurs spontaneously and efficiently until at least some cells in the cocultures contain more than one provirus. The amplified Epo sequences in these proviruses lack introns, as expected from the retroviral transmission mechanism *via* an RNA intermediate (Fig. 5). The Epo-encoding viruses released from the amplified cocultures caused abundant erythroblastosis and erythrocytosis in infected mice (see Table 3).

This gene amplification method has been previously used to express other recombinant proteins including mouse erythropoietin receptor (Hoatlin *et al.*, 1990; Li *et al.*, 1990), human growth hormone (Kozak and Kabat, 1990), mouse ecotropic retroviral receptor (Wang *et al.*, 1991), human interleukin 2 receptor β chains (Li and Baltimore, 1991), human granulocyte-macrophage colony stimulating factor (G. Bagby, personal communication), and a transcription factor, PU.1 (Schuetze *et al.*, 1992). These results suggest that it is widely applicable for expressing recombinant proteins and for obtaining high-titer helper-free viruses that can transfer this expression into cells of different types from different species or into animals for *in vivo* analyses. Bodine *et al.* (1990) described evidence that this method can be useful for gene therapy in primates.

We have attempted to design and to construct a more effective vector for ping-pong amplifications. Our strategy was to evaluate different retroviral LTRs for their activities and potential abilities to target gene expression into specific tissues and to use the information to improve the pSFF retroviral vector. Transient transfection assays using LTR-CAT constructs suggested that the promotor-enhancer region of the MCF-13 LTR was approximately 10 times more active in fibroblasts than the SFFV LTR that occurs in the pSFF vector (see Fig. 2). Consequently, we constructed the pSFY vector by replacing the enhancer-promotor sequences from the 3' LTR of pSFF with the corresponding sequences from an MCF-13 molecular clone (see Fig. 1). During reverse transcription from transcribed pSFY RNA, the 3' LTR enhancer-promotor sequences of MCF-13 should become duplicated in both proviral LTRs (Even *et al.*, 1983; Gilboa *et al.*, 1979; Temin, 1982). Increased transcription of the SFY-Epo provirus was expected to cause higher yields of Epo from the same number of proviral copies in the cocultures. Although the pSFY-Epo vector was amplified approximately as extensively as pSFF-Epo as indicated by the proportion of cells in the cocultures that synthesized Epo (see Fig. 3 and Table 1), the SFY-Epo proviruses were not more active than the SFF-Epo proviruses as indicated by the amounts of Epo produced by these cocultures. On the contrary, our results suggest that the chromosomally integrated SFF-Epo proviruses are approximately 1.2 times as expressive as the integrated SFY-Epo proviruses. Furthermore, these two cocultures contained similar quantities of Epo-specific genomic and subgenomic RNAs (Fig. 6). Thus, the two vectors were amplified and expressed to similar extents and the transcribed RNAs were also processed with approximately equal efficiencies.

Transient transfection assays of LTR-CAT constructs differ from ping-pong amplification assays in several respects. Specifically, transient assays are considered to be measurements of transcription of unintegrated plasmid DNAs (Gorman *et al.*, 1982; Hollen and Yoshimura, 1989; Linney *et al.*, 1984; Overhauser and Fan, 1985; Spiro *et al.*, 1988; Yoshimura *et al.*, 1985), whereas expression in our cocultures involves transcription of chromosomally-integrated proviruses. Previous workers have found that a substantial proportion of chromosomally-integrated proviruses are expressed unstably due to deletions during cell replication, due to methylation, or due to chromosomal position effects (reviewed in Hoeben *et al.*, 1991; Kozak and Kabat, 1990). Although such factors would presumably have no influence on the short-term expression that occurs in the transient assays, they may have a determining influence on expression of chromosomally integrated proviruses. Other recent results are compatible with this conclusion (Archer *et al.*, 1992; Paludan *et al.*, 1989).

The factors that control tissue specificities of retroviral expression are also poorly understood. Previous studies have shown that transient transfection assays of LTR-CAT constructs in different cell cultures correlate at least roughly with tissue specificities of retrovirus replication and pathogenesis *in vivo* (Celander and Hazeltine, 1984; Chatis *et al.*, 1983; Hollen and Yoshimura, 1989; Ishimoto *et al.*, 1987; Rosen *et al.*, 1985; Short *et al.*, 1987; Spiro *et al.*, 1988; Yoshimura *et al.*, 1985), in agreement with our results (Fig. 1). Moreover, switching the LTRs of Friend and Moloney MuLVs has indicated that these sequences control the target tissues for viral pathogenesis (Chatis *et al.*, 1983; Ishimoto *et al.*, 1987; Rosen *et al.*, 1985). However, Friend and Moloney MuLV replications *in vivo* are not stringently tissue specific, in contrast to the fastidious tissue specificity of MCFs (Cloyd, 1983; Evans and

Morrey, 1987). It is also striking that all retroviral LTRs that have been examined are expressed substantially in transient transfection assays in all cell lines (Celander and Hazeltine, 1984; Chatis *et al.*, 1983; Hollen and Yoshimura, 1989; Ishimoto *et al.*, 1987; Rosen *et al.*, 1985; Short *et al.*, 1987; Spiro *et al.*, 1988; Yoshimura *et al.*, 1985) (see also Fig. 1), and that all mouse retroviruses can also replicate promiscuously in fibroblasts and other cultured cells.

The discrepancy between fastidious tissue-specific expression *in vivo* and promiscuity in cell cultures is especially evident in the case of thymotropic MCFs, which cause thymic lymphoma and can be recovered only from the thymic epithelium and not from the spleens or other tissues of infected mice (Cloyd, 1983). These same MCFs replicate readily in cells cultured from different tissues. Strikingly, our SFY-Epo virus, which contains the thymotropic MCF-13 LTR enhancer-promotor sequences, not only causes erythroblastosis but also clearly replicates within the proliferating splenic erythroblasts. Indeed, we were able to recover SFY-Epo and SFF-Epo viruses with similar pathogenic activities from the splenic erythroblasts (Table 3). This agrees with evidence of Wolff and Ruscetti (1986) that the erythrotropism of SFFV is substantially determined by the pathogenic gene product rather than by its LTR. Because retroviral infection and release of progeny virions both require cell proliferation, retroviral replication becomes selectively amplified in neoplastic cells. Our results suggest that the LTR of a stringently thymotropic MCF may actually be promiscuous in its potential for expression in different tissues, and are compatible with the idea that the target tissue specificity of a retrovirus can be redirected by a gene (Epo) that causes erythroblast proliferation. A corollary is that the target tissue specificity of retroviral replication may be influenced only slightly by the viral LTR.

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Figure 1. Map of retroviral constructs showing the major restriction endonuclease landmarks, relative sizes and genetic features in the vectors. Lightly hatched areas represent pSP64 vector sequences. The black areas in the region containing the human genomic Epo gene (ghEpo) represent the exons. A. pSFF Epo encodes human Epo using an LTR derived from Friend spleen focus forming virus (SFFV) (Bestwick *et al.*, 1988). B. pSFY Epo encodes human Epo using a 3' LTR derived from MCF-13 (darkly hatched area).

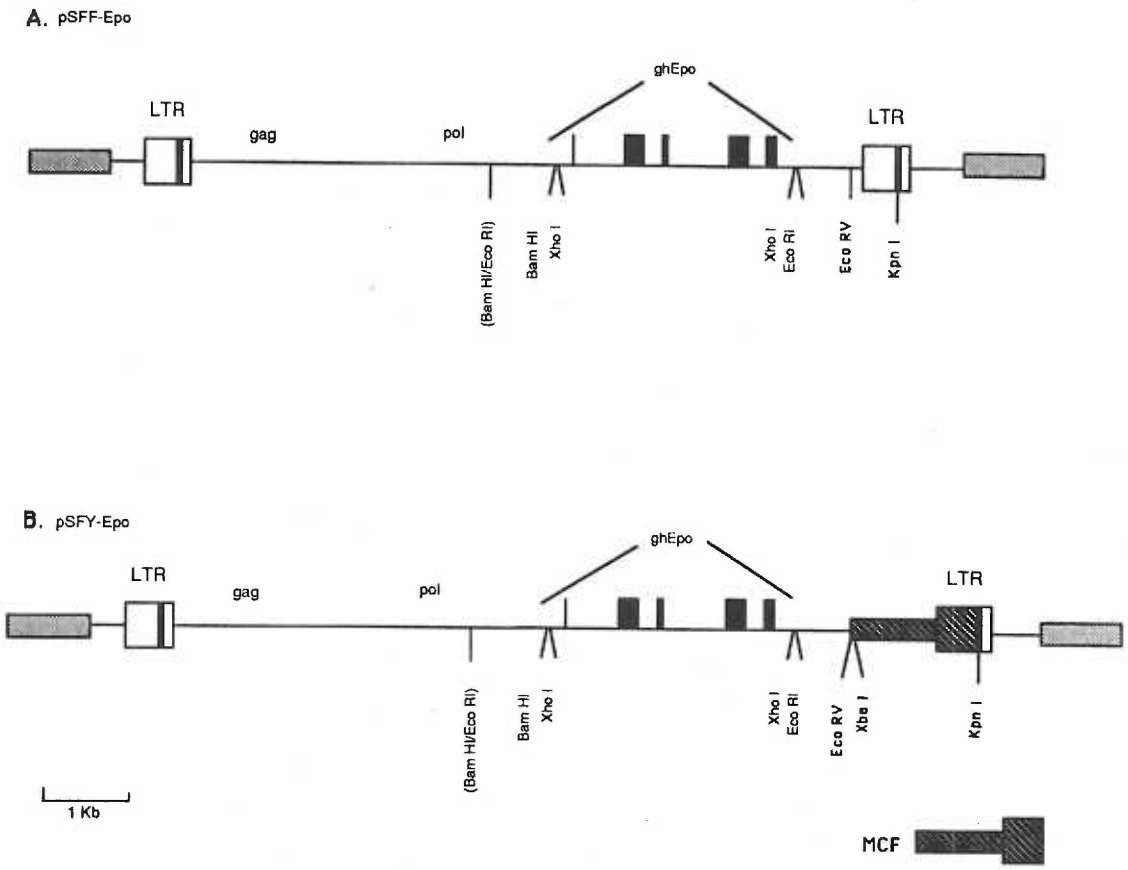
A. pSFF-Epo
ghEpo
LTR
gag
pol
LTR
(Bam HI/Eco RI)
Bam HI
Xho I
Xho I
Eco RI
Eco RV
Kpn I
B. pSFY-Epo
ghEpo
LTR
gag
pol
LTR
(Bam HI/Eco RI)
Bam HI
Xho I
Xho I
Eco RI
Eco RV
Xba I
Kpn I
1 Kb
MCF

Figure 2. Relative strengths of various LTRs measured by transient transfections of LTR-CAT constructions into NIH/3T3 fibroblasts, FVT/A erythroleukemia cells and S49 thymoma cells. The CAT activities were normalized to activities obtained with an SV40 viral enhancer-promoter, pSV2 CAT (Gorman *et al.*, 1982).

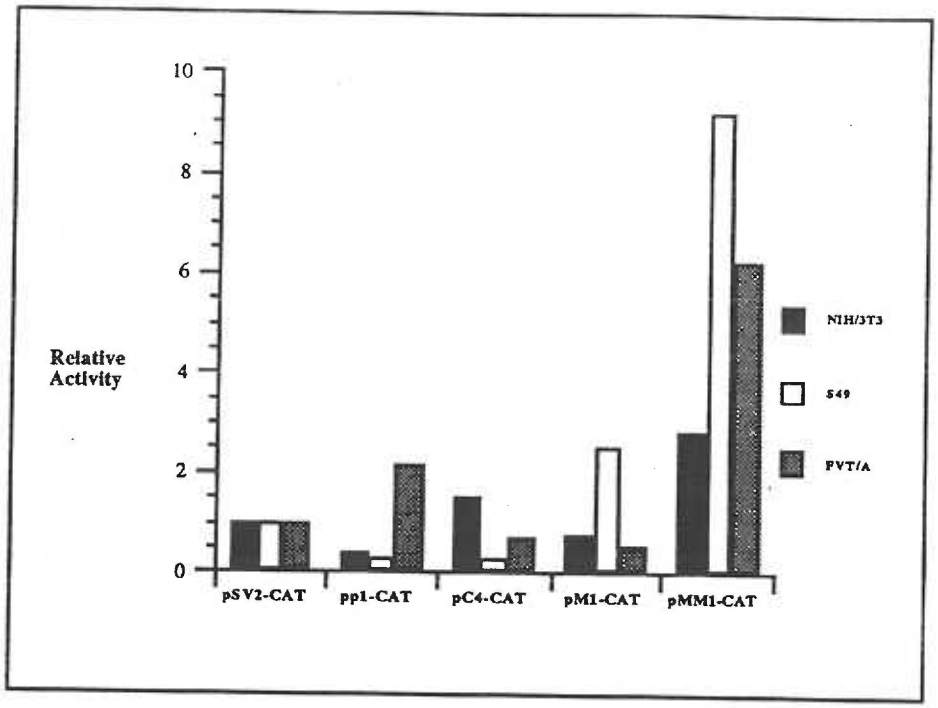
Relative Activity
10
8
6
4
2
0
pSV2-CAT
pp1-CAT
pC4-CAT
pM1-CAT
pMM1-CAT
NIH/3T3
S49
PVT/A

Figure 3. Results of a typical time course of Epo production in amplifying cocultures measured by RIA. Duplicate cocultures were transfected with either pSFF Epo (filled symbols) or pSFY Epo (outline symbols). At the time points shown, cells were seeded into culture flasks and allowed to grow until confluent (approximately $2 \times 10^6$ cells after 96 hrs of growth). The medium was then removed and assayed for Epo by RIA as described previously (Bestwick *et al.*, 1988).

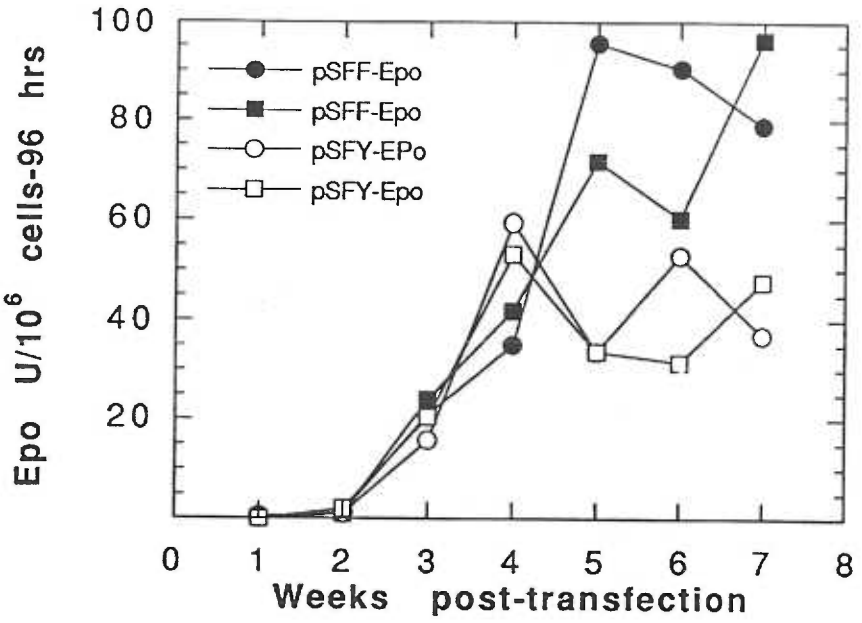
100
80
60
40
20
Epo U/10^6 cells-96 hrs
pSFF-Epo
pSFF-Epo
pSFY-EPo
pSFY-Epo
0
1
2
3
4
5
6
7
8
Weeks post-transfection

Figure 4. Detection of hEpo in ping-pong cocultures by indirect immunofluorescence. Panel A shows a coculture of ψ2 and PA12 cells transfected with pSFF Epo and allowed to ping pong for several weeks. A rabbit anti Epo antibody (HCC-3b at a dilution of 1:1000) (Terry Fox Labs, Vancouver, B.C.) was used followed by a second FITC-conjugated swine antirabbit antibody (Swine anti rabbit FITC at 1:500 dilution) (Tago, Burlingame, CA). Panel C shows an untransfected ψ2 and PA12 coculture using these same antibodies. Panels B and D show the corresponding bright phase views of these cells.

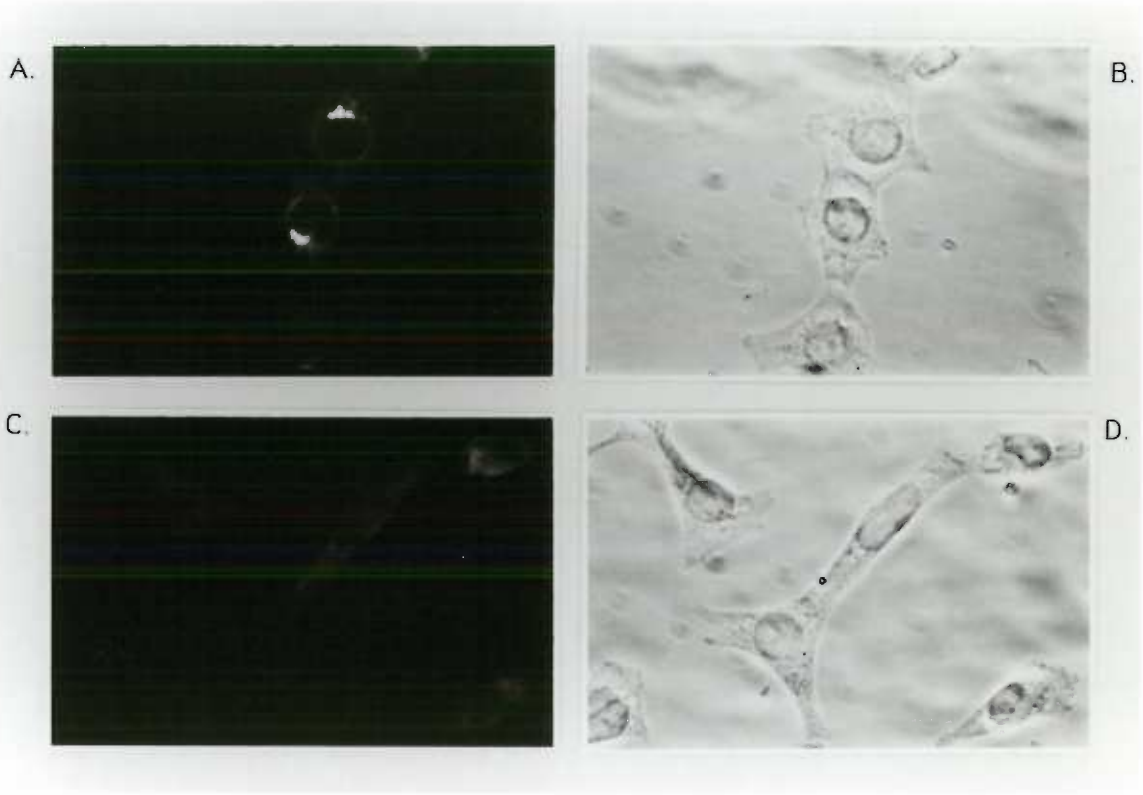
A.
B.
C.
D.

Figure 5. Detection of hEpo in a ping-pong amplified clone by Southern blot analysis. pSFF Epo clone U4 genomic DNA was restricted with EcoR I (lane 6) or Xho I (lane 4). Genomic DNA isolated from untransfected cells was restricted with EcoR I (lane 5) or Xho I (lane 3). A 2.4 kbp Xho I fragment encoding ghEpo was loaded on lane 2 (1 pg) and lane 1 (20 pg). This same 2.4 kbp fragment was used as a $^{32}P$ labeled probe. Sizes of molecular weight standards (kbp) are shown at the left. The horizontal lines are artifacts introduced during automated film development.

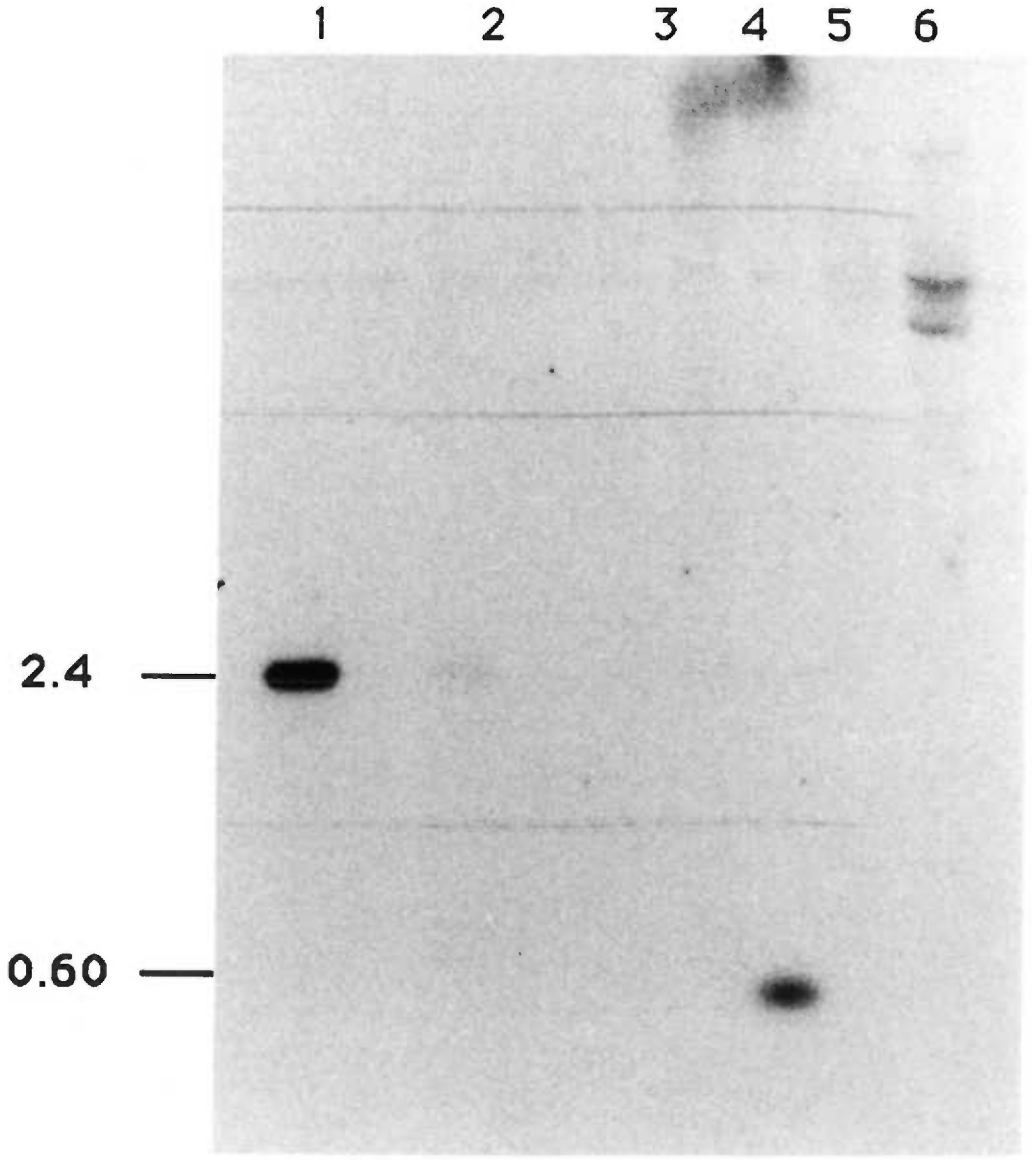
1
2
3
4
5
6
2.4
0.60

Figure 6. Northern blot analysis of total RNA isolated from ping pong cocultures transfected with pSFF Epo (lane 3) and pSFY Epo (lane 4). Total RNA (10 μg/lane) from a coculture expressing an unrelated protein is shown in lane 1. Poly A RNA from PA12 negative control cells is shown in lane 2. A $^{32}P$ labeled, 2.4 kbp Xho I fragment encoding ghEpo was used as probe. Molecular weight markers (kbp) are shown at the right.

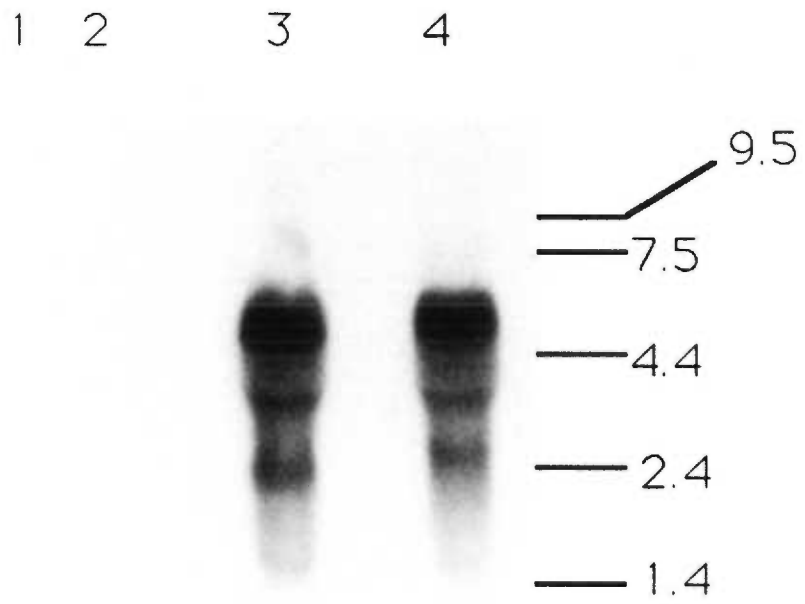
1
2
3
4
9.5
7.5
4.4
2.4
1.4

**Table 1. Comparison of multiplicity of infection of duplicate packaging-cell cocultures transfected with pSFF-Epo or pSFY-Epo**

| Coculture | % Positive cells* | Multiplicity of infection§ |
|---|---|---|
| pSFF-Epo A | 33 | 0.39 |
| pSFF-Epo B | 50 | 0.68 |
| pSFY-Epo A | 73 | 1.29 |
| pSFY-Epo B | 45 | 0.59 |

* Based on indirect immunofluorescence as described in text at thirty days post-transfection.

§ The multiplicity of infection was determined as $-\log P_0/0.44$, where $P_0$ is equal to the fraction of cells lacking Epo immunofluorescence (Kozak and Kabat, 1990).

**Table 2. Radioimmunoassay of Epo production from single cell clones**

| Cell clone* | Epo U/$10^6$ cells-96 h[§] |
|---|---|
| U4 | 12.5 |
| U8 | 73.5 |
| U18 | 70.0 |
| U26 | 246.5 |

*The cell clones in this study were from a coculture that had been transfected with the pSFF-Epo vector.

[§]The activity in units can be used to calculate concentration based on the conversion estimate of 70U/ug Epo (Krystal *et al.*, 1986).

**Table 3. Splenomegaly and polycythemia in NIH/Swiss mice caused by passaged Epo-encoding retroviruses**

| Virus Used[a] | Hematocrit | Spleen weight (g.) |
|---|---|---|
| **SFY-Epo** | | |
| Mouse #1[b] | ND* | 2.5 |
| #2[c] | ND | ND |
| #3 | 88 | 4.0 |
| #4 | 67 | 4.2 |
| #5 | 50 | 0.2 |
| #6 | 68 | 3.4 |
| **SFF-Epo** | | |
| #1[d] | ND | 1.5 |
| #2 | 82 | 3.2 |
| #3 | 77 | 1.6 |
| #4 | 87 | 2.6 |
| #5 | 86 | 3.0 |
| #6 | 56 | 2.2 |
| Uninfected | 39-43 | 0.09-0.12 |

*ND, not done.

[a] Viable mice were examined 20 d post-infection.

[b] Moribund mouse was killed and examined at 15 d post-infection.

[c] Mouse with obvious splenomegaly found dead 18 d post-infection.

[d] Mouse found dead 15 d post-infection.

Manuscript #3

# Erythropoietin Receptors do not Mediate Infections by Dualtropic Murine Leukemia Viruses

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Abstract Recent evidence suggested that envelope glycoproteins encoded by the dualtropic host-range class of murine leukemia viruses (MCFs) bind as agonists to erythropoietin receptors (EpoR) and possibly to other members of the hemopoietin receptor superfamily [Li, J.-P., D'Andrea, A.D., Lodish, H.F., and Baltimore, D. (1990) Nature (London) 343, 762-764; Li, J.-P. and Baltimore, D. (1991) J. Virol. 65, 2408-2414; Tschilis, P.N. and Bear, S.. (1991) Proc. Natl. Acad. Sci. USA 88, 4611-4615] Moreover it was proposed that these adsorptions might also mediate specific virus entry into pathologically relevant target cells. We found that hamster fibroblasts which are resistant to MCFs lack any post-penetration block to infection by these viruses. Hamster fibroblasts that express EpoR remain resistant to MCFs.

Dualtropic viruses (MCFs) have been implicated in murine retroviral pathogenesis (1-9). These viruses form *de novo* when ecotropic murine leukemia viruses (MuLVs) replicate in mice and recombine with inherited nucleic acids (3, 9-11). Moreover, MCFs specifically occur in the affected tissues in the cases of thymic lymphomas, B cell leukemias, erythroleukemias and central nervous system degeneration (9, 10, 12-14). Mice that fail to form MCFs appear to be resistant to these diseases (9, 15). MCFs differ from ecotropic MuLVs in the amino terminal portions of their envelope glycoproteins and often in the sequences of their long terminal repeats (LTRs) (16-20). MCF isolates also appear to be relatively stringent in the tissue-specificities of their replication *in vivo* compared with ecotropic MuLVs (21-24). Recent studies suggest that MCF envelope glycoproteins may be mitogens that bind to erythropoietin receptors (EpoR) and possibly to other members of this hemopoietin receptor superfamily (25-27). Friend spleen focus-forming virus (SFFV), a highly pathogenic member of the MCF family, encodes a glycoprotein (gp55) that causes virus-induced erythroblastosis by this mechanism (28-32). Replication competent MCFs also encode glycoproteins (gp70s) that bind to EpoR as shown by coimmunoprecipitation (26) and by crosslinking studies (33). These MCFs have been implicated in mitogenic activation of EpoR and interleukin-2 receptors (26, 27). Mitogenic activation of cells susceptible to infection provides a powerful enhancement to retroviral replication, thus driving selection of oncogenic retroviruses (34).

Previous evidence (26, 27) does not establish whether cell surface receptors for MCF mitogenesis can also function as receptors for infection. Although the cell surface receptors that mediate MCF infection of mouse fibroblasts appear to be encoded by a genetic locus distinct from that of EpoR

(35-38), it is conceivable that MCFs could use different receptors to infect different tissues. HIV-1 appears to use different receptors on different cells (39-42).

Because hamster fibroblasts are resistant to all mouse retroviruses (43-45) it was difficult to know whether their block to MCFs was due to an absence of functional cell surface receptors. To address this issue, we used two derivative cell lines that express receptors for ecotropic MuLVs. One (BM3C3) is a derivative of E36 hamster fibroblasts that contains a translocation fragment of mouse chromosome 5 (35, 36) and the other (CERDCL9) is a Chinese hamster ovary cell line that expresses recombinant mouse ecotropic receptors (46). Although completely resistant to MCF infections, these cells were readily infected by MCF pseudotypes that had ecotropic envelope glycoproteins. These pseudotypes were harvested from the media of ψ–2 ecotropic packaging murine cells that had been infected with MCFs. The infected hamster cells expressed MCF envelope glycoproteins and released MCF host-range virions. Consequently, resistance of hamster cells to MCFs must be due to absence of cell surface receptors.

A retroviral vector encoding EpoR (pSFF-EpoRPA11) was constructed by inserting a Bgl II- Sty I restriction fragment containing the entire coding region of EpoR but not the polyadenlyation signal from the plasmid pXM190 (47) into the retroviral vector pSFF (48). When pSFF-EpoRPA11 was transfected into a coculture of ψ–2 (49) and PA-12 (50) packaging cells it spontaneously amplified (48, 51). The transfected cultures strongly bound [$^{125}$I] Epo (300-400 Ci/mmol, Amersham-Searle, Arlington Heights, IL) and released helper-free EpoR-encoding virions that enabled interleukin 3 (IL-3)-dependent BaF3 cells (52) to grow without IL-3 in the presence of Epo. These virions were used to infect the BM3C3 and CERDCL9 hamster cells that

contain mouse ecotropic receptors, and we thereby obtained clones of hamster cells that strongly bound [$^{125}$I] Epo (see Table 1).

The MCFs that we used (MCF-1E and MCF-13) (1, 2) have been well-characterized as causes of erythroleukemia and thymic lymphoma, respectively. Our preparations of these viruses efficiently infected CCL64 mink lung fibroblasts and BALB/c 3T3 mouse fibroblasts, as indicated by immunofluorescence microscopy using an antiserum (53) that reacts strongly with MCF envelope glycoproteins. All of our hamster cell lines (CHO, CERDCL9, CERDCL9/EpoR-24, BM3C3, BM3C3/EpoR-4) were completely resistant to infection by these MCFs.

Elsewhere we showed by crosslinking that the F-MCF gp70 (54) forms a cell surface complex with EpoR (33). However, we conclude that mouse EpoR cannot function as a receptor for infection of hamster cells by MCFs. Although this implies that MCF receptors for infection differ from those for mitogenesis, several qualifications must be considered. Conceivably, mouse EpoR could mediate MCF infection of mouse but not of hamster cells. For example, a mouse-specific cofactor might be required. Furthermore, the EpoR family of receptors apparently functions in signal transducing cells in association with other subunits (55-57). If these proteins were required for mediation of MCF infections, EpoR might only facilitate MCF entry into hematopoietic cells.

## Acknowledgments

We are grateful to Alan D'Andrea (Dana-Farber Cancer Institute, Boston, MA) for pXM190. We thank Scott Schuetze and Frank Ferro for encouragement and Evelyn Jackson for assistance. This research was supported by NIH grant CA25810. M.E.H. was the recipient of an N.L. Tartar fellowship from the Medical Research Foundation of Oregon.

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Table 1. [$^{125}$I] Epo binding[b] to hamster cells infected[a] with the EpoR-encoding retrovirus.

| Cell Line | [$^{125}$I] Epo binding, cpm |
|---|---|
| CERDCL9 | 156 |
| CERDCL9/EpoR-24 | 7500 |
| BM3C3 | 90 |
| BM3C3/EpoR-4 | 1200 |

[a] Fresh or frozen viral harvest from ψ–2 and PA-12 cocultures transfected with pSFF-EpoRPA11 was used to infect BM3C3 cells and CERDCL9 cells seeded out 16-24 hours earlier at a concentration of 5 x $10^4$ cells / 25 $cm^2$. Viral harvests were obtained by incubating half-confluent monolayers of the transfected coculture in a minimum amount (0.1 ml/$cm^2$) of fresh medium for 16-24 hours, removing the medium and passing it through a 0.2 μm filter. BM3C3 and CERDCL9 cells were infected with 2 ml of viral harvest by incubating for 2 hours at 37°C in the presence of polybrene (8 μg/ml) (48).

[b] Cells were tested for the presence of EpoR after one week by [$^{125}$I] Epo binding. Cells were seeded out in 2 $cm^2$ dishes. The medium was removed and 0.1 μCi [$^{125}$I] Epo (300-400 Ci/mmol, Amersham-Searle, Arlington Heights, IL) diluted in 1 ml DMEM plus 10% FBS was added to approximately 1 x $10^6$ cells. The cells were incubated for one hour at 37°C, washed once with complete medium and two times with phosphate buffered saline. Sodium dodecyl sulfate (1%) was added to lyse the cells and the lysate was

then counted in a Packard Instruments (Downer's Grove, IL) model 5005 gamma counter. The binding on this population of cells was approximately two-fold over that of the uninfected cells. The infected cell populations were then cloned out by limiting dilution (51) and screened by [$^{125}$ I] Epo binding to obtain the two clones listed above.

Manuscript #4

# Origin and Rapid Evolution of a Novel Murine Erythroleukemia Virus of the Spleen Focus-Forming Virus Family

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Running title: Evolution of a new SFFV

## Abstract

The Friend spleen focus-forming virus (SFFV) *env* gene encodes a glycoprotein with apparent Mr 55,000 that binds to erythropoietin receptors (EpoR) to stimulate erythroblastosis. A retroviral vector (pSFF) that does not encode any *env* glycoprotein was packaged into retroviral particles and was coinjected into mice in the presence of a weakly pathogenic helper virus. Although most mice remained healthy, one mouse developed splenomegaly and polycythemia at 67 days; and the virus from this mouse reproducibly caused the same symptoms in secondary recipients by two to three weeks post-infection. This disease, which was characterized by extramedullary erythropoietin-independent erythropoiesis in the spleens and livers, was also reproduced in long-term bone marrow cultures. Viruses from the diseased primary mouse and from the secondary recipients converted erythropoietin-dependent cells into factor-independent cell lines; and this enabled us to isolate the novel pathogenic virus at its initial stages of formation. Interestingly, our earliest virus isolate contained a mixture of components that were all capable of activating EpoR. These components included a replication competent virus of the mink cell focus-inducing class and a derivative virus that encoded a truncated Mr 60,000 glycoprotein. During further *in vivo* passage, a virus that encodes a Mr 55,000 glycoprotein became predominant. The *env* gene that encodes the Mr 60,000 glycoprotein provided evidence for an intermediate in evolution of an MCF into an SFFV. The results strongly suggest that some MCFs can activate EpoR, and that such MCFs can rapidly evolve *in vivo* to form increasingly pathogenic SFFVs.

## Introduction

The independently isolated Friend and Rauscher erythroleukemia viruses (20, 57) are complexes of a replication competent murine leukemia virus (MuLV) and a replication-defective spleen focus-forming virus (SFFV) (44, 46, 55). The SFFVs encode *env* glycoproteins (gp55) that are inefficiently processed to form larger cell surface derivatives ($gp55^P$) (21). The gp55 binds to erythropoietin receptors (EpoR) to cause erythroblast proliferation and splenomegaly in susceptible mice (17, 34, 36, 49, 60). Recent evidence has suggested that the critical mitogenic interaction probably occurs exclusively on cell surfaces (6, 17).

SFFVs are structurally closely-related to mink cell focus-inducing viruses (MCFs) (1, 4, 10, 59), a class of replication competent murine retroviruses that has a broad host-range called dualtropic (or polytropic) (16, 23). Although MCFs are not inherited as replication-competent intact proviruses, the mouse genome contains numerous dispersed dualtropic *env* gene sequences (7, 18, 30). MCFs apparently readily form *de novo* by recombination when ecotropic host-range MuLVs replicate in mice (15, 16, 29, 48). MCF *env* genes typically are hybrid recombinants that contain a 5' dualtropic-specific region and a 3' ecotropic-specific portion (29). They encode a gPr90 *env* glycoprotein that is cleaved by partial proteolysis to form the products gp70 surface (SU) glycoprotein plus p15E transmembrane (TM) protein (37, 44, 55). In addition, MCFs often differ from ecotropic MuLVs in their long terminal repeat sequences (7, 19, 25, 27, 28, 45).

Conceivably, SFFVs could have derived from MCFs by a 585 base pair deletion and by a single base pair addition in the ecotropic-specific portion of the *env* gene (10). Evidence suggests that both the 585 base pair deletion and the frameshift mutation probably contribute to SFFV pathogenesis (2, 58). Several pathogenic differences among SFFV strains have also been ascribed to amino

acid sequence differences in the ecotropic-specific portion of the *env* glycoproteins (9, 47).

This paper describes the origin and rapid stepwise evolution of a new SFFV. This new pathogenic virus initially formed in a mouse that had been injected with an ecotropic strain of MuLV in the presence of a retroviral vector that does not encode any *env* glycoprotein. The mouse developed erythroleukemia, splenomegaly and polycythemia after a long lag phase. At that time the spleen contained an MCF that was able to activate EpoR plus several derivatives with *env* gene deletions. Serial passage of this initial virus isolate resulted in selection of an SFFV that encodes a gp55 glycoprotein. This experimental system provides a method for isolating new SFFVs, and for mapping the stages in their evolution from MCFs.

## Materials and Methods

### Plasmid Construction

The retroviral vector pSFF-EpoRPA11 was constructed from the ligation of an EcoR I/ BamH I fragment of pSFF (5), a Bgl II /Nhe I 800 bp fragment from pXM190 ( generous gift of A. D'Andrea, Dana-Farber Cancer Institute, Boston, MA), and a Nhe I/EcoR I 790 bp fragment of pXM190 containing the 3' end of the EpoR gene without its polyadenylation signal. The EcoR I site of the latter fragment was constructed by treating a Sty I site ( EpoR nucleotide position 1547 (12)) with Klenow polymerase which fortuitously introduced a stop codon at the natural stop site in the EpoR sequence, and also created an EcoR I restriction site in the proper orientation to insert the fragment into the BamH I/EcoR I cloning site of pSFF.

### Viruses and cells

Retroviral packaging cell lines ψ-2 (39) and PA12 (42) were used to produce helper-free virions encoding EpoR and gp55 by ping-pong amplification after transfection with retroviral vectors pSFF-EpoRPA11 and pL26K, respectively, as previously described (5, 32). The EpoR-encoding virions were used to infect the interleukin-3 (IL-3) dependent hematopoietic cell line BaF3 (40) to produce BaF3/EpoR cells used in this study. The pL26K retroviral vector encoding gp55 of wild-type SFFV (Lilly-Steeves polycythemic strain) has been described (35). ψ-2, PA12 and mink lung CCL64 cells were maintained in Dulbecco's modified Eagle's Medium, supplemented with 10% fetal bovine serum (FBS). BaF3 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and $5 \times 10^{-5}$ M β-mercaptoethanol with10% WEHI-3 as an IL-3 source. BaF3/EpoR cells were maintained in the same medium with Epo at 0.5

unit/ml (Boeringer-Mannheim, Indianapolis, IN) instead of IL-3. Preparation of passaged virus from spleens was described previously (24).

**Pathogenic Assays**

Helper-free virus encoding wild-type EpoR was mixed with ecotropic helper virus B4 (43) for injection as described previously (24). 4-8 week-old Female NIH/Swiss mice were used for all *in vivo* pathogenic characterization, except for the analysis of *Fv-2* restriction. For that study, DBA/2J ($Fv\text{-}2^s$) and the $Fv\text{-}2^{rr}$ congenic strain D2.R was used (14).

**Proliferation assays**

For $^3$H-Thymidine incorporation assays, spleen cells were collected from the spleens of normal, anemic, and virus-infected mice. Mice were made anemic by subcutaneous injection of phenylhydrazine hydrochloride (Eastman Kodak, Rochester, NY) in phosphate buffered saline (PBS) (Gibco BRL, Gaithersburg, MD) at a dosage of 60 mg/kg for two consecutive days (22). The $^3$H-Thymidine incorporation assay method previously described by Krystal was used with slight modifications (33). Briefly, spleen cells were resuspended at 8 X $10^6$ cells/ml, then aliquoted into a 96 well plate containing serial dilutions of Epo. The cells were then incubated for 22 hrs at 37°C and 5% $CO_2$. Ten µCi of [Methyl-$^3$H]-Thymidine ( 6.7 Ci/mmol, DuPont NEN, Boston, MA) was then added to each well and incubated for 2 hrs as above. The reaction was stopped by addition of trichloracetic acid (TCA) so that the final concentration of TCA in the wells was 10%. Adherent cells were dissolved in 0.1M NaOH and the total precipitate was washed three times in 10% TCA. The pellets were counted in a gamma counter. Colony assays to detect CFU-E were performed using standard methods (26). Briefly, bone marrow cells taken from femurs and tibias of control

and virus-infected mice were suspended in alpha medium (Gibco Laboratories, Grand Island, NY), sedimented by centrifugation and resuspended in semisolid medium containing methyl cellulose purchased from Terry Fox Laboratory (catalog number HCC-3230, Vancouver, BC) without Epo or in the presence of 0.5 unit/ml Epo. The cell suspension was plated in triplicate in 35 mm Lux dishes (cat. #5221, Nunc Inc., Naperville, IL) at 2 X $10^5$ cells/plate. After incubation for three days in a humidified atmosphere containing 5% $CO_2$ at 37°C the plates were examined for hemoglobinized bursts. For factor-independent growth assays BaF3 cells or BaF3/EpoR cells were infected with passaged virus for 2 hrs at 37°C in the presence of 8 μg/ml polybrene. The cells were pelleted by centrifugation and resuspended in medium containing growth factor for 48 hrs. The cells were then sedimented by centrifugation and washed twice with PBS and resuspended in complete medium without growth factors to allow for selection of factor-independent cells.

**Long term bone marrow culture**

Long term bone marrow (LTBM) cultures were made using a modification of the original Dexter LTBM culture method (13) that allowed for an extended period of erythropoiesis without addition of Epo to the cultures (41). Bone marrow cells taken from the tibias and femurs of 6-8 week-old female NIH/Swiss control and virus-infected mice were gently suspended in Iscove's modified Dulbecco's medium (IMDM, JRH Biosciences), sedimented by low-speed centrifugation and resuspended in IMDM supplemented with 25% horse serum (ICN Biochemicals, Costa Mesa, CA), 125 U/ ml penicillin G, 125 μg/ml streptomycin and 0.5 μg/ml hydrocortisone succinate (Sigma, St. Louis, MO). The combined bone marrow from three mice was plated into three 6-well tissue culture plates (Becton Dickenson, Lincoln Park, NJ) in a volume of 3.0 ml/well.

After incubation for one week at 37°C in a humidified atmosphere containing 5% $CO_2$ the supernatant was completely removed and the cultures were reseeded with fresh bone marrow suspensions prepared as described above. The cultures were subsequently fed weekly by removing half of the medium and replacing it with fresh medium.

**Western, northern blots**

For western blotting, cell lysates were immunoprecipitated with an anti-Friend Leukemia virus gp70 antiserum that crossreacts with *envs* encoded by SFFV and MCFs (21, 50, 51) and electrophoresed on polyacrlyamide gels under reducing conditions in the presence of 1% sodium dodecyl sulfate. The proteins were then transferred to nitrocellulose membranes , incubated with the same antibody and detected with [$^{125}$ I] protein A as described previously (21, 35). For northern blotting, total RNA was extracted from cells and spleens by the guanidinium thiocyante-cesium chloride procedure (8, 52), electrophoresed and transferred to a Nytran membrane (Schleicher and Schuell, Keene, N.H.) and hybridized with a probe recognizing MCF-related nucleic acid sequences as previously described (53).

# Results

## Origin of a new virus that causes rapid-onset splenomegaly and polycythemia

This work was serrendipitously initiated by injecting mice with a weakly pathogenic clone of ecotropic MuLV (43) plus a helper-free preparation of a retroviral vector (pSFF-EpoRPA11) that encodes wild-type mouse EpoR (see Materials and Methods). Although wild-type EpoR is apparently nonpathogenic, certain EpoR mutations can constitutively activate its mitogenic signaling to cause leukemia (38, 61). We reasoned that such activating mutations might occur *in vivo*, and that pathogenic selection might produce a maximally mitogenic form of EpoR that would help to define the properties of this hemopoietin receptor family (3, 11). Among eleven NIH/Swiss mice injected with this virus, three had massive splenomegaly by 62-83 days , whereas the five mice injected only with helper virus had only slightly enlarged spleens (see Table 1). Passaged virus prepared from the mouse that had splenomegaly and polycythemia at 67 days post-infection reproducibly caused these symptoms within 2-3 wks in secondary recipients (see Table 1).

## Unexpected properties of the newly-formed pathogenic virus

Using RNA, DNA and protein blotting methods (see Materials and Methods), we were unable to detect any EpoR-encoding virus in the enlarged spleens of the initially infected mouse or in the diseased mice that had been infected with passaged virus. Furthermore, PCR amplifications using total RNA and genomic DNAs from these diseased spleens did not enable us to detect viral-encoded EpoR nucleic acids in significant amounts.

Fig. 1 shows analysis of *env*-related glycoproteins in the original spleen samples from infected mice. The small spleen from a mouse injected only with

helper virus contained a negligible amount of *env* glycoproteins (see lane 1). In contrast, the spleen from the original 67 day mouse with transmissible disease (see Table 1) contained a relatively large concentration of MuLV and/or MCF (see below) gPr90 and gp70 glycoproteins plus traces of smaller components including one with apparent Mr 60,000 (see lane 2). The latter sample did not contain a glycoprotein that coelectrophoresed with gp55 (compare lanes 2 and 3).

**Further characterization of the viral disease**

Although splenic architecture and histology remained normal in mice infected only with helper virus, the grossly enlarged spleens of mice infected with the new pathogenic virus lacked recognizable follicles and appeared to be completely engorged with proliferating erythroblasts and their differentiating progeny (frames B and C, Fig 2). Microscopic examination of fragments from the enlarged spleens revealed the presence of structures typical of erythroid islands (Fig 2, frame I). Benzidine staining confirmed the presence of hemoglobin in the erythroid cells. The livers of these mice also appeared to contain erythropoietic islands (compare frames D to E and F, Fig. 2) and blood from these polycythemic mice contained relatively large proportions of reticulocytes ( Fig. 2, compare frame G to frame H).

Although erythropoiesis in normal adult mice is substantially confined to bone marrows, anemia induces some erythroblast migration to the spleens and livers (22). As shown in Fig 3B, this results in a slight elevation of Epo-dependent cell proliferation by the cultured spleen cells. In contrast, cells from the grossly enlarged spleens of mice infected with the new virus proliferated at a relatively amplified rate in an Epo-independent manner (see Fig 3A).

Bone marrow cells from helper virus-injected control mice and from mice infected with the new virus were plated in semisolid medium, and the plates

were examined three days later for hemoglobin-containing bursts. As shown in Table 2, the burst-forming erythroblasts from control mice were Epo-dependent, whereas those from the mice infected with the new virus were Epo-independent.

Preparations of the new virus that had been passaged once in adult NIH/Swiss mice were tested for their pathogenic activities in DBA/2J (Fv-$2^s$ strain) mice compared with the congenic D2.R (Fv-$2^r$ homozygous) mice. All infected DBA/2J mice developed palpable splenomegaly by 2-3 weeks, whereas the D2.R mice reproducibly did not (see Table 3).

**Effects of the new virus in long term bone marrow cultures**

Long-term bone marrow cultures are generally made in stages by first establishing an adherent layer consisting principally of stromal cells, and by later adding fresh marrow onto this stromal support (13, 41). Whenever marrow cells from mice infected with the new virus were added into cultures that contained uninfected marrow, nonadherent islands of active erythropoiesis formed in relatively large numbers (see Fig 4). This did not occur when we used only bone marrow cells from diseased mice, presumably because the erythroblasts in these mice had migrated *in vivo* to the spleens and livers. Our results suggest that erythroblasts remain viable in the uninfected long-term cultures maintained without Epo, and that their Epo-independent proliferation and differentiation are induced by virus after the cultures are seeded with infected cells. In this way, the virus-induced disease appears to be reproduced in cell cultures.

**Isolation of distinct new viruses that activate EpoR**

BaF3 is a line of interleukin 3 (IL-3) dependent hematopoietic cells whereas BaF3/EpoR is a derivative that expresses EpoR and can grow in either IL-3 or Epo (35). The newly-formed pathogenic virus (see Table 1) and its

progeny that had been passaged in secondary recipient mice were able to convert BaF3/EpoR but not BaF3 cells to factor-independent proliferation, suggesting that the viruses cause mitogenesis by activating EpoR. Thereby, we obtained cell lines that contain the EpoR-activating viruses.

Fig 5 shows a protein immunoblot analysis of *env*-related glycoproteins that were encoded by these pathogenic viruses. In agreement with Fig 1, the spleen from the 67 day mouse that originally developed the disease (see Table 1) contained helper-virus encoded glycoproteins plus a low abundance Mr 60,000 component (Fig 5, lane 1). Factor-independent cell lysates from BaF3 cells infected with passaged virus 1218 contained the Mr 60,000 component plus a minor proportion of a component that comigrated with gp55 (see lane 3 in comparison to the gp55 standard in lane 4). Moreover, after additional *in vivo* passages (passaged virus 429), the virus encoded the gp55 comigrating component but not the Mr 60,000 component (lane 2).

The population of BaF3/EpoR cells that had been analyzed in Fig 5, lane 3 was used to isolate single cell clones by limiting dilution (32), and the analysis of *env* glycoproteins in eleven of the resulting cell clones is shown in Fig 6 (lanes 3-13). The results suggest that the early passage 1218 virus that was used to infect the BaF3/EpoR cells probably contained at least three different viruses that were able to activate EpoR. The most abundant of these viruses encoded a Mr 60,000 glycoprotein that was present in most of the factor independent cell clones, whereas a gp55-encoding virus was evidently present in one cell clone (lane 6). Interestingly, one of the factor-independent cell clones appeared to lack both gp60 and gp55 glycoproteins, and to contain additional components with sizes expected for the gPr90 and gp70 glycoproteins encoded by a replication-competent helper virus (see lane 11). As described below, this virus is an MCF.

Total RNAs that were isolated from the cell clones used in Fig 6 (lanes 6, 11, and 12) and from control cell lines and spleens of normal and infected mice were studied by electrophoretic blot analysis, using a radioactive probe specific for MCF-related nucleic acid sequences (see Fig 7). The results indicate that the diseased spleens and factor independent BaF3/EpoR cells contained MCF-related *env* sequences. These MCF-related RNAs appeared to consist of full-length viral transcripts of approximately 8.0 kilobases plus subgenomic processed *env* mRNAs of approximately 3.5 kilobases (for viruses that encode gPr90 or gp60 components) or 2.5 kilobases (for viruses that encode gp55). The implication is that the new viruses that activate EpoR are members of the MCF and SFFV family.

The results in Fig 7 (lane 6) imply that the factor-independent BaF3/EpoR cells studied in Fig 6 (lane 11) contain an MCF-related virus that encodes glycoproteins with sizes consistent with the gPr90 and gp70 expected of a replication competent MCF. However the idea that replication competent MCFs can activate EpoR has remained controversial (31, 34). Consequently we harvested virus that was released from this cell clone (termed 1218-3F) and used it to infect mink CCL64 fibroblasts at a low multiplicity. As expected for a replication competent MCF, the mink cells all became infected and their growth became inhibited. After two passages in mink cells, the virus retained its ability to convert BaF3/EpoR cells to factor independent proliferation. Thus, this new replication competent MCF appears to be reproducibly able to activate EpoR. These same BaF3/EpoR cells reproducibly remained growth factor-dependent after infection with other MCFs.

## Discussion

### Origin and evolution of a new lineage of MCF and SFFV-related erythroleukemia viruses

This paper documents the origin and rapid pathogenic evolution of a new lineage of SFFV. This new viral lineage initially formed after a long latency in an adult female NIH/Swiss mouse that had been injected with a weakly pathogenic, biologically cloned ecotropic helper MuLV plus a replication-defective virus that encodes wild-type EpoR. Our expectation was that the latter virus might mutate *in vivo* to encode a constitutively active EpoR derivative; and that this virus mutant might cause a leukemia (38). On the contrary, our results suggest that the viral-encoded EpoR gene was eliminated or lost by dilution during *in vivo* replication, and that the pathogenic virus that formed in this mouse was a replication-competent MCF that could activate EpoR to stimulate proliferation and differentiation of infected erythroblasts. Furthermore, this MCF apparently evolved in stages to form increasingly pathogenic derivatives. Before sacrifice at 67 days, the original mouse contained an MCF derivative virus that encodes a gp60 (see Figs 1 and 5, lanes 2 and 1, respectively). After passage of this virus mixture into a secondary recipient , virus formed that encodes a gp55 (see Figs 5 and 6); and this gp55-encoding SFFV then rapidly overgrew its progenitors during subsequent *in vivo* passage.

These results have several major implications for our understanding of murine retroviral leukemogenesis. First, they support previous indications that certain replication-competent MCFs can activate EpoR and /or perhaps other members of this hemopoietin receptor superfamily (34, 56). Further studies will be required to determine whether our new replication-competent MCF differs substantially from the MCFs that we previously found to be unable or less able to

activate EpoR (31). Second, our results strongly support the idea that SFFVs can derive from MCFs. Our evidence that the intermediates in this derivation are all able to activate EpoR is compatible with the rapid succession of pathogenic viruses that we have detected. If the SFFV intermediates were nonpathogenic we would not have been able to detect or to isolate them, and the formation of an active SFFV would have been much less likely. Thus, our results support the conclusion that SFFV formation from MCFs may be a surprisingly facile and high probability process. In this process, successive intermediates are probably increasingly pathogenic. Third, the fact that we can readily isolate viruses that activate EpoR has enabled us to identify the intermediates in evolution of an MCF into an SFFV. Complete characterization of all intermediates may eventually provide a detailed understanding of this lineage of viruses. Fourth, our results suggest that it may now be relatively easy to isolate new lineages of viruses that activate EpoR or other hemopoietin receptors. Although we initiated this study using a retroviral vector that encodes EpoR, it seems unlikely that the EpoR sequences were essential. Rather, we would anticipate that any vector that could recombine with endogenously inherited dualtropic *env* sequences might suffice to initiate MCF formation and subsequent SFFV evolution. In this context, we wish to emphasize that the pSFF vector used for this work was initially derived from a 5.9 kilobase SFFV molecular clone that had large deletions in its *gag* and *pol* regions (5). The vector construction involved deletion of *pol*-related and MCF-related *env* sequences, and creation of a multicloning site. Additional studies will be required to learn how this vector might have participated in formation of the replication competent MCF, and whether other vectors would also function in this process. Clearly, mice harbor in their genomes nonfunctional retroviral sequences that can readily recombine to generate highly pathogenic viruses.

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**Table 1. Pathogenicity of SFFV mutants in susceptible mice[1]**

| Virus | day post-infection | spleen weight (g) [Hematocrit] |
|---|---|---|
| SFF-EpoRPA11 | 32 | 0.18 [43], 0.16 [42] |
| | 62 | 2.98 [ND] [2] |
| | 67 | 2.81 [75] [3] |
| | 83 | 0.2 [47], 0.2 [47], 0.2 [46], 0.13 [43], 0.97 [38], 0.14 [39] |
| passaged virus [4] | 18 | 3.4 [74] [3], ND [5] |
| passaged virus [4] | 26-31 | 4.67 [50], 3.64 [82], 2.1 [59] |
| Control [6] | 32 | 0.15 [42] |
| | 67 | 0.12 [46] |
| | 83 | 0.27 [50], 0.29 [45], 0.14 [43] |

[1] NIH/Swiss mice 4-6 wks old

[2] ND, not done

[3] spleen lysates from these animals examined by western blot (see Figs 1 and 5)

[4] spleen from the day 67 mouse was used to prepare passaged virus as previously described (24).

[5] dead mouse with massive hepatosplenomegaly

[6] ecotropic helper virus-injected only

**Table 2. Bone marrow colony assay**

| Bone marrow | 3-day bursts (CFU-E) | |
|---|---|---|
| | -Epo | +Epo |
| infected mouse | 30, 21, 20 | 23, 34, 14 |
| helper injected control | 0, 0, (1*) | 14, 13 |

* possible burst

**Table 3 . Splenomegaly and polycythemia caused by passaged virus in *Fv*-2 restricted and susceptible mice**

| Mice | Mice with splenomegaly* | | | |
|---|---|---|---|---|
| | 813 virus preparation | | | |
| | d21 | d29 | d35 | d42 |
| DBA/2 | 0/3 | 2/3 | 3/3 | 3/3 |
| D2.R | 0/3 | 0/3 | 0/3 | 0/3 |
| | 1118 virus preparation | | | |
| | d14 | d20 | d27 | |
| DBA/2 | 0/3 | 3/3 | 3/3 | |
| D2.R | 0/3 | 0/3 | 0/3 | |

* d, day post-injection

**Figure 1. Detection of *env* glycoproteins in spleen lysates of infected mice by immunoblot.** Lysates from spleen cells or cell lines were immunoprecipitated, run on electrophoresis gels under reducing conditions and transferred to nitrocellulose membranes. The membranes were incubated with anti-*env* antibody followed by [$^{125}$ I] Protein A. Lane 1, spleen lysate from a control animal infected with ecotropic helper virus; lane 2, spleen lysate from infected day 67 mouse with massive splenomegaly (see Table 1); lane 3, cell lysate from IP/IR erythroleukemic cell line containing helper-free SFFV (54).

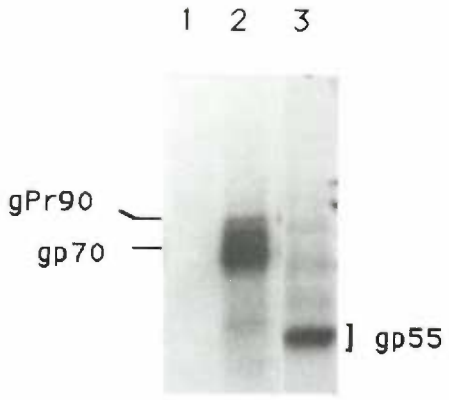

1 2 3
gPr90
gp70
gp55

**Figure 2. Pathology consistent with extramedullary erythropoiesis in infected mice.** A-F are paraffin-embedded tissue sections stained with hematoxylin and eosin. (A) normal spleen, magnification, 214x; (B and C) virus-infected spleen, magnifications, 428x and 857x, respectively, showing loss of normal splenic architecture due to proliferating erythroid precursors; (D) liver from helper virus-injected control mouse, magnification 214x; (E and F) liver from an infected mouse showing erythropoietic foci (arrow), magnifications 428x and 857x, respectively; G and H are peripheral blood smears stained with new methylene blue to detect reticulocytes, magnification, 857x; (G) helper virus-injected control mouse; (H) infected mouse showing a striking increase in reticulocytes; (I) A cytocentrifuged cell suspension from an infected mouse spleen showing an erythroid island composed of macrophages attached to erythroid precursors, magnification, 857x. A benzidine stain confirmed the presence of hemoglobin in the immature erythroid cells.

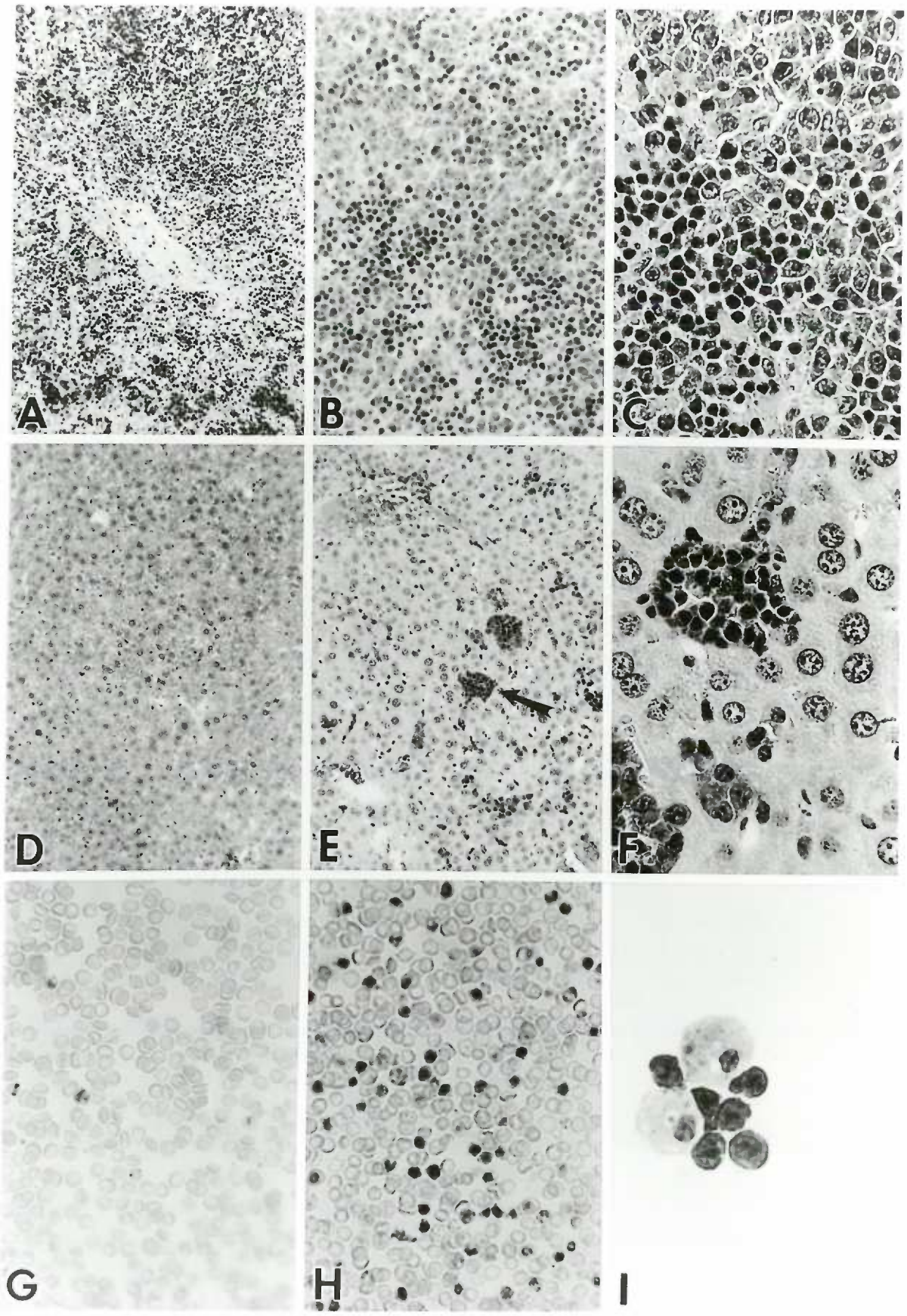
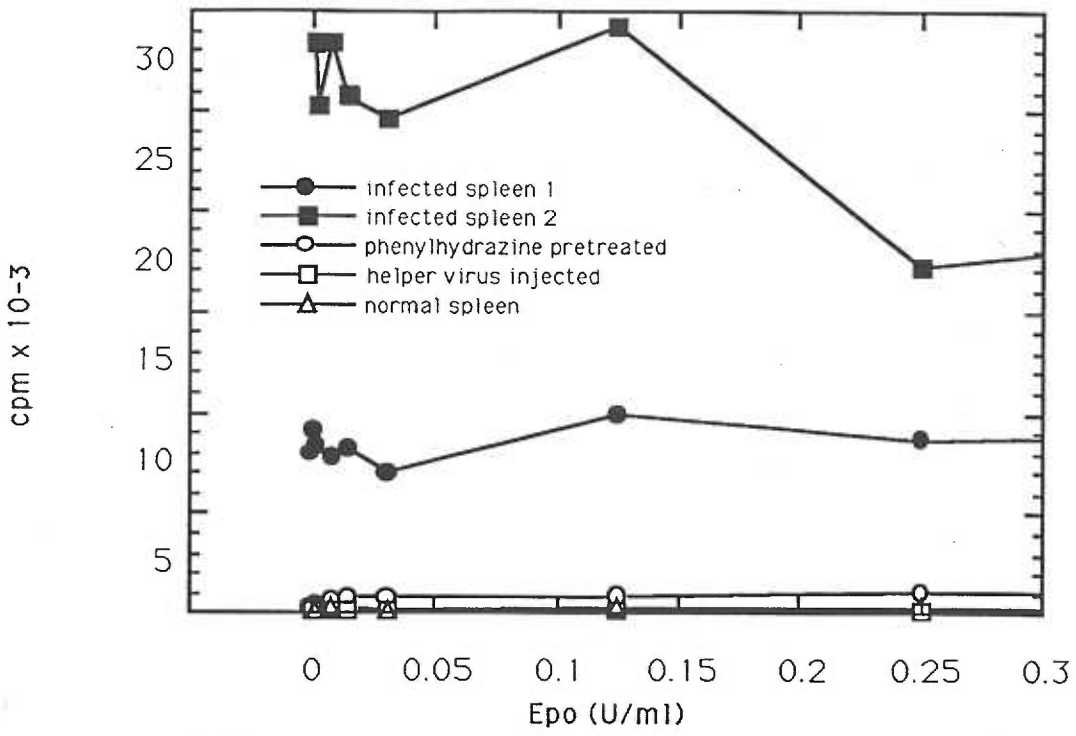

A
B
C
D
E
F
G
H
I

**Figure 3. Proliferation assay of spleen cells from mice demonstrating amplified Epo-independent growth.** Spleen cells from control and infected animals were incubated in serial dilutions of Epo for 22 hrs followed by addition of $^{3}$H-Thymidine for 2 hrs. The cells were then assayed for $^{3}$H-Thymidine incorporation (see Materials and Methods).

A


30
25
20
15
10
5
cpm x 10-3
infected spleen 1
infected spleen 2
phenylhydrazine pretreated
helper virus injected
normal spleen
0
0.05
0.1
0.15
0.2
0.25
0.3
Epo (U/ml)

B

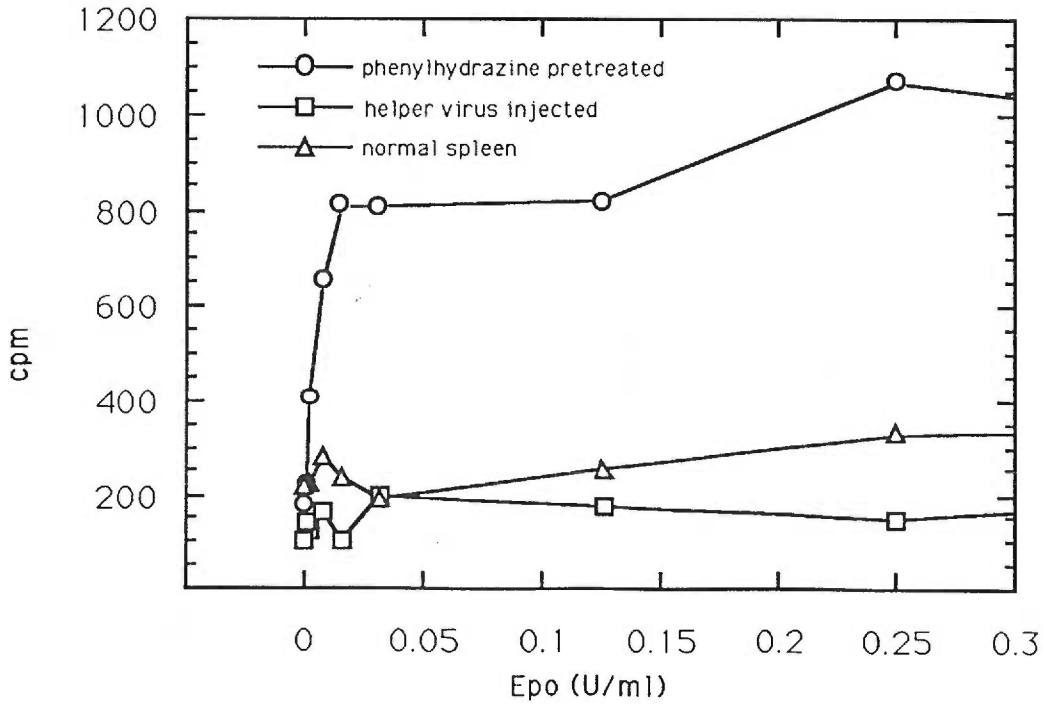

1200
1000
800
600
400
200
cpm
phenylhydrazine pretreated
helper virus injected
normal spleen
0
0.05
0.1
0.15
0.2
0.25
0.3
Epo (U/ml)

**Figure 4. Cytospin preparation of non-adherent cells in long term bone marrow cultures of normal NIH/Swiss mice after addition of infected bone marrow.** Normal long term bone marrow cultures (LTBM) were recharged with normal bone marrow or with bone marrow from mice infected with the new virus. Non-adherent cells were collected from the medium each week and examined. The samples shown are cytospin preparations taken from the cultures four weeks after recharge and stained with Wright-Giemsa, magnification, 428x. A, normal LTBM culture recharged with normal bone marrow; B, normal LTBM culture recharged with bone marrow from an infected mouse. Benzidine staining for hemoglobin confirmed that the cells surrounding the central macrophages were erythroid.

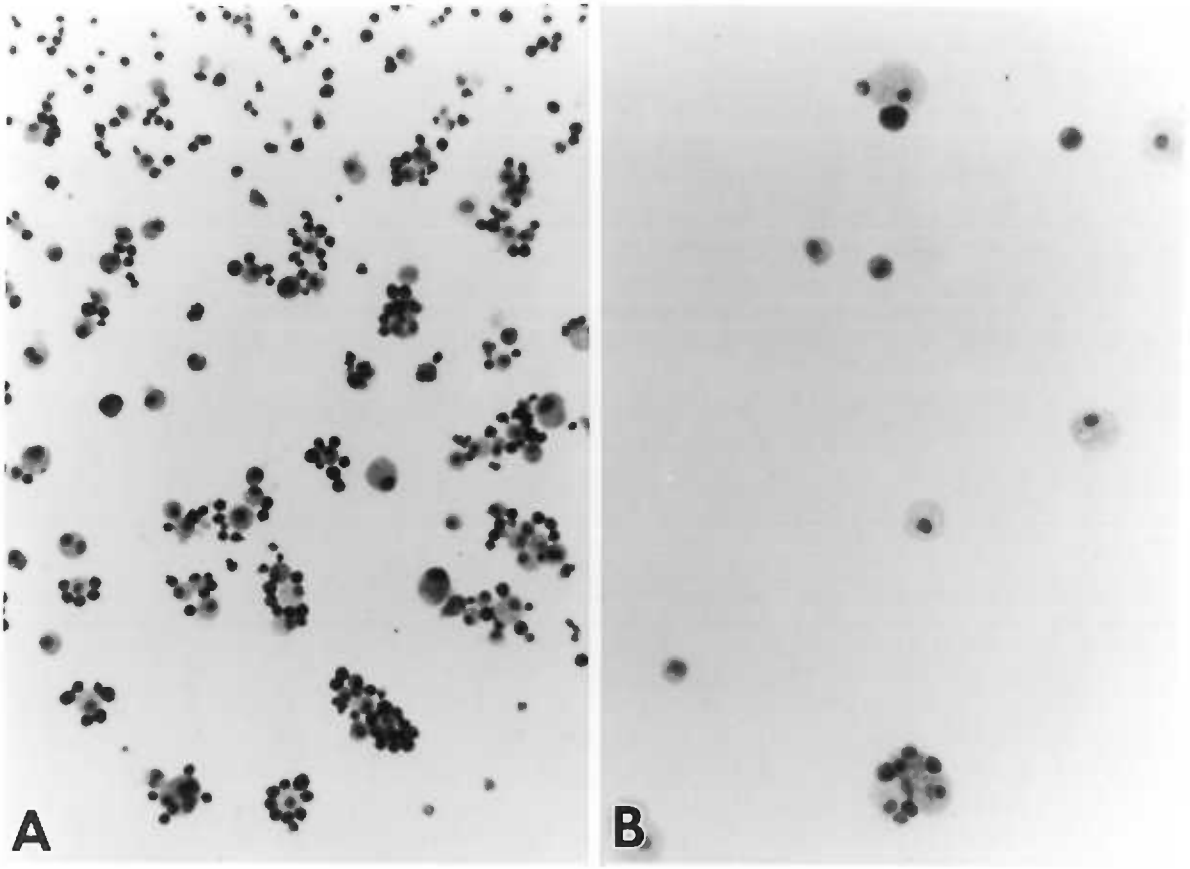

A
B

**Figure 5. *Env* glycoproteins in growth factor-independent BaF3/EpoR cells infected with passaged virus.** Protein immunoblot methods were as described for Fig. 1. The original spleen lysate from the infected day 67 mouse is shown in lane 1; BaF3/EpoR/passaged virus 429, lane 2; BaF3/EpoR/passaged virus 1218, lane 3; BaF3/EpoR/SFFV wild-type, lane 4. BaF3 cells express a Mr 85,000 protein (17, 31).

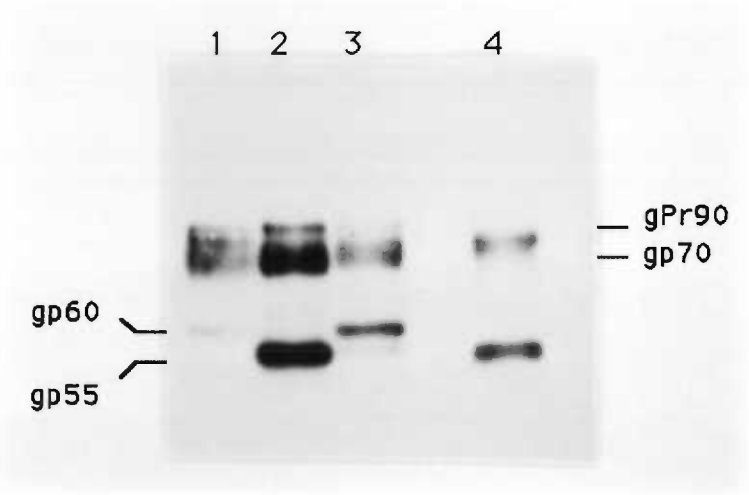

1
2
3
4
gPr90
gp70
gp60
gp55

**Figure 6. *Env* glycoproteins in growth factor-independent clones of BaF3/EpoR cells infected with passaged virus.** Proteins were prepared and blotted as described in Fig. 1. BaF3/EpoR cells infected with wild-type SFFV, lane 1; BaF3/EpoR cells infected with passaged virus 1218 (from which 11 clones analyzed in this blot were obtained by limiting dilution), lane 2; clone 5S, lane 3; clone 4S, Lane 4; clone 3S, lane 5; clone 2S, lane 6; clone 1S, lane 7; clone 6F, lane 8; clone 5F, lane 9; clone 4F, lane 10, clone 3F, lane 11; clone 2F, lane 12; clone 1F, lane 13.

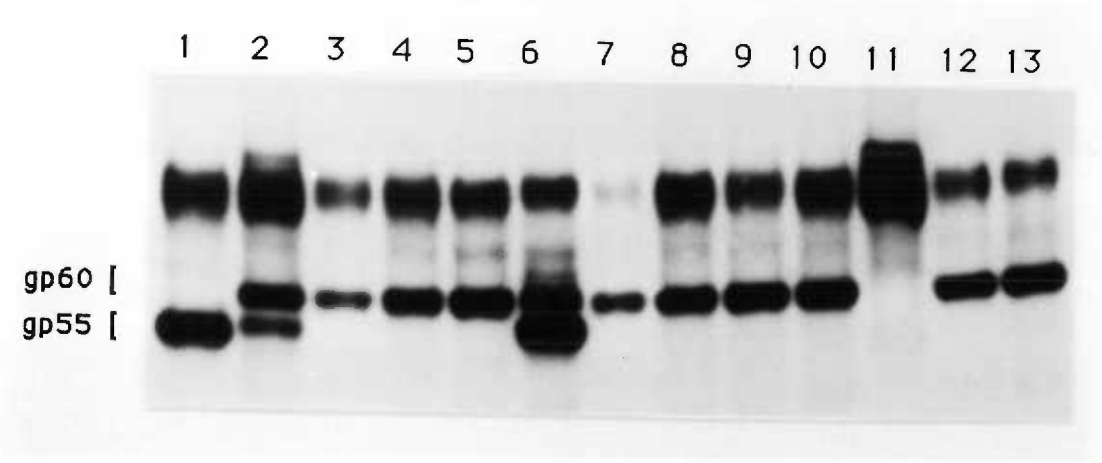
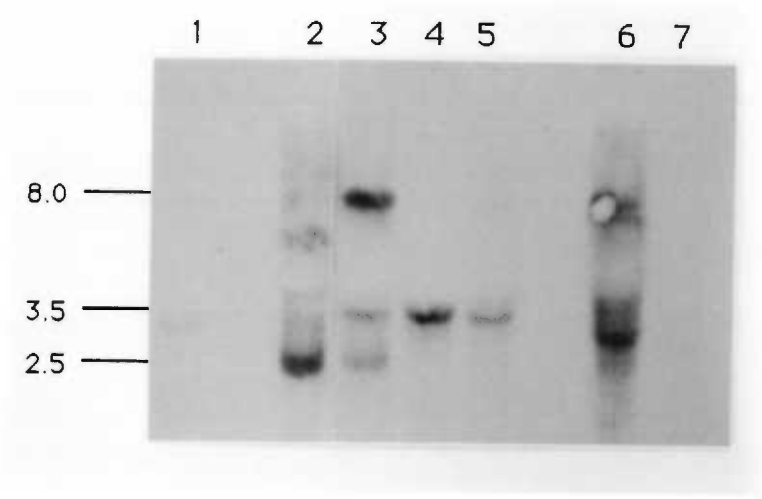

1 2 3 4 5 6 7 8 9 10 11 12 13
gp60 [
gp55 [

**Figure 7. Detection of MCF-related nucleic acids in mouse spleen and three growth factor-independent clones containing passaged virus by northern blot.** The total RNA of three of the clones shown in Fig. 6 and RNA isolated from the spleen of the day 67 mouse were hybridized with radiolabeled probe recognizing MCF-related nucleic acid sequences. BaF3, lane 1; BaF3/EpoR/SFFV, lane 2; BaF3/EpoR/(1218-2S), lane 3; BaF3/EpoR/(1218-2F), lane 4; BaF3/EpoR/(1218-3F), lane 5; infected day 67 mouse spleen, lane 6; normal mouse spleen, lane 7. Sizes are listed to the left of the figure in kilobase pairs.


1
2
3
4
5
6
7
8.0
3.5
2.5

Manuscript #5

# A Friend Virus Mutant Encodes a Small Glycoprotein that Activates Erythropoietin Receptors

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## Abstract

The PvuΔ mutant of Friend spleen focus-forming virus encodes the smallest *env* glycoprotein (apparent Mr 41,000) that is known to activate erythropoietin receptors. *In vivo,* PvuΔ causes rapid erythroblastosis and development of erythroleukemia. We isolated two leukemic cell lines that contain PvuΔ; both synthesize hemoglobin in response to dimethylsulfoxide. The PvuΔ *env* gene contains a 204 base deletion in the ecotropic-specific region.

Recent studies have indicated that the *env* glycoprotein (gp55) encoded by Friend spleen focus-forming virus binds to erythropoietin receptors (EpoR) to stimulate erythroblastosis (11, 28). Although only a small percentage (3-5%) of gp55 is processed to plasma membranes (6), it appears that the mitogenic interaction occurs exclusively on cell surfaces (5). In addition, gp55 and erythropoietin bind to nearby but nonoverlapping sites on EpoR (2, 5, 8).

The site(s) on gp55 necessary for EpoR binding and activation remain uncertain. Different studies have suggested the dualtropic domain, the ecotropic domain, or the carboxyl terminal hydrophobic membrane anchor region may be critical (1, 4, 8-10, 12, 23-25, 29). One approach toward resolving this matter is to identify substantially altered forms of gp55 that retain ability to activate EpoR. For example, a gp42 glycoprotein encoded by the BB6 *env* mutant of SFFV activates EpoR, yet it contains a large deletion in its ecotropic region (8, 13). An EpoR-gp55 fusion protein that lacks dualtropic *env* sequences can activate wild-type EpoR, perhaps by a mechanism that involves the remaining *env* sequences (20). Although there had been controversy regarding initial evidence that *env* glycoproteins of dualtropic murine retroviruses (MCFs) can activate EpoR (8, 9), we recently isolated a novel replication-competent MCF that reproducibly has this activity ( M. Hoatlin, unpublished results); these results imply that dualtropic *env* sequences must be necessary for activation of EpoR.

Recently, during another investigation we isolated an SFFV mutant, PvuΔ, that activates EpoR in a cell culture system and encodes a gp41 glycoprotein (5). Since this is the smallest SFFV *env* glycoprotein known to activate EpoR, we have further analyzed its biological and structural properties. When the PvuΔ virus was coinjected with helper virus into mice, spleen foci and mild erythrocytosis occurred by ten days post-infection (see Table 1). By 59 days, the mice developed mild to severe splenomegaly that correlated with polycythemia. Disease

diminished by 80 days, with the exception of one mouse that had splenomegaly and severe anemia. Anemia is generally associated with the transplantable stage of Friend disease, regardless of the SFFV strain (22). The spleen of this diseased 80-day mouse was found by protein immunoblotting to contain a gp41 that coelectrophoresed with the original gp41 (compare Fig. 1, lanes 3 and 6). Two cell lines were also derived by intraperitoneal transplantation of cells from this spleen into sublethally irradiated secondary recipients and growth of omental tumors (21, 26). Protein immunoblot analysis of the resulting samples established that they both synthesized gp41 (Fig. 1, lanes 4 and 5). There were, therefore, no obvious *env* gene modifications that occurred during prolonged pathogenic selection of PvuΔ virus *in vivo*. The two cell lines are morphologically similar to standard Friend erythroleukemia cell lines (see Fig. 2). Approximately 2% of the cells stain positively for hemoglobin with benzidine; and this percentage increases to approximately 10% following exposure to dimethylsulfoxide for 72-96 hours.

Although BaF3 cells require interleukin-3 (14), derivative cells that express EpoR (i.e., BaF3/EpoR cells) survive and grow in either interleukin-3 or erythropoietin (11). Furthermore, SFFV or PvuΔ both convert BaF3/EpoR cells but not BaF3 cells to factor independence (5, 11). Total RNA was isolated from these factor-independent cells by standard methods, and it was analyzed by electrophoresis and blotting using dualtropic-specific *env* sequences as a probe (see Fig. 3). The subgenomic *env* messenger RNA of PvuΔ (lane 3), was smaller than that of wild-type SFFV (lane 2), suggesting a possible partial deletion in the PvuΔ *env* gene. The PvuΔ *env* gene was then amplified from total RNA by reverse transcriptase-PCR (7). The PvuΔ DNA was cloned and sequenced; and the sequence is compared with that of the wild-type SFFV *env* in Fig 4. The PvuΔ sequence is substantially smaller due to a 204 base deletion in the ecotropic-

specific region between nucleotides 1149 and 1354 compared to the wild-type *env* (27). The PvuΔ sequence has a two amino acid insertion at amino acid 194 due to a linker insertion that has been described previously (10).

These sequence results and pathologic studies strongly suggest that the *env* gene sequences of PvuΔ are sufficient for EpoR activation and for induction of erythroleukemia *in vivo*. Consequently, the deleted ecotropic-specific region of gp55 cannot be essential for pathogenic activity. The previously- analyzed BB6 SFFV mutant has a large overlapping deletion between nucleotides 1221 and 1380 (13). In addition, PvuΔ has several other amino acid sequence changes compared to SFFV (see Fig. 4). These results agree with previous evidence that the dualtropic and membrane anchor regions of gp55 may be necessary for SFFV pathogenesis.

Comparison of the different glycoproteins that activate EpoR allows several important conclusions. The fact that at least some MCFs can activate EpoR, whereas ecotropic glycoproteins cannot, would seem to establish a critical role for the dualtropic region, consistent with mutagenesis data (10). However, MCFs do not cause rapid extensive erythroblastosis *in vivo* . The implication is that the ecotropic sequences present in MCF *env* glycoproteins but absent from gp55 must have a negative modulatory role in disease. These sequence changes are not involved in the primary pathogenic mechanism ( i.e., activation of EpoR). However, their removal from gp55 increases the inherent pathogenic potential of the *env* glycoprotein. The sequence deletions described here and in the BB6 *env* mutant further eliminate some of the ecotropic sequences without eliminating pathogenesis. The hypothesis that these ecotropic sequences can serve as a negative control region is compatible with the observation that the BB6 *env* deletion overcomes the reistance to Friend virus-induced erythroblastosis that occurs in *Fv-2*$^{r}$ homozygous mice.

## Acknowledgments

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**Table 1. Pathogenicity of SFFV mutant PvuΔ in susceptible mice [1]**

| Virus | day post-infection | spleen weight (g) [Hematocrit] |
|---|---|---|
| PvuΔ | 10 | 0.11 [49] [2], 0.09 [49] [2] |
| | 59 | 0.12 [40], 1.8 [70], 0.14 [45], 0.3 [48], 0.36 [48], 1.0 [ND][3] |
| | 80 | 0.18 [40], 1.7 [14][4, 5, 6], 0.12 [46], 0.12 [47], 0.12 [48] |
| Passaged Virus[7] | 30 | 0.27 [46], 0.38 [53], 0.17 [45], 0.11 [50], 1.0 [85] |
| | 36 | 1.7 [ND] |
| | 47 | 2.4 [47], 3.4 [66], 1.1 [70], 1.2 [44] |
| Control [8] | 10 | 0.14 [46] |
| | 59 | 0.12 [40] |

[1] NIH/Swiss mice 4-6 wks old

[2] At least 30 foci observed on spleens after fixing in Bouin's solution as described previously (8).

[3] ND, not done

[4] spleen lysates from this animal examined by western blot (see Fig. 1)

[5] spleen cells from this animal were injected intraperitoneally into sublethally irradiated mice to develop the cell lines described in the text.

[6] passaged virus was prepared from this spleen.

[7] passsaged virus was prepared by homogenizing infected spleens in 10 volumes of lysate solution (0.02 M Tris-HCl ,pH 7.4/0.1 M NaCl/ 0.001 M EDTA) and centrifuging at 10,000 x g for 15 min. The supernatant was then passed through a 0.45 μ filter before injection.

[8] ecotropic helper virus-injected control

**Figure 1. Protein immunoblot analysis of PvuΔ-encoded *env* glycoprotein .** Cell lysates were immunoprecipitated with an anti-Friend Leukemia virus gp70 antiserum (6, 15, 16) and electrophoresed on polyacrlyamide gels under reducing conditions in the presence of 1% sodium dodecyl sulfate. The proteins were then transferred to nitrocellulose membranes, incubated with the same antibody and detected with [$^{125}$ I] protein A as described previously (6, 10). BaF3/EpoR/PvuΔ (5), BaF3/EpoR and BaF3/EpoR/SFFV have been described (11). BaF3/EpoR, lane 1; BaF3/EpoR/SFFV, lane 2; BaF3/EpoR/PvuΔ, lane 3; cell line 46NIH7PvuΔ, lane 4; cell line 56NIH7PvuΔ, lane 5; spleen lysate from 80-day mouse used to produce cell lines (see Table 1), lane 6. Lysates of BaF3/EpoR negative control cells express an endogenous *env*-related glycoprotein of approximate Mr 85,000. Sizes of molecular mass standards are indicated in kDa on the right.

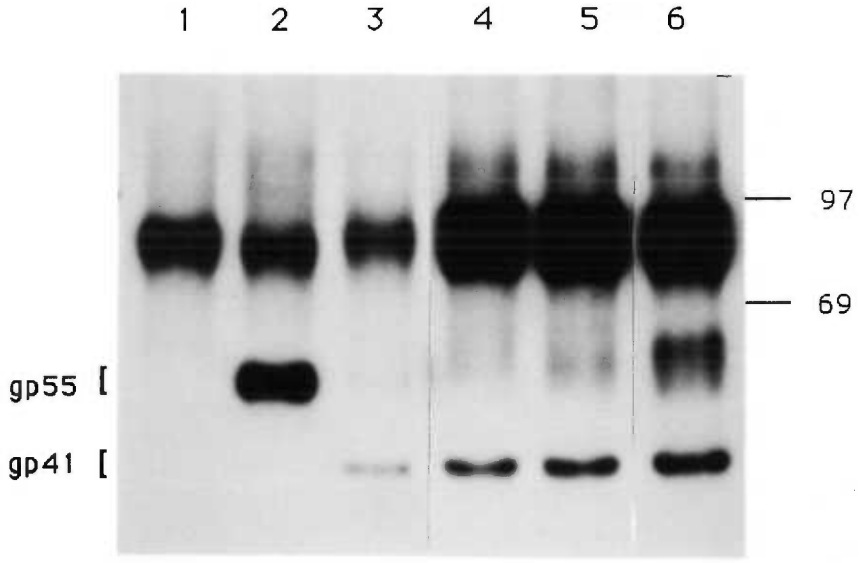
1
2
3
4
5
6
97
69
gp55
gp41

**Figure 2. Growth factor-independent PvuΔ cell lines have erythroid morphology.** Two cell lines were produced from the original 80-day PvuΔ-injected mouse (see Table 1) by intraperitoneal injection of the infected spleen cells into sublethally-irradiated recipient mice. Cells from omental tumors that developed grew without growth factor and expressed PvuΔ-encoded *env* glycoprotein (see Fig 1, lanes 4 and 5). Cytospin preparations from each cell line were treated with Wright's stain (magnification 857x). (A), 46NIH7PvuΔ; (B), 56NIH7PvuΔ; (C) IP/IR erythroleukemia cell line (22). Approximately 2% of the cells in each cell line stain positively for hemoglobin with benzidine. Following exposure to 1%-2% dimethylsulfoxide for 72-96 hours this percentage increases to approximately 10%.

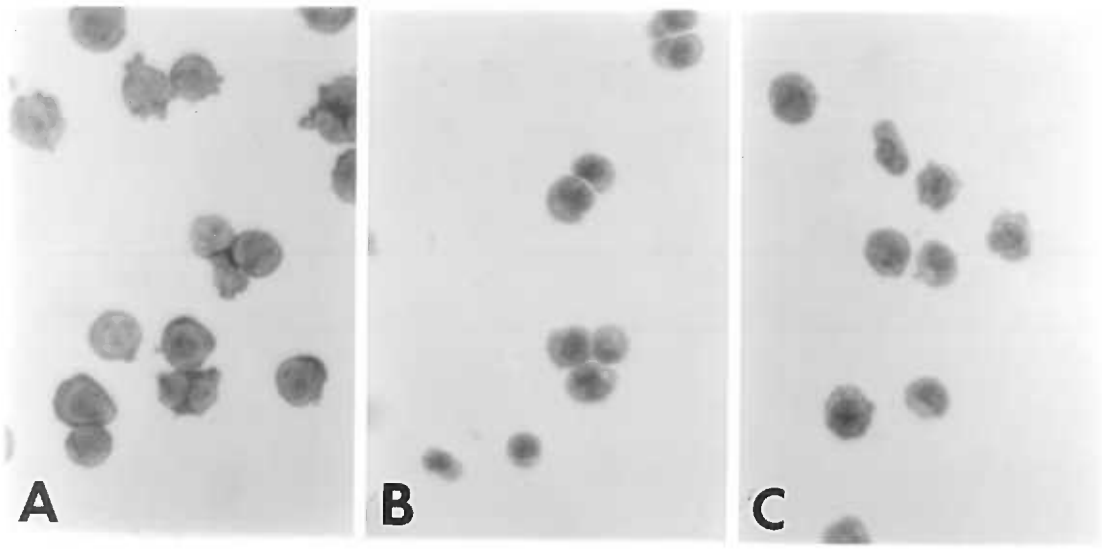
A
B
C

**Figure 3. Northern blot analysis of PvuΔ RNA.** Total RNA was extracted from factor-independent BaF3/EpoR/PvuΔ cells by the guanidinium thiocyante-cesium chloride procedure (3, 17), electrophoresed, transferred to a Nytran membrane and hybridized with a probe recognizing MCF-related nucleic acid sequences as previously described (19). BaF3/EpoR, lane 1; BaF3/EpoR/SFFV, lane 2; BaF3/EpoR/PvuΔ, lane 3.

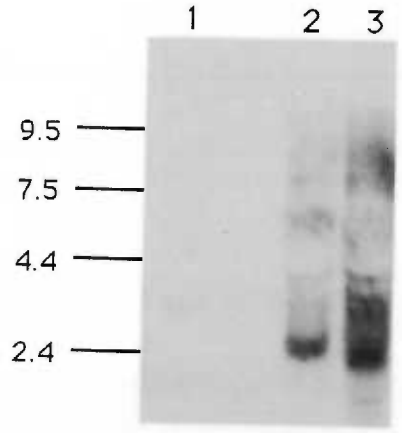
1
2
3
9.5
7.5
4.4
2.4

**Figure 4. Sequence of PvuΔ *env* compared to that of SFFV.** Amplification of *env* genes from total RNA was done by reverse transcriptase-PCR (7) using a Thermolyne Temptronic thermocycler (Dubuque, IA) and reagents purchased from Cetus Corp (Emeryville, CA). Standard buffers and reagent concentrations were used as recommended by Cetus for PCR. The PCR profile used was 30 cycles consisting of a 95°C 1.0 min., 55°C 1.0 min., 72°C 2.0 min. pattern. This was followed by a 5 min. extension at 72°C. Primers used were (5' to 3') CGT**GGATCC**ACGCCGCTCACGT and CGTTACAGCG**GGATCC**GGCTAAGC (positions 162-184 and 1619-1642 relative to Wolff and Ruscetti numbering for SFFV (27)). The residues represented in bold type form internal BamH I sites. The amplified products were cloned using the TA cloning vector system (Invitrogen, San Diego, CA) as recommended by the manufacturer. The DNA sequence of the cloned material was subsequently determined by the dideoxy DNA sequencing method (18). The sequence of SFFV (27), top line, is compared to the sequence of PvuΔ shown on the bottom line.

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  1 MEGPASSKPLKDKTNPWGPLIILGILIRAGVSVQLDSPHQVSNVTWRVTN 50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 MEGPASSKPLKDKTNPWGPLIILGILIRAGVSVQLDSPHQVSNVTWRVTN 50

 51 LMTGQTANATSLLGTMTEAFPKLYFDLCDLMGDDWDETGLGCRTPGGRKR 100
    ||||||||||||||||||||||||||||||:||||||||||||||||||||
 51 LMTGQTANATSLLGTMTEAFPKLYFDLCDLIGDDWDETGLGCRTPGGRKR 100

101 ARTFDFYVCPGHTVPTGCGGPREGYCGKWGCETTGQAYWKPSSSWDLISL 150
    ||||||||||||||||||||||||||||||||.||||||||||||||||||
101 ARTFDFYVCPGHTVPTGCGGPREGYCGKWGCETAGQAYWKPSSSWDLISL 150

151 KRGNTPKDQGPCYDSSVSSGVLGATPGGRCNPLVLEFTDAGRKA..SWDA 198
    ||||||||||||||||||||||||||||||||||||||||||  ||||
151 KRGNTPKDQGPCYDSSVSSGVLGATPGGRCNPLVLEFTDAGRKASSSWDA 200

199 PKVWGLRLYRSTGTDPVTRFSLTRQVLDIGPRVPIGSNPVTTDQLPLSRP 248
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 PKVWGLRLYRSTGTDPVTRFSLTRQVLDIGPRVPIGSNPVTTDQLPLSRP 250

249 VQTMPPRPLQPPPPGAASIVPETAPPPQQPGAGDRLLNLVDGAYQALNLT 298
    ||||  ||||||||||||||||||||||||||||||||||||||||||||
251 VQTMLPRPLQPPPPGAASIVPETAPPPQQPGAGDRLLNLVDGAYQALNLT 300

299 NPDKIQECWLCLVSGPPYYEGVVVLGTYFNHTIALKEKCCFYADHTGLVR 348
    |||||||||
301 NPDKIQECW..................................... 309

349 DSMAKLRKRLTQRQKLFESSRGWFEGSSNRSPWFTTLISAIMGSLIILLL 398
                                |||||||||||||||||||||||
310 ........................SNRSPWFTTLISAIMGSLIILLL 332

399 LLILLIWTLYS* 410
    ||||||||||||
333 LLILLIWTLYS* 344
```

Manuscript #6

# Activation of Erythropoietin Receptors by the Friend Virus-encoded Glycoprotein (gp55) Involves Cytoplasmic Regulatory Factors

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running title: Viral Activation of Erythropoietin Receptors

## Abstract

The Friend virus-encoded membrane glycoprotein (gp55) activates erythropoietin receptors (mEpoR) only in mice that have the *Fv-2*$^{s}$ susceptibility gene, but not in *Fv-2*$^{rr}$ homozygotes or in other species. The *Fv-2* and EpoR genes are unlinked. Previous studies have shown that certain truncated derivatives of gp55 (e.g., gp42 encoded by the BB6 viral mutant) can overcome *Fv-2*$^{rr}$ resistance, establishing that this resistance is not absolute and suggesting that it occurs at the level of EpoR activation. To study factors that may contribute to resistance of non-murine species, we expressed human EpoR (hEpoR) in mouse cells (*Fv-2*$^{s}$ cells) and analyzed activation by Friend viral glycoproteins. Although hEpoR can be activated by erythropoietin in mouse cells, it is resistant to activation by gp55. Surprisingly, hEpoR is easily activated in these cells by the same truncated derivatives that overcome *Fv-2*$^{rr}$ resistance. Therefore, hEpoR can interact with gp55-related glycoproteins and we infer that its resistance to activation by gp55 may be influenced by proteins other than hEpoR that are encoded by endogenous *Fv-2* genes. Thus, hEpoR in *Fv-2*$^{s}$ cells appears to be functionally similar to mEpoR in *Fv-2*$^{rr}$ cells. This cell culture model provides an approach for understanding the roles of regulatory subunits in hematopoietin receptor function and participation of the receptors in host resistance to an oncogenic protein.

## Introduction

Spleen focus- forming virus (SFFV), the replication-defective component of the Friend viral complex, encodes a gp55 glycoprotein that activates mEpoR (23, 35) and causes mitogenic stimulation of erythroblasts in infected susceptible animals (13, 18). Recent work has suggested that the mitogenic EpoR-gp55 interaction occurs exclusively on cell surfaces (5, 11). Mice homozygous for the *Fv-2* $^{r}$ gene are resistant to the erythroblastosis caused by Friend SFFV, although they are susceptible to all other retroviral diseases (34, 40). *Epor* and *Fv-2* are unlinked (4, 16, 24).

Murine EpoR is a 507 amino acid protein with a single transmembrane domain (7). The human homologue contains 508 amino acids (17). The EpoR extracellular domain has several structural features in common with other members of the recently identified and expanding hemopoietin receptor superfamily: four cysteine residues in conserved positions in the N-terminal extracellular region and a WSXWS motif in the extracellular region adjacent to the membrane (2). There is no known kinase domain in the intracellular region. However, closely related members of this family (EpoR, IL-2Rβ, IL-3R, IL-4R and the gp130 signal transducer for IL-6R) share two highly homologous cytoplasmic regions in the first 60 amino acids after the end of the transmembrane domain (32). It has been proposed that these highly conserved segments interact with structurally similar signaling molecule(s) (32).

The existence of EpoR subunits has been proposed based on crosslinking studies of [$^{125}$ I] labeled Epo (5, 7, 11, 17, 20, 29) ,experiments with an Epo-encoding retrovirus (16) and by analogy to the large family of cytokine receptors to which EpoR belongs. For example, the IL-2Rβ chain cytoplasmic region interacts with the *src*-family kinase p56$^{lck}$ to transduce a signal (15). Association of EpoR with phosphorylated proteins, including a soluble cytoplasmic tyrosine

kinase, has been demonstrated (25, 45). EpoR can form high affinity and low affinity binding sites for Epo (for review see (9)). However the nature of the differences between the high and low affinity forms is not yet understood. It is likely that EpoR associates with other proteins to form receptor complexes of high and low affinity similar to other hemopoietin receptors in the cytokine receptor family. A third IL-2R subunit, IL-2Rγ, was recently discovered that participates in the formation of high and low affinity complexes with the IL-2Rβ (41). A common β subunit is shared between the GM-CSF, IL-3, and IL-5 receptors in the mouse to form the corresponding high affinity receptors (19, 42). Highlighting the subtle functional differences between mouse and human hemopoietin receptor homologues that have been recently studied, the mouse has two distinct functional high affinity receptors for IL-3 consisting of an α chain and two distinct β chains, one of which is homologous to a unique human IL-3Rβ chain (14).

Deletion mapping has revealed several non-overlapping regulatory domains in the mEpoR C-terminal domain (8, 31, 46). Additionally, EpoR expression in FDC-P1 cells has been shown to down-modulate responsiveness of the cells to GM-CSF. This down modulation was relieved by C-terminal deletions in EpoR, suggesting that GM-CSFR and the C-terminal region of EpoR may compete for limiting regulatory factor(s) (33).

It is uncertain how *Fv-2*$^{rr}$ homozygotes resist gp55-induced erythroblastosis. However, *Fv-2* and *Epor* genes are unlinked (4, 16, 24). We recently reported that a truncated SFFV mutant (BB6) obtained by forced passage of SFFV in *Fv-2*$^{rr}$ congenic mice is able to overcome *Fv-2* resistance (26). BB6 encodes a gp42 glycoprotein with a large deletion in its membrane-proximal domain. Our experiments revealed that gp42 specifically activates mEpoR at the surface of the cell, and that accessory proteins associated with mEpoR are

displaced differentially by gp42 and gp55. Consequently, we proposed that *Fv*-2 - encoded protein(s) interact closely with mEpoR to control its activation by the SFFV family of *env* glycoproteins (20).

It is generally believed that Friend disease is restricted to certain strains of mice and that rats and humans are resistant (10). To investigate the molecular basis for the resistance to Friend disease, we expressed hEpoR in murine cells and tested SFFV and BB6 viruses for activation. The human and murine EpoRs are 82% similar (17). Our results suggest that hEpoR activation is also controlled by *Fv*-2 -encoded protein(s).

## Materials and Methods

### Cells

Retroviral packaging cell lines ψ-2 (27) and PA12 (30) cells were maintained in Dulbecco's modified Eagle's Medium, supplemented with 10% fetal bovine serum (FBS). Interleukin-3 (IL-3) dependent hematopoietic cell line BaF3 (28) were maintained in RPMI 1640 medium supplemented with 10% FBS and $5 \times 10^{-5}$ M β-mercaptoethanol with10% WEHI-3 as an IL-3 source. ψ-2 and PA12 cells were transfected with retroviral vectors encoding EpoRs and *env* glycoproteins to produced helper-free virions by ping-pong amplification as previously described (3, 21). The retroviral vectors encoding gp55 of wild-type SFFV (Lilly-Steeves polycythemic strain) (22), and mutant SFFV *env* genes BB6 (20) and PvuΔ (11) have been described. BaF3 cells that were infected with the EpoR-encoding virions and selected for growth in Epo were maintained in medium containing recombinant mouse Epo at 0.5 unit/ml (Boeringer-Mannheim, Indianapolis, IN).

### Plasmid Construction

The retroviral vector encoding mEpoR (pSFF-EpoRPA11) was constructed from the ligation of an EcoR I/ BamH I fragment of pSFF (3), a Bgl II /Nhe I 800 bp fragment from pXM190 ( generous gift of A. D'Andrea, Dana-Farber Cancer Institute, Boston, MA), and a Nhe I/EcoR I 790 bp fragment of pXM190 containing the 3' end of the EpoR gene without its polyadenylation signal. The EcoR I site of the latter fragment was constructed by treating a Sty I site ( EpoR nucleotide position 1547 (7)) with Klenow polymerase which fortuitously introduced a stop codon at the natural stop site in the EpoR sequence, and also created an EcoR I restriction site in the proper orientation for insertion into the BamH I/EcoR I cloning site of pSFF. pSFF-hEpoR was constructed from a three-

way ligation of the following fragments: a BamH I to Xho I pSFF vector fragment, a Bgl II to Nhe I and a Nhe I to Xho I fragment from the hEpoR-encoding plasmid p18 (a gift from G. Wong, Genetics Inst., Cambridge, MA). A Bgl II site common to both receptors (nucleotide position 866, mEpoR numbering (7)) was used to make the in-frame connection between the human and murine EpoR for the construction of the chimeras (Fig. 1).

**[$^{125}$ I] Epo Binding Assays**

Cells (1-8 x$10^6$) were incubated with 0.03 μCi of [$^{125}$ I] Epo (300-900 Ci/mmol) (Amersham Corp., Arlington Heights, IL.) in 0.1 ml of DMEM containing 10% FBS and 0.2% sodium azide for 3 hrs at 20°C. Cells with bound [$^{125}$ I] Epo were separated from excess unbound [$^{125}$ I] Epo by pelleting through 0.15 ml dibutylphalate oil. The cell pellets were clipped from the bottoms of the tubes and assayed for radioactivity in a gamma counter.

**Factor-Independent Growth Assays**

For factor-independent growth assays BaF3 cells or BaF3/EpoR cells were infected with SFFV, PvuΔ or BB6 virus for 2 hrs at 37°C in the presence of 8 μg/ml polybrene. The cells were pelleted by centrifugation and resuspended in medium containing growth factor for 48 hrs. The cells were then sedimented by centrifugation and washed twice with phosphate buffered saline (PBS) (Gibco BRL, Gaithersburg, MD) and resuspended in complete medium without growth factors and seeded into microtiter wells or flasks to allow for selection of factor-independent cells.

**Analysis of Proteins**

For detection of SFFV, BB6 and PvuΔ *env* glycoproteins by western blotting, cell lysates were immunoprecipitated with an anti-Friend Leukemia virus gp70 antiserum that crossreacts with *envs* encoded by SFFV and MCFs (12, 36, 37) and electrophoresed on polyacrlyamide gels under reducing conditions in the presence of 1% sodium dodecyl sulfate. The proteins were then transferred to nitrocellulose membranes, incubated with the same antibody and detected with [$^{125}$ I] protein A as described previously (12, 22). EpoR protein was detected similarly using an antibody made by injecting rabbits with a synthetic peptide (APSPSLPDPKFESKC) matching the amino terminus of the mature mEpoR sequence (7).

## Results

### Production of BaF3 derivatives expressing human or murine EpoR

The first step in our strategy to analyze the resistance of nonmurine species to Friend disease was to express human EpoR in murine cells to first determine if a mitogenic signal could be transduced in the presence of Epo.

Human EpoR and murine EpoR cDNAs were ligated into the retroviral vector pSFF (3) (see Fig. 1; A, B) and transfected into cocultures of ψ-2(27) and PA12 (30) retroviral packaging cell lines as described earlier (3, 21). Thirteen days after transfection, cocultures were examined by immunoprecipitation followed by western blot using a polyclonal anti-mEpoR antibody. The transfected cocultures expressed EpoRs of the expected Mr of 66,000 ( Fig. 2, lanes 2 and 3).

Helper-free human and murine EpoR-encoding virions harvested from each ψ-2 and PA12 coculture were then used to infect the murine interleukin-3 (IL-3) dependent hematopoietic cell line BaF3 (28). Infection with mEpoR or hEpoR-encoding retroviruses enabled BaF3 cells to grow in the presence of Epo as the only added growth factor. As shown in Table 1, both of the EpoR containing derivatives bound [$^{125}$ I] Epo. BaF3/hEpoR cells consistently bound approximately two to three times as much labeled hEpo as the BaF3/mEpoR cells in all binding experiments performed (see also below). Additionally, BaF3/hEpoR cells grew slowly at the beginning of selection in Epo, suggesting that an increase in hEpoR expression and/or some other modification might be required before proliferation approaching that of the BaF3/mEpoR cells was achieved.

We conclude that hEpoR can transduce a signal in murine cells in the presence of Epo as the only added growth factor, confirming the results of Jones

*et al.* (17). However the adaptation that occurs during selection in Epo implies that the hEpoR may not function as well as mEpoR in the mouse cell context.

**Interaction of mEpoR and hEpoR with glycoproteins encoded by wild-type SFFV and SFFV mutants BB6 and PvuΔ**

The BaF3/hEpoR cells we produced enabled us to study the interaction of gp55 with hEpoR in cells permissive for mEpoR activation by gp55 (BaF3 cells were derived from a *Fv*-$2^s$ mouse strain). BaF3/mEpoR cells (but not BaF3 cells) become factor independent after expression of SFFV-encoded gp55 (11, 20, 23, 44) or after infection with a virus encoding a truncated form of gp55 ( BB6) that is able to overcome *Fv*-2 resistance (20) We also tested a gp55 mutant (PvuΔ) we have recently isolated that encodes the smallest gp55-related protein known that is able to activate mEpoR (11).

BaF3 cells expressing human EpoR became factor-independent when infected with BB6 and PvuΔ viruses but not with wild-type SFFV in nine consecutive factor-independent growth assays (see Table 2). We observed in several assays that BaF3/hEpoR cells infected with SFFV seemed to be partially stimulated, but these cells ultimately failed to proliferate even when Epo was re-added at several time points in an attempt to rescue BaF3/hEpoR/SFFV cells.

Western blot analysis of the SFFV, BB6 and PvuΔ -infected factor-independent BaF3/EpoR cells using an anti-*env* antibody (Fig. 3) detected a Mr 55,000 protein expressed by BaF3/mEpoR/SFFV cells (lane 2). The BaF3/mEpoR and BaF3/hEpoR cells that became factor-independent after infection with the mutant SFFVs expressed the *env* glycoproteins expected for BB6 (Mr 42,000; lanes 3 and 7) and PvuΔ (Mr 41,000; lanes 4 and 6). BaF3 cells express a Mr 85,000 endogenous *env*-related protein (lane 1).

We conclude that although hEpoR is capable of transducing a mitogenic signal in murine cells in the presence of Epo, hEpoR is resistant to activation by gp55. On the contrary, *env* glycoproteins expressed by the BB6 and PvuΔ mutants are capable of easily and quickly converting BaF3/hEpoR cells to vigorous factor-independent growth.

**Critical role of the cellular context in activation of EpoR by SFFV-related *env* glycoproteins**

One derivative line of BaF3/hEpoR cell became marginally competent for activation by SFFV after it had been grown in Epo for longer than three months. In this case, the cells infected with SFFV were minimally stimulated and gradually began to grow after three weeks (see Fig. 4). On the contrary, SFFV infection reproducibly had no effect on newly prepared BaF3/hEpoR cells. We presume that this adapted line of BaF3/hEpoR cells developed a secondary alteration that enabled it to respond weakly to gp55. We mention it here because it suggests that the resistance of hEpoR to activation by gp55 is not absolute and can depend on the cellular context.

We also made the hEpoR-mEpoR reciprocal chimeras described in Fig 1. As expected, the BaF3/chimeric EpoR derivative cells bound [$^{125}$ I] Epo (Fig 5). When super-infected with SFFV, cells with both chimeric EpoRs slowly became factor-independent for survival and proliferation, after lag periods of only marginal survival and proliferation (Table 3). In contrast, BaF3/mEpoR cells infected with SFFV always became factor-independent rapidly. Independently made BaF3/chimeric EpoR cells also behaved somewhat differently in their responses to SFFVs, consistent with the conclusion that different adaptations can occur during culture in Epo prior to SFFV infections, and that these

preadaptations affect activation by SFFV (see above). The factor-independent cells that formed synthesized gp55s (see Fig. 6).

## Discussion

Human EpoR was expressed in murine BaF3 cells to test if superinfection with SFFV would enable the cells to become factor-independent. We found that hEpoR was able to transduce a signal in murine hematopoietic cells, and, consistent with another recent report, that BaF3/human EpoR cells bound 2-3 times as much labeled hEpo as did BaF3/mEpoR cells (Table 1, Fig. 5) (39). We noticed that the newly made BaF3/hEpoR cells grew slowly in Epo compared to the BaF3/mEpoR cells, and that an adaptation period precedes their vigorous proliferation. We infer that hEpoR functions only marginally in mouse BaF3 cells and that selection for increase in receptor number and/or myriad other factors involved in the signaling pathway may precede rapid proliferation of BaF3/hEpoR cells in the presence of Epo. One implication of this preselection is that independently adapted BaF3/hEpoR and BaF3/chimeric EpoR cell lines might differ somewhat in their properties.

BaF3/hEpoR cells are efficiently and reproducibly converted to factor-independence by superinfection with BB6, a virus that encodes a retroviral *env* that overcomes *Fv*-2 resistance (Table 2) (26). This implies that hEpoR might be resistant to gp55 activation by proteins that are encoded by endogenous *Fv*-2 genes. Our results show unambiguously that hEpoR has an inherent capability to be activated by SFFV-related *env* glycoproteins.

After long-term growth and adaptation in Epo, one line of BaF3/hEpoR cells became able to be converted to factor-independent growth by SFFV infection and selection. When the altered cells were infected with SFFV, they survived without Epo and then over the course of 6-8 days began to slowly proliferate. Gradually, an adaptation occurred and the cells eventually grew actively. Similar lag periods were observed in experiments involving mouse-human interspecies GM-CSF receptor and receptor-subunit mixing experiments

in BaF3 cells (38). This suggests that a change in the receptor, the cellular factors that participate in signaling, or in the activating *env* glycoprotein might be required to enable factor-independent growth of these cells. Support for a change in the *env* glycoprotein is suggested by the slower mobility of the gp55 band (Fig 4). This could have been caused by an alteration in the cell's glycosylation of gp55 or by a mutation in the gp55 gene. That BaF3/hEpoR cells could eventually adapt to allow activation by SFFV supports our results with BB6 and PvuΔ SFFVs that the resistance of hEpoR to activation by SFFVs is not absolute and can be influenced by viral and cellular changes. These results also demonstrate that assays with BaF3/EpoR cells can be critically affected by adaptations and alterations (presumably mutations) that occur during growth in selective conditions. Our evidence suggests that the resistance of non-murine species to SFFV may be controlled in part by *Fv*-2 -encoded homologues.

Our studies with hEpoR-mEpoR chimeras are compatible with these conclusions. gp55 lacks a cystolic domain, and is basically an extracellular protein with a carboxyl terminal hydrophobic membrane anchor (1, 6, 43). The fact that gp55 can activate both of our chimeras (one of which contains mEpoR sequences only in the intracellular region) in at least some cellular contexts implies also that mEpoR activation by gp55 must be mediated or restricted by accessory components of an EpoR complex. Therefore, our basic conclusion is that EpoR functions as a multisubunit assemblage in its activation by ligands including the SFFV family of *env* glycoproteins.

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**Table 1. [$^{125}$ I] Epo Binding of BaF3 and BaF3/EpoR Derivatives**

| Cells | [$^{125}$ I] Epo bound* cpm | growth factor required |
|---|---|---|
| BaF3 | 521 | IL-3 |
| BaF3/murineEpoR | 12,394 | IL-3 or Epo |
| BaF3/humanEpoR | 26,223 | IL-3 or Epo |

*Binding was done on triplicate samples of 5 x $10^6$ cells as described previously (20).

**Table 2. Factor-independent growth assays of BaF3 and BaF3/EpoR derivatives after infection with SFFV, BB6 and PvuΔ viruses***

| Experiment | Cells | Number of wells (in a 24-well plate) with factor-independent cells (number of cells plated/well) | | | |
|---|---|---|---|---|---|
| | | (10,000) | (1000) | (100) | (10) |
| 1 | BaF3/mEpoR/SFFV | 24 | 15 | | |
| | BaF3/mEpoR/BB6 | 24 | 24 | | |
| | BaF3/hEpoR/SFFV | 0 | 0 | | |
| | BaF3/hEpoR/BB6 | 24 | 22 | | |
| 2 | BaF3/SFFV | 0 | 0 | 0 | 0 |
| | BaF3/mEpoR/SFFV | 24 | 24 | 14 | 1 |
| | BaF3/hEpoR/SFFV | 0 | 0 | 0 | 0 |
| 3 | BaF3/mEpoR/SFFV | 24 | 24 | 4 | 1 |
| | BaF3/mEpoR/BB6 | 24 | 24 | 24 | 5 |
| | BaF3/mEpoR/PvuΔ | 24 | 22 | 9 | 2 |
| | BaF3/hEpoR/SFFV | 0 | 0 | 0 | 0 |
| | BaF3/hEpoR/BB6 | 24 | 15 | 7 | 2 |
| | BaF3/hEpoR/PvuΔ | 24 | 24 | 21 | 7 |

* Fresh or frozen viral harvests were used to infect cells for 3 hrs at 37°C in the presence of 8 μg/ml polybrene. After growth for 48 hrs in medium with growth factors, cells were suspended in medium without growth factors and serially diluted into micotiter culture plate wells. The wells were observed for proliferating factor-independent cells.

**Table 3. Factor-independent growth assays of BaF3 cells and BaF3/EpoR derivatives after infection with SFFV and BB6 viruses**

| Cells | Number of wells (in a 24-well plate) with factor-independent cells* (number of cells plated/well) | | | |
|---|---|---|---|---|
| | (10,000) | (1000) | (100) | (10) |
| BaF3/mEpoR/SFFV | 24 | 24 | 24 | 10 |
| BaF3/hEpoR/SFFV | 0 | 0 | 0 | 0 |
| BaF3/pUC-HM/SFFV | 24 | 20 | 5 | 0 |
| BaF3/SR3-MH/SFFV | 24 | 21 | 3 | 0 |
| BaF3/mEpoR/BB6 | 24 | 24 | 24 | 17 |
| BaF3/hEpoR/BB6 | 24 | 15 | 0 | 0 |
| BaF3/pUC-HM/BB6 | 24 | 24 | 9 | 2 |
| BaF3/SR3-MH/BB6 | 24 | 24 | 3 | 0 |

* BaF3 cells expressing chimeric receptors and infected with SFFV were not convincingly proliferating until day 6-8 after removal of growth factors. This judgment is based on the number, refractility and shape of the cells and the appearance of doublets. BaF3/mEpoR cells infected with SFFV were proliferating by day 3-4 after removal of growth factors.

Figure 1. Maps of retroviral constructs. The relative sizes and restriction sites used to produce the retroviral constructs encoding murine EpoR (mEpoR), human EpoR (hEpoR) and chimeric EpoRs are shown. The pSFF retroviral vector has been described (3). Murine and human sequences are shown as black and striped boxes, respectively. (A) pSFF-mEpoRPA11, (B) pSFF-hEpoR. The mouse and human EpoR restriction fragments used to construct pSFF-EpoR reciprocal chimeric receptors are shown: pUC-HM (C) encodes the human EpoR up to 36 amino acids beyond the end of the transmembrane region, the remainder of the C-terminal protein is murine. SR3-MH encodes the reciprocal receptor (D). The plasmids encoding EpoRs were transfected into cocultures of packaging cells to produce helper-free EpoR-encoding virions (see Materials and Methods).

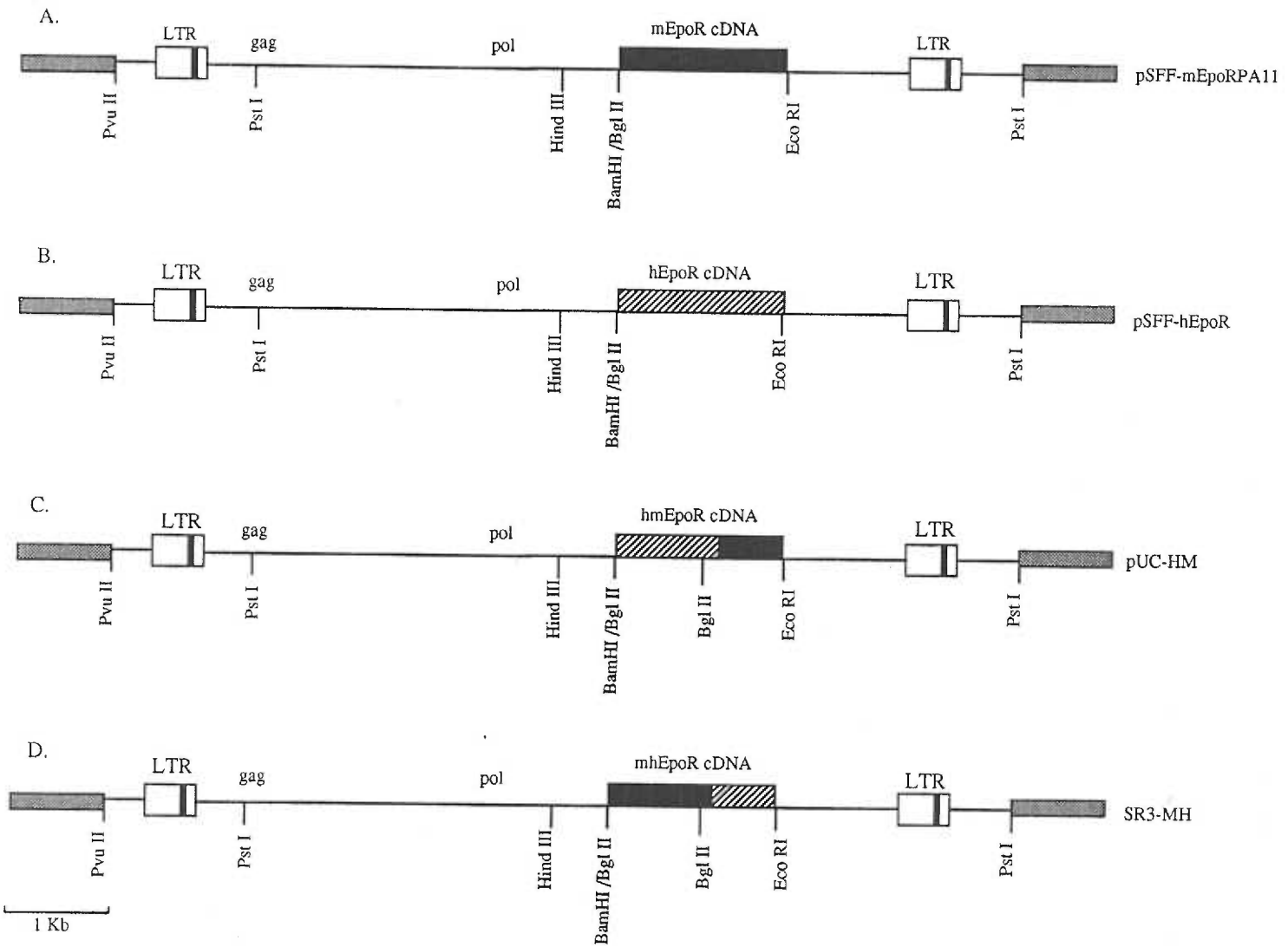

A.
LTR
gag
pol
mEpoR cDNA
LTR
pSFF-mEpoRPA11
Pvu II
Pst I
Hind III
BamHI /Bgl II
Eco RI
Pst I
B.
LTR
gag
pol
hEpoR cDNA
LTR
pSFF-hEpoR
Pvu II
Pst I
Hind III
BamHI /Bgl II
Eco RI
Pst I
C.
LTR
gag
pol
hmEpoR cDNA
LTR
pUC-HM
Pvu II
Pst I
Hind III
BamHI /Bgl II
Bgl II
Eco RI
Pst I
D.
LTR
gag
pol
mhEpoR cDNA
LTR
SR3-MH
Pvu II
Pst I
Hind III
BamHI /Bgl II
Bgl II
Eco RI
Pst I
1 Kb

Figure 2. Murine and human EpoRs expressed in murine retroviral packaging cells. Cell lysates from ψ-2 and PA12 cocultures transfected with pSFF-mEpoRPA11 and pSFF-hEpoR were immunoprecipitated with an anti-mEpoR antibody (see Materials and Methods). The lysates were run on electrophoresis gels under reducing conditions and transferred to nitrocellulose membranes. The membranes were incubated with anti-EpoR antibody followed by [$^{125}$ I] Protein A. Lane 1, untransfected coculture; lane 2, ψ-2 and PA12 cells expressing mEpoR; lane 3, ψ-2 and PA12 cells expressing hEpoR. Sizes of molecular mass standards are indicated in kDa on the left.

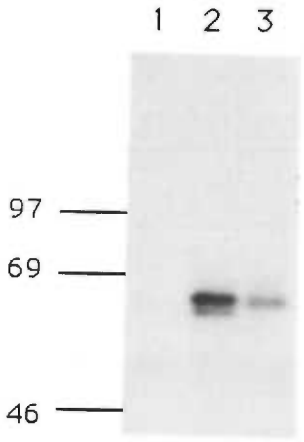

1
2
3
97
69
46

Figure 3. SFFV, BB6 and PvuΔ *env* glycoproteins expressed by factor-independent BaF3/EpoRs. The proteins were immunoprecipitated, electrophoresed and transferred to nitrocellulose membranes for detection by immunoblotting using an *env* antiserum (see Materials and Methods). Lane 1, BaF3/mEpoR; lane 2, BaF3/mEpoR/SFFV; lane 3, BaF3/mEpoR/BB6; lane 4, BaF3/mEpoR/PvuΔ; lane 5, BaF3/hEpoR; lane 6, BaF3/hEpoR/PvuΔ; lane 7, BaF3/hEpoR/BB6. BaF3 cells express an endogenous Mr 85,000 protein (11, 20).

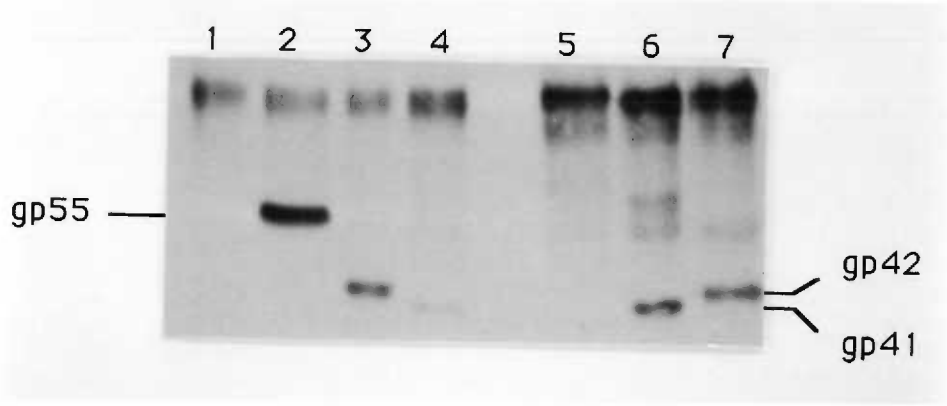

1
2
3
4
5
6
7
gp55
gp42
gp41

Figure 4. Immunoblot of adapted BaF3/hEpoR cells that became factor-independent after SFFV infection. Cell lysates were analyzed with *env* antiserum as described in the legend of Fig 3. Lanes 1 and 3 show an endogenous Mr 85,000 protein expressed by BaF3 cells; lane 2, BaF3/mEpoR/SFFV; lane 4, adapted [BaF3/hEpoR] /SFFV.

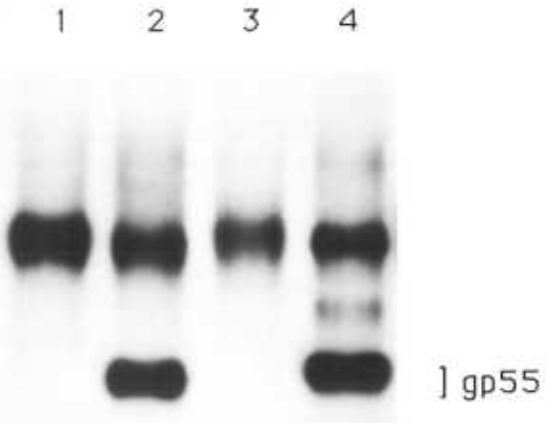

1
2
3
4
] gp55

Figure 5. [$^{125}$ I] Epo Binding of BaF3 and BaF3/EpoR derivatives. Cells were incubated with 0.03 μCi of [$^{125}$ I] Epo (300-900 Ci/mmol) for 3 hrs at 20°C in a volume of 0.1 ml. Cells with bound [$^{125}$ I] Epo were assayed for radioactivity in a gamma counter. BaF3/hEpoR superscript notations refer to (1) BaF3/hEpoR that were adapted by long term growth in Epo and became able to be converted to factor independence by SFFV, (2) BaF3/hEpoR cells freshly selected in Epo. These latter cells failed to become factor-independent after SFFV infection (see text).

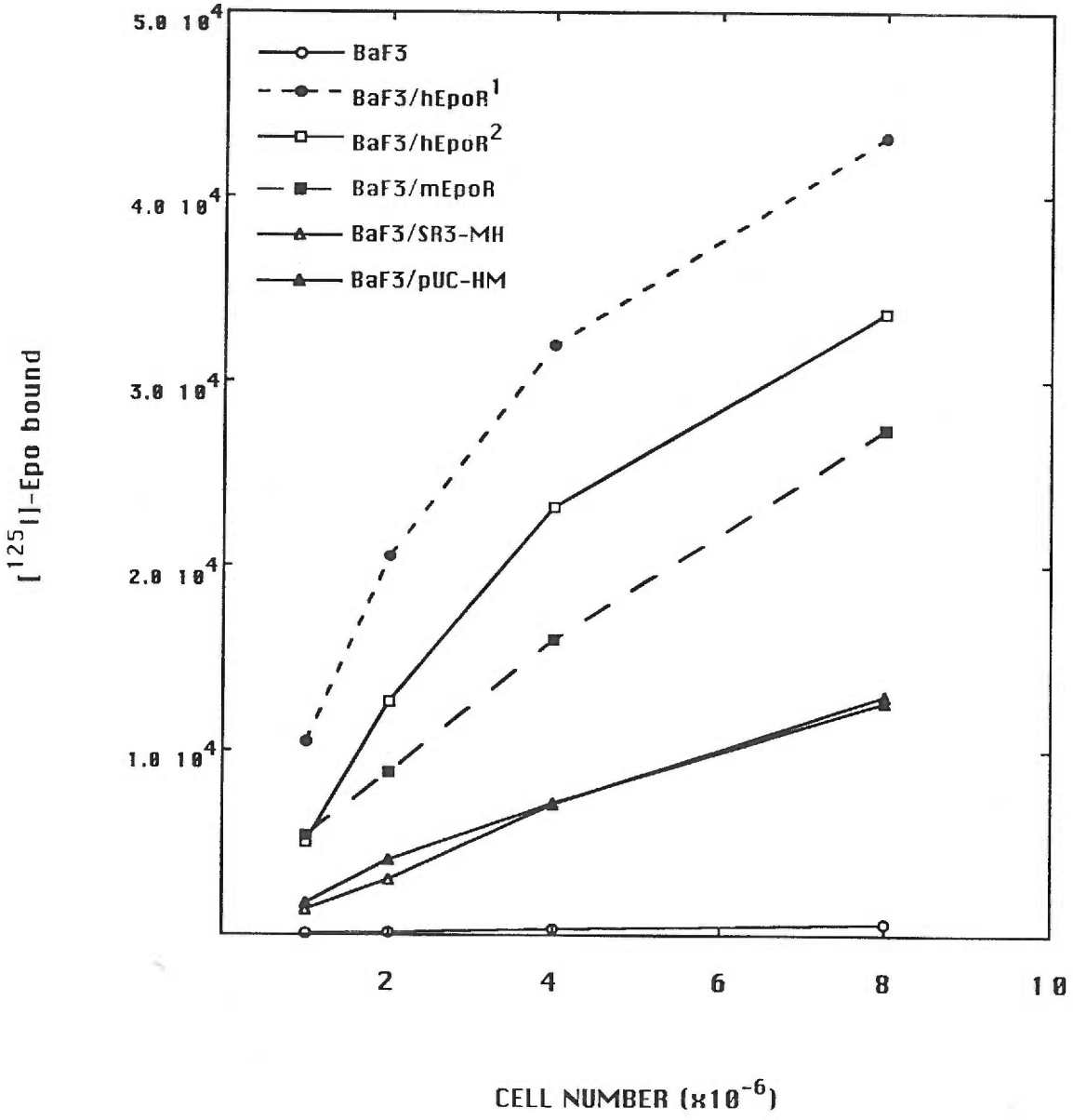
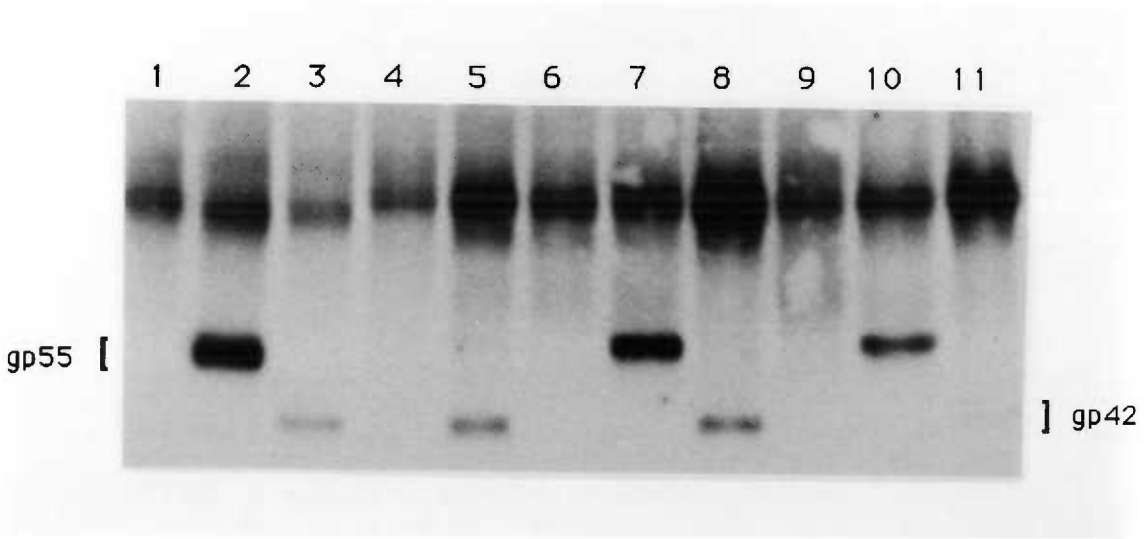

5.0 10^4
4.0 10^4
3.0 10^4
2.0 10^4
1.0 10^4
BaF3
BaF3/hEpoR^1
BaF3/hEpoR^2
BaF3/mEpoR
BaF3/SR3-MH
BaF3/pUC-HM
[125I]-Epo bound
2
4
6
8
10
CELL NUMBER (x10^-6)

Figure 6. Immunoblot of factor-independent BaF3/EpoR derivatives expressing PvuΔ and BB6 and SFFV-encoded *envs*. Cell lysates were analyzed with *env* antiserum as described in the legend of Fig 3. Lane 1, BaF3/mEpoR; lane 2, BaF3/mEpoR/SFFV; lane 3, BaF3/mEpoR/BB6; lane 4, BaF3/hEpoR; lane 5, BaF3/hEpoR/BB6; lane 6, BaF3/SR3-MH; lane 7, BaF3/SR3-MH/SFFV; lane 8, BaF3/SR3-MH/BB6; lane 9, BaF3/pUC-HM; lane 10, BaF3/pUC-HM/SFFV; lane 11, BaF3/pUC-HM/BB6 (the light gp42 band in lane 11 was clearly seen in a longer exposure of this blot).


1 2 3 4 5 6 7 8 9 10 11
gp55
gp42

## Discussion and Conclusions

A retrovirus that encodes erythropoietin was used to directly compare the effects of erythropoietin and gp55 on EpoR. An IL-3 dependent hematopoietic murine cell line that had been converted to Epo dependence by infection with EpoR-encoding virus became factor-independent after infection with either the Epo-encoding virus or SFFV. This *in vitro* assay supported and extended previous evidence that Epo and gp55 have overlapping mechanisms of EpoR activation (69, 102) . The Epo virus also mimicked Friend erythroleukemia in susceptible mice. Additionally, congenic mice homozygous for the *Fv-2*$^{r}$ gene were resistant to the Epo virus. *Fv-2*$^{rr}$ homozygotes are resistant to SFFV-induced disease, but are susceptible to all other naturally occurring retroviral diseases (101, 108). One possible explanation for these results is that *Fv-2* encodes the EpoR, and that the *Fv-2*$^{r}$ allele encodes a version that is relatively sluggish in transducing its signal. We ruled out this possibility by mapping the *Epor* gene to a site on murine chromosome 9 that is unlinked to *Fv*-2. (70). These results suggest that *Fv*-2-encoded protein(s) interact with EpoR to regulate its activation.

Recent studies of Phillips and others suggest that the maintenance of specific sizes of murine stem cell pools and cycling kinetics are under genetic control. The primitive hematopoietic cell compartment in C57Bl/6J mice (*Fv-2*$^{rr}$) is restricted in size compared to the stem cell pools of DBA/2J (*Fv-2*$^{s}$) mice (90). This hematopoietic cell pool-size restriction may explain why cells from *Fv-2*$^{rr}$ mice are responsive to Epo but cannot be constitutively activated by the inappropriate autocrine stimulation engendered by Epo virus or SFFV infection. According to this idea, the BFU-E and CFU-E compartments in *Fv-2*$^{rr}$ mice cannot expand because there is restriction of the hematopoietic

precursor pool. In contrast, SFFV-infected BFU-E and CFU-E from *Fv*-$2^s$ mice proliferate and are readily replenished. Consistent with this notion, *Fv*-$2^{rr}$ BFU-E and CFU-E have been shown to cycle less frequently than those isolated from *Fv*-$2^s$ mice (114). It is unfortunate that *Fv*-$2^{rr}$ congenic mice (those differing in only the *Fv*-2 locus from a susceptible strain) were not analyzed in the experiments of Phillips *et al.* (90).

The Epo virus described above was made by inserting Epo genomic sequences into the retroviral vector pSFF, a derivative of SFFV lacking *env* -encoding sequences. A second version of the Epo-encoding retroviral vector was constructed by substituting the SFFV 3' LTR with that encoded by an MCF virus. Although the MCF virus is strictly thymotropic, both versions of the Epo virus resulted in indistinguishable diseases in susceptible mice. Our results are consistent with the idea that expression of a heterologous gene can influence the target cell specificity of a retroviral vector.

Recent studies suggested that MCF *env* glycoproteins may be mitogens that bind to EpoR and possibly to other members of the hemopoietin receptor superfamily (67, 69, 119). We expressed EpoR in hamster cells to test this hypothesis. Hamster cells are resistant to infection by all mouse retroviruses (45, 84, 94). Hamster cells that had been engineered to express the ecotropic receptor were infected with an ecotropic host-range virus encoding EpoR. Hamster cells that expressed EpoR were completely resistant to MCF infection, suggesting that EpoR does not function as a receptor for these viruses. Ecotropic pseudotypes of MCF viruses were able to infect these cells demonstrating that the cells were otherwise susceptible to productive infection by MCF virus. We cannot rule out that a mouse-specific cofactor might be required for MCF infection, or that EpoR might only facilitate MCF entry into hematopoietic cells.

Certain EpoR activating mutations have been selected *in vitro* (72, 132). Additionally, the murine myeloproliferative leukemia virus encodes an *env* gene with striking similarity to the EpoR receptor and is able to immortalize hematopoietic progenitors (110). We hoped to exploit retroviral genomic drift and pathogenic selection *in vivo* (55) to isolate an EpoR with maximal mitogenic activity. Injection of mice with a preparation of a retrovirus encoding wild-type mouse EpoR in the presence of a weakly pathogenic MuLV helper virus resulted in a long latency disease characterized by extramedullary erythropoietin-independent hematopoiesis. However northern, Southern and western blots using material from the infected animals did not reveal viral EpoR transcript, integrated EpoR in genomic DNA, or EpoR protein expressed in the infected spleen lysates above the detection limit. Furthermore, amplification of EpoR sequences from total RNA and genomic DNA by PCR suggested that the presence of virally-encoded EpoR sequences was less than one copy per cell in the infected spleens. Virus prepared from infected mice enabled BaF3/EpoR cells (but not BaF3 cells) to become factor-independent. When lysates from the infected cells were examined by western blot with an antiserum that cross reacts with *env* glycoproteins encoded by SFFVs and MCFs, we discovered that we had captured several different SFFV-related viruses that apparently arose by recombination of the retroviral vector with endogenous MCF-related sequences. The passage history of the viruses and relative molecular weights of the encoded *env* glycoproteins suggested that we had isolated SFFV evolutionary intermediates. One of the viruses we isolated was a member of the mink cell focus-inducing class. This result strongly suggests that some MCFs can activate EpoR, and supports evidence that SFFVs are MCF derivatives. The question of MCF activation of EpoR is an important issue in

the understanding of retroviral-mediated leukemogenesis and has been a subject of some controversy (63, 67). Nucleotide sequence analysis and comparison of the *envs* of these three novel isolates will be useful for understanding the molecular evolution of these pathogenic viruses.

One useful technique for studying the erythropoietic lineage arose from the pathologic characterization of the disease *in vitro*. We found that long term bone marrow cultures using a modification (77) of the original Dexter method (24) allowed for extended erythropoiesis without an exogenous source of Epo. There is currently no explanation for this surprising observation. It is compelling to imagine that a source of Epo or a factor with biological properties that overlap with Epo is produced in these cultures.

During another investigation, we recently isolated an SFFV mutant, PvuΔ, that activates EpoR in a cell culture system and encodes a gp41 glycoprotein (31). Since this is the smallest SFFV *env* glycoprotein known to activate EpoR, we analyzed its biological and structural properties. We found that even though PvuΔ has a 204 base deletion in the ecotropic-specific domain compared to SFFV, injection of PvuΔ into susceptible mice caused a rapid-onset erythroleukemia. Different studies have suggested that the dualtropic domain, the ecotropic domain, or the carboxyl terminal hydrophobic membrane anchor region of gp55 may be critical for EpoR activation (2, 12, 63, 67, 68, 73, 112, 125, 126, 133). The previously- analyzed BB6 SFFV mutant has an overlapping 159 base deletion (75). Interestingly, BB6 not only activates EpoR in *in vitro* assays, but is able to overcome *Fv-2*$^{r}$ restriction *in vivo*. The structural characteristics and pathologic properties of the *env* glycoproteins encoded by the BB6 and PvuΔ mutants suggest that the

ecotropic-specific domain is not essential for pathogenic activity. It will be interesting to determine if PvuΔ is able to overcome *Fv*-2$^{r}$ restriction.

To analyze the resistance of nonmurine species to Friend disease we expressed human EpoR in murine cells to determine if a mitogenic signal could be transduced in the presence of Epo. We found that hEpoR was able to transduce a signal in murine hematopoietic cells but that an initial preselection for increase in receptor number and/or other factors involved in the EpoR signaling pathway appeared to be required before rapid proliferation in the presence of Epo. Interestingly, mEpoR expressed in human IL-3 dependent cells and selected in Epo also functioned marginally compared with those expressing hEpoR. Furthermore, our preliminary experiments suggest that human cells expressing mEpoR do not become factor-independent after infection with amphotropic host-range pseudotyped SFFV.

The BaF3/hEpoR cells we produced enabled us to study the interaction of gp55 with hEpoR in cells permissive for mEpoR activation by gp55 (BaF3 cells were derived from a *Fv*-2$^{s}$ mouse strain). BaF3/mEpoR cells (but not BaF3 cells) become factor-independent after expression of SFFV-encoded gp55 (31, 63, 69, 130) or after infection with SFFV deletion mutants BB6 (63) or PvuΔ (31). BaF3 cells expressing human EpoR became factor-independent when infected with BB6 and PvuΔ viruses but not with wild-type SFFV. Our results show unambiguously that hEpoR has an inherent capability to be activated by SFFV-related *env* glycoproteins.

After long-term growth and adaptation in Epo, one line of BaF3/hEpoR cells became able to be converted to factor-independent growth by SFFV infection and selection. When the altered cells were infected with SFFV, they survived without Epo and then began to slowly proliferate. Gradually, an adaptation occurred and the cells eventually grew actively. Similar lag

periods were observed in experiments involving mouse-human interspecies GM-CSF receptor and receptor-subunit mixing experiments in BaF3 cells (103). This suggests that a change in the receptor, the cellular factors that participate in signaling, or in the activating *env* glycoprotein might be required to enable factor-independent growth of these cells. That BaF3/hEpoR cells could eventually adapt to allow activation by SFFV supports our results with BB6 and PvuΔ SFFVs that the resistance of hEpoR to activation by SFFVs is not absolute and can be influenced by viral and cellular changes. These results also demonstrate that assays with BaF3/EpoR cells can be critically affected by adaptations and alterations (presumably mutations) that occur during growth in selective conditions. Our evidence suggests that the resistance of non-murine species to SFFV may be controlled in part by *Fv-2* -encoded homologues.

Our studies with hEpoR-mEpoR chimeras are compatible with these conclusions. gp55 lacks a cytosolic domain, and is essentially an extracellular protein with a carboxyl terminal hydrophobic membrane anchor (1, 14, 129). The fact that gp55 can activate both of our chimeras (one of which contains mEpoR sequences only in the intracellular region) in at least some cellular contexts, implies also that mEpoR activation by gp55 must be mediated or restricted by accessory components of an EpoR complex. Therefore, our basic conclusion is that EpoR functions as a multisubunit assemblage in its activation by ligands including the SFFV family of *env* glycoproteins. Consistent with this idea, EpoR is associated with many other cellular proteins (8, 19, 31, 53, 63, 71, 76, 131).

Two soluble versions of the EpoR were expressed in retroviral packaging cell lines (see Appendix I, fig. 1). The first version encodes extracellular EpoR up to four amino acids before the start of the

transmembrane region. Although the transfected cells expressed solEpoR-1 and solEpoR-1 was detected in the medium, the soluble receptor failed to bind [$^{125}$ I] Epo. Because of this result, we suspected that solEpoR-1 was truncated in a region critical for Epo binding. We then made a second version of the soluble receptor that included an additional eight amino acids (ending at EpoR nucleotide position 786) called solEpoR-24. We established that solEpoR-24 protein was expressed by immunofluorescence microscopy, and showed that the protein was secreted from the cells by immunoprecipitation from the culture medium with an EpoR antiserum (Appendix I, fig. 2). The secreted solEpoR-24 also failed to bind [$^{125}$ I] Epo. Since immunofluorescence microscopy on fixed, permeabilized cells expressing all versions of EpoR had detected relatively similar amounts of intracellular receptor, we developed a [$^{125}$ I] Epo binding and immunoprecipitation assay using solubilized full-length Epo receptors as a positive control. Comparison of the binding of the solubilized full-length EpoR with the binding of the solEpoRs suggests that the truncated soluble receptors do not bind Epo (Appendix I, Table 2) . The soluble receptors also failed to compete for [$^{125}$ I] Epo binding to cells expressing full-length EpoR (Appendix I, Table 1). Two groups have reported that soluble versions of EpoR similar to the ones we have described here bind Epo (65, 133), however they do not show data that led to this conclusion, so it is difficult to find these results very convincing. It may be that the concentrations of the soluble proteins we expressed are below the threshold of detection for the assays we used. Nevertheless, our results clearly show that solEpoRs do not bind Epo with the same affinity as the full-length receptor. Another possible explanation for our results is that EpoR requires accessory proteins for Epo binding. Truncation of the receptor may reduce or prevent participation of

these accessory factors. The interleukin-2 receptor-β chain shares a particular homology with the EpoR (18, 47). Interestingly, IL-2Rβ binds IL-2 with extremely low affinity when expressed in fibroblasts, but binds IL-2 with intermediate affinity when expressed in large granular lymphocytes, oligodendrocytes and certain T-cells, suggesting that cell-specific factors can change the binding affinity of IL-2R for IL-2 (85, 120, 121). A soluble IL-2Rβ–chain was detected in the supernatants of human lymphoid cell lines that overexpress the IL-2Rβ–chain by affinity labeling with recombinant IL-2 followed by fluorescent sandwich-ELISA (50). It may be possible to demonstrate solEpoR•Epo binding by i) increasing expression levels of the solEpoRs, ii) partial purification of soluble receptors, iii) Epo affinity labeling and detection by ELISA using a procedure similar to that described for the soluble IL-2Rβ chain.

Conclusions of the thesis:

(1) Epo and SFFV-encoded gp55 both specifically stimulate EpoR.

(2) Epo virus causes a disease in susceptible mice that mimics Friend erythroleukemia.

(3) *Fv-2*$^{rr}$ homozygotes are resistant to both Epo and Friend viral erythroleukemia, but they are susceptible to all other natural retroviral diseases.

(4) *Fv-2* and *Epor* genes are unlinked.

(5) *Fv-2* encodes protein(s) that control EpoR mitogenic signaling.

(6) Expression of a growth factor in a responsive cell can redirect target tissue specificity of a retrovirus.

(7) The EpoR is not a cell surface receptor for MCF virus.

(8) A series of pathogenic intermediates in the evolution of SFFV were isolated, including a replication competent MCF virus. Each of these intermediates were able to activate EpoR.

(9) The ecotropic-specific region of gp55 is not essential for EpoR activation.

(10) EpoR activation by gp55 is mediated or restricted by accessory components of the EpoR complex. EpoR functions as a multisubunit assemblage.

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# Appendix I. Soluble Versions of the Erythropoietin Receptor Fail to Bind Erythropoietin

Figure 1. Retroviral constructs used for expression of soluble Epo receptors. The retroviral vector pSFF has been described previously (1). (A) Schematic of EpoR coding region. The extracellular-, transmembrane-, and intracellular-encoding regions are depicted by black, dotted, and striped boxes, respectively. (B) solEpoR-1 was constructed from a Bgl II to a (Klenow polymerase-treated) Nhe I fragment from EpoR cDNA-containing plasmid pXM190 (a gift from A. D'Andrea, Dana-Farber Cancer Institute, Boston, MA). The Nhe I site was treated with Klenow polymerase and ligated to a pSFF vector fragment cut with BamH I and EcoR I (Klenow polymerase-treated) . The Nhe I site is at EpoR nucleotide position 757 of the extracellular-encoding region, 4 amino acid residues before the start of the transmembrane domain (3). (C) solEpoR-24 was constructed from a EpoR PCR product using the antisense primer 5'-GTGC**GAATTC**CTACGTCAAGATGAGAGGGTCCAG-3' and sense primer 5'-GCAC**GGATCC**TGAAGCTAGGGCTG-3'. The bold letters represent EcoR I and BamH I sites, respectively, created to insert the PCR product into the polylinker of the retroviral vector pSFF. Standard buffers and reagent concentrations were used as recommended by Cetus (Emeryville, CA) for PCR. The PCR profile used was 30 cycles consisting of a 95°C 1.0 min., 55°C 1.0 min., 72°C 2.0 min. pattern. This was followed by a 5 min. extension at 72°C. solEpoR-24 encodes the receptor up to 4 amino acids after the start of the transmembrane region, EpoR nucleotide position 786.

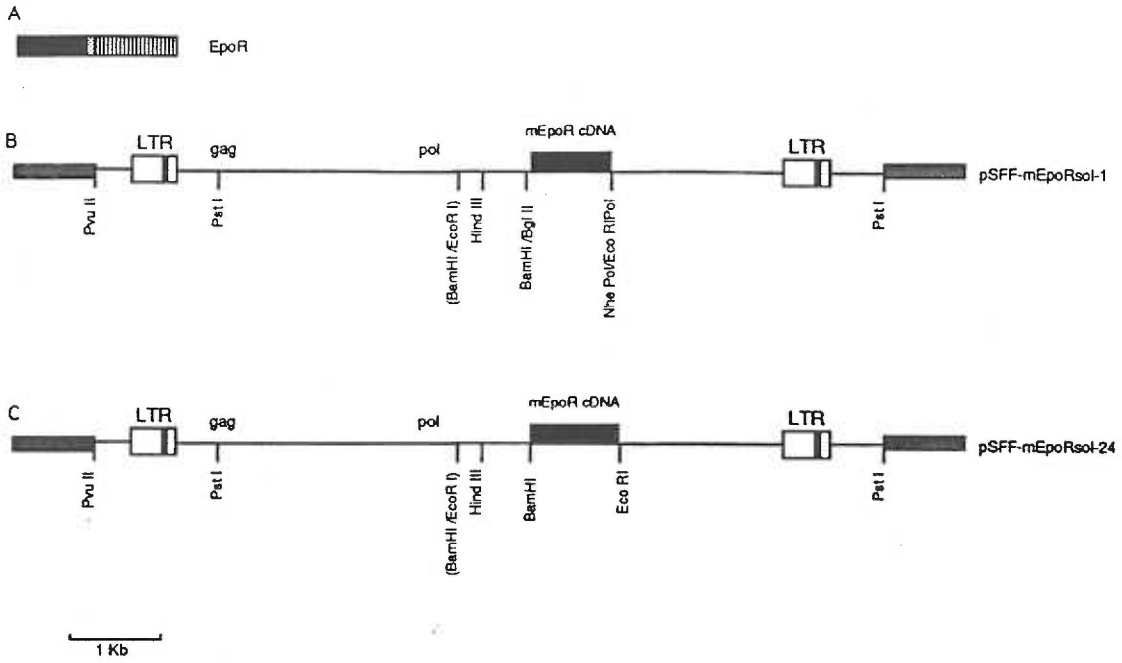
A
EpoR
B
LTR
gag
pol
mEpoR cDNA
LTR
pSFF-mEpoRsol-1
Pvu II
Pst I
(BamHI /EcoR I)
Hind III
BamHI /Bgl II
Nhe PolEco RIPol
Pst I
C
LTR
gag
pol
mEpoR cDNA
LTR
pSFF-mEpoRsol-24
Pvu II
Pst I
(BamHI /EcoR I)
Hind III
BamHI
Eco RI
Pst I
1 Kb

Figure 2. Soluble and full-length EpoRs synthesized in retroviral packaging cell lines. Molecular mass markers in kDa are indicated to the left of the figures. (A) Cell lysates and supernatants from confluent 10 cm culture dishes were analyzed by immunoprecipitation followed by electrophoreses and then by blotting using an EpoR antiserum directed to the amino terminal portion of the EpoR. solEpoR-1 supernatant, lane 1; cell lysate, lane 2; full-length EpoR cell lysate, lane 3. (B) Analysis of [$^{35}$S]-labeled proteins of cocultures expressing soluble EpoRs. Cells were grown in 10 cm culture dishes to 75% confluency and then labeled by incorporation overnight with L-[$^{35}$S] methionine and L-[$^{35}$S] cysteine (Tran$^{35}$S-label; ICN Biochemicals Inc., Costa Mesa, CA) as previously described (4). The supernatants and cell lysates were analyzed as described in part A of this figure. solEpoR-24 supernatant, lane 1; cell lysate, lane 2; solEpoR-1 supernatant, lane 3; cell lysate, lane 4; negative control cell supernatant, lane 5; cell lysate, lane 6. The positions of soluble EpoRs are indicated by arrows. The slower mobility of solEpoR-24 compared to that of solEpoR-1 is consistent with the eight amino acids added in the construction.

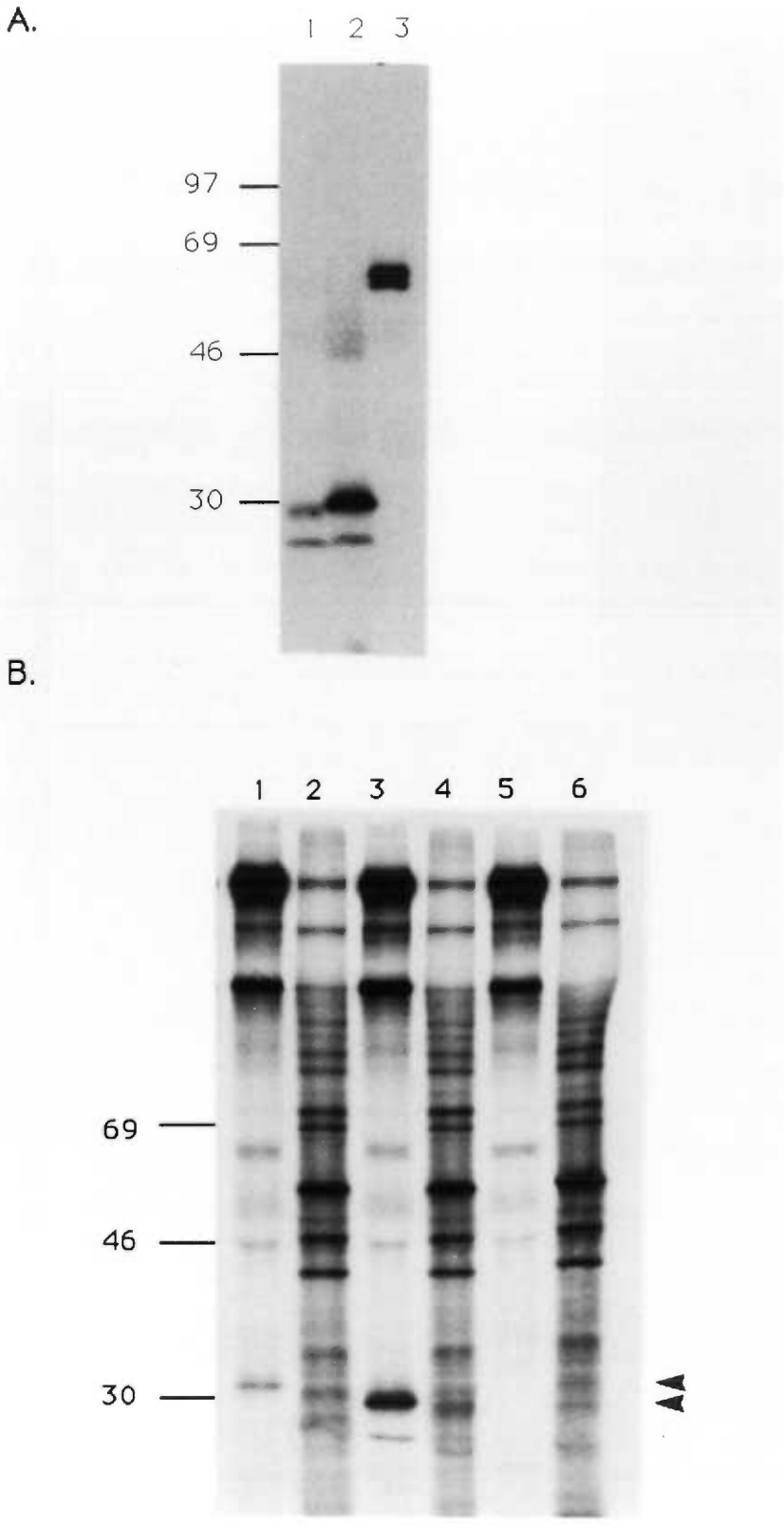
A.
1 2 3
97
69
46
30
B.
1 2 3 4 5 6
69
46
30

Table 1. Competition between soluble EpoRs and full-length EpoR expressed on cell surfaces for [$^{125}$ I] Epo binding.

| Supernatant added[1] | [$^{125}$ I] Epo bound (cpm)[2] |
|---|---|
| PA12 | 3972, 4878, 3916 |
| DMEM | 4134, 3804, ND |
| sEpoR-24 | 3940, 4213, 4559 |
| sEpoR-1 | 4134, 4053, 3452 |
| sEpoR-1[3] | 5504, 5539, 3744 |
| DMEM[3] | 4525, 5476, 5490 |

[1] Fresh DMEM, the fresh supernatants obtained from confluent layers of cells expressing soluble EpoRs, or PA12 (negative control) cells were filtered through a 0.45 μ filter and sodium azide was added (final concentration of 0.2%). The supernatants (0.5 ml, 1.0 ml and 3.0 ml ) were then added to 6-well tissue culture dishes containing a nearly confluent monolayer of fibroblasts expressing EpoR. 0.03 μCi [$^{125}$ I] Epo (300-900 Ci/mmol) was added and the cells were incubated at 25°C for three hours. The supernatants were removed, and the cell monolayers were washed twice with DMEM containing 10% fetal bovine serum. The cells were then solubilized in a 1% solution of sodium dodecyl sulfate, and the cell lysates were assayed for radioactivity.

[2] The numbers in series represent counts obtained from wells with of 0.5 ml, 1.0 ml or 3.0 ml of DMEM, control or sEpoR supernatant added. All volumes were adjusted to a total volume of 3.0 ml with DMEM.

[3] [$^{125}$I] Epo binding as described in footnote 1 except incubation was performed at 37°C for three hours in the absence of azide.

Table 2. Binding of [$^{125}$ I] Epo to EpoR-expressing cell lysates[1]

| Cells | [$^{125}$ I] Epo bound (cpm) |
|---|---|
| control | 82; 128; 68 |
| EpoR | 9471; 12,237; 8739 |
| sEpoR-24 | 142; 126; 151 |
| sEpoR-17[2] | 172; 175; 197 |

[1] ψ2 and PA12 cocultures of retroviral packaging cell lines expressing full-length EpoR or soluble EpoRs were solubilized for 15 minutes at 4°C in 1.5% Triton X-100 in 25 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, sodium azide 0.02% (w/v), 1 μg/ml each of aprotinin, leupeptin, and pepstatin as previously described (2). Insoluble material was pelleted by centrifugation. 0.03 μCi [$^{125}$ I] Epo (300-900 Ci/mmol) was added and the lysates were incubated at 10°C for four hours. An anti-EpoR antibody recognizing the amino terminal portion of EpoR was added (1:100) and allowed to bind for 1 hour on ice. Protein A-Sepharose was then added and incubation continued for 1 hour. The samples were centrifuged and the pellets were washed twice with fresh lysis buffer before the pellets were assayed for radioactivity. Each data point represents a lysate from a confluent 10 cm culture dish. Examination of these EpoR-expressing cell lines by immunofluorescence microscopy showed that levels of EpoR expression were within a 3-5 fold range.

[2] sEpoR-17 is a high-expressing clone isolated from the solEpoR-1 cell population.

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