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

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The Friend spleen focus-forming virus (SFFV) *env* gene encodes a glycoprotein with apparent M_r of 55,000 that binds to erythropoietin receptors (EpoR) to stimulate erythroblastosis. A retroviral vector that does not encode any Env glycoprotein was packaged into retroviral particles and was coinjected into mice in the presence of a nonpathogenic helper virus. Although most mice remained healthy, one mouse developed splenomegaly and polycythemia at 67 days; the virus from this mouse reproducibly caused the same symptoms in secondary recipients by 2 to 3 weeks postinfection. 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 secondary recipients converted an erythropoietin-dependent cell line (BaF3/EpoR) into factor-independent derivatives but had no effect on the interleukin-3-dependent parental BaF3 cells. Most of these factor-independent cell clones contained a major Env-related glycoprotein with an M_r of 60,000. During further *in vivo* passaging, a virus that encodes an M_r -55,000 glycoprotein became predominant. Sequence analysis indicated that the ultimate virus is a new SFFV that encodes a glycoprotein of 410 amino acids with the hallmark features of classical gp55s. Our results suggest that SFFV-related viruses can form in mice by recombination of retroviruses with genomic and helper virus sequences and that these novel viruses then evolve to become increasingly pathogenic.

The independently isolated Friend and Rauscher erythroleukemia viruses (18, 48) are complexes of a replication competent murine leukemia virus (MuLV) and a replication-defective spleen focus-forming virus (SFFV) (39, 42, 47). The SFFVs encode Env glycoproteins (gp55) that are inefficiently processed to form larger cell surface derivatives (gp55^p) (19). The gp55 binds to erythropoietin receptors (EpoR) to cause erythroblast proliferation and splenomegaly in susceptible mice. Evidence has suggested that the critical mitogenic interaction occurs exclusively on cell surfaces (7, 16).

SFFVs are structurally closely related to mink cell focus-inducing viruses (MCFs) (2, 5, 10, 50), a class of replication-competent murine retroviruses that has a broad host range termed polytropic (15, 21). Although MCFs are not inherited as replication-competent intact proviruses, the mouse genome contains numerous dispersed polytropic *env* gene sequences (8, 17, 27). MCFs apparently readily form *de novo* by recombination when ecotropic host range MuLVs replicate in mice (14, 15, 26, 43). MCF *env* genes typically are hybrid recombinants that contain a 5' polytropic-specific region and a 3' ecotropic-specific portion (26). 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 (32, 39, 47). In addition, MCFs often differ from ecotropic MuLVs in their long terminal repeat (LTR) sequences (8, 20, 26, 28, 29, 45).

Based on their sequences, SFFVs could have derived from MCFs by a 585-base deletion and by a single-base addition in the ecotropic-specific portion of the *env* gene (10). Evidence suggests that both the 585-bp deletion and the frameshift mutation probably contribute to SFFV pathogenesis (3, 49). 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, 41).

This report 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 viruses with *env* genes that were able to activate EpoR. Serial passage of this initial virus isolate resulted in selection of a novel SFFV that encodes a gp55 glycoprotein of 410 amino acids. This experimental system provides a method for isolating new SFFVs and for mapping the stages in their genesis.

MATERIALS AND METHODS

Viruses and cells. Retroviral packaging cell lines ψ -2 (34) and PA12 (37) 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 (6, 29). The EpoR-encoding virions were used to infect the interleukin-3 (IL-3)-dependent hematopoietic cell line BaF3 (35) to produce the BaF3/EpoR cells (BER) used in this study as previously described (22). The pL26K retroviral vector encoding gp55 of wild-type SFFV (Lilly-Steeves polycythemic strain) has been described elsewhere (31). ψ -2 and PA12 cells were maintained in Dulbecco's modified Eagle's medium sup-

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plemented with 10% fetal bovine serum BaF3 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 5×10^{-5} M β -mercaptoethanol with 10% WEHI-3 as an IL-3 source. BER cells were maintained in the same medium with erythropoietin (Epo; Boehringer Mannheim, Indianapolis, Ind.) at 0.5 U/ml instead of IL-3. Preparation of passaged virus from spleens was described previously (23).

Pathogenic assays. Helper-free virus encoding wild-type EpoR was mixed with ecotropic helper virus B4 (38) for injection as described previously (23). Female NIH/Swiss mice (4 to 8 weeks old) were used for all in vivo pathogenic characterization except the analysis of *Fv-2* restriction. For that study, DBA/2J (*Fv-2*⁻) and the *Fv-2*⁺ congenic strain D2.R were used (13).

Proliferation assays. For [³H]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, N.Y.) in phosphate-buffered saline (Gibco BRL, Gaithersburg, Md.) at a dosage of 60 mg/kg for 2 consecutive days (20). The [³H]thymidine incorporation assay method previously described by Krystal (30) was used, with slight modifications. Briefly, spleen cells were resuspended at 8×10^6 cells/ml and then aliquoted into a 96-well plate containing serial dilutions of Epo. The cells were then incubated for 22 h at 37°C and 5% CO₂. Ten microcuries of [³H]methyl-³H]thymidine (6.7 Ci/mmol; DuPont NEN, Boston, Mass.) was then added to each well, and the plates were incubated for 2 h as described above. The reaction was stopped by addition of trichloroacetic acid (TCA) so that the final concentration of TCA in the wells was 10%. Adherent cells were dissolved in 0.1 M 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 (erythroid) were performed using standard methods (25). Briefly, bone marrow cells taken from femurs and tibias of control and virus-infected mice were suspended in alpha medium (Gibco Laboratories, Grand Island, N.Y.), sedimented by centrifugation, and resuspended in semisolid medium containing methylcellulose purchased from Terry Fox Laboratory (Vancouver, British Columbia, Canada) without Epo or in the presence of 0.5 U of Epo per ml. The cell suspension was plated in triplicate in 35-mm-diameter Lux dishes (Nunc Inc., Naperville, Ill.) at 2×10^5 cells/plate. After incubation for 3 days in a humidified atmosphere containing 5% CO₂ at 37°C, the plates were examined for hemoglobinized bursts. For factor-independent growth assays, BaF3 cells or BER cells were infected with passaged virus for 2 h at 37°C in the presence of Polybrene (8 μ g/ml). The cells were pelleted by centrifugation and resuspended in medium containing growth factor for 48 h. The cells were then sedimented by centrifugation, washed twice with phosphate-buffered saline, and resuspended in complete medium without growth factors to allow for selection of factor-independent cells.

LTBM culture. Long-term bone marrow (LTBM) cultures were made by using a modification of the original Dexter LTBM culture method (12) that allowed for an extended period of erythropoiesis without addition of Epo to the cultures (36). Bone marrow cells taken from the tibias and femurs of 6- to 8-week-old female NIH/Swiss control and virus-infected mice were gently suspended in Iscove's modified Dulbecco's medium (IMDM; JRH Biosciences, Lenexa, Kans.), sedimented by low-speed centrifugation, and resuspended in IMDM supplemented with 25% horse serum (ICN Biochemicals, Costa Mesa, Calif.) and with 125 U of penicillin G, 125 μ g of streptomycin, and 0.5 μ g of hydrocortisone succinate (Sigma, St. Louis, Mo.) per ml. The combined bone marrow from three mice was plated into three six-well tissue culture plates (Becton Dickinson, Lincoln Park, N.J.) in a volume of 3.0 ml/well. After incubation for 1 week at 37°C in a humidified atmosphere containing 5% CO₂, 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 blotting. For Western blotting, cell lysates were immunoprecipitated with an anti-Friend MuLV gp70 antiserum that cross-reacts with Envs encoded by SFFV and MCFs (19, 44, 45) and electrophoresed on polyacrylamide 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 [¹²⁵I] protein A as described previously (19, 31).

RT-PCR and DNA sequencing. Reverse transcription-PCR (RT-PCR) was performed by standard methods, using total RNA obtained from the diseased spleens and two primers: forward primer U5 (5'-TCAGCGGGGTCTTTCAT TGT-3'), located in the 5' LTR (40), and PV3 (5'-CGTTACAGCGGATCtCG GCTAAGC-3'), located in the 3' LTR. Lowercase letters indicate point mutations substituted to create restriction sites for PCR cloning. Either Elongase (Gibco BRL) or PCR SuperMix (Gibco BRL) were used for amplification. PCR products were TA cloned into the pCR 2.1 vector by using T4 ligase and transformed into INVαF' (Invitrogen, San Diego, Calif.) as instructed by the manufacturer. Qiagen minipreps were cycle sequenced with ABI PRISM Dye Terminator (Perkin-Elmer, Branchburg, N.J.). The sequence was analyzed by using the computer program MacVector (Oxford Molecular Group, Ltd.). The sequences obtained were compared to others in the databases by using the BLAST network service (1).

Nucleotide sequence accession number. The accession number for the DE410 sequence is AF030182.

TABLE 1. Pathogenicity of SFFV mutants in susceptible 4- to 6-week-old NIH/Swiss mice

Virus	Day postinfection	Spleen wt (g) [hematocrit]
SFF-EpoRPA11	32	0.18 [43], 0.16 [42]
	62	2.98 [ND] ^a
	67	2.81 [75] ^b
	83	0.2 [47], 0.2 [47], 0.2 [46], 0.13 [43], 0.97 [38], 0.14 [39]
Passaged virus ^c	18	3.4 [74], ^b ND ^d
	26-31	4.67 [50], 3.64 [82], 2.1 [59]
Control ^e	32	0.15 [42]
	67	0.12 [46]
	83	0.27 [50], 0.29 [45], 0.14 [43]

^a ND, not done.

^b Spleen lysates examined by Western blotting (Fig. 2 and 5).

^c Spleen from the day 67 mouse was used to prepare passaged virus as previously described (23).

^d Dead mouse with massive hepatosplenomegaly.

^e Ecotropic helper virus injected only.

RESULTS

Origin of a new virus that causes rapid-onset splenomegaly and polycythemia. This work was serendipitously initiated by injecting mice with a nonpathogenic clone of ecotropic Friend MuLV (38) plus a helper-free preparation of a retroviral vector (pSFF-EpoRPA11) that encodes wild-type mouse EpoR (see Materials and Methods). Although a previously made retrovirus that encodes wild-type EpoR is apparently nonpathogenic, certain EpoR mutations can constitutively activate its mitogenic signaling to cause leukemia (33, 51). 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 signal transduction properties of this hemopoietin receptor (4, 11). Among 11 NIH/Swiss mice injected with this virus, three developed massive splenomegaly by 62 to 83 days (Table 1). Passaged virus prepared from the mouse that had splenomegaly and polycythemia at 67 days postinfection reproducibly caused these symptoms within 2 to 3 weeks in secondary recipients (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 the

FIG. 1. Detection of Env glycoproteins in spleen lysates of infected mice by immunoblotting. 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 [¹²⁵I]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 (Table 1); lane 3, cell lysate from the IP/IR erythroleukemic cell line containing helper-free SFFV (46).

FIG. 2. Pathology consistent with extramedullary erythropoiesis in infected mice. (A to F) Paraffin-embedded tissue sections stained with hematoxylin and eosin. Comparable pathology was observed in animals injected with first- and second-passage viruses, examples of which are shown here. (A) Normal spleen; magnification, $\times 214$. (B and C) Virus-infected spleen (magnifications, $\times 428$ and $\times 857$, respectively) showing loss of normal splenic architecture due to proliferating erythroid precursors: (D) Liver from helper virus-injected control mouse; magnification, $\times 214$. (E and F) Liver from an infected mouse showing erythropoietic foci (arrow); magnifications, $\times 428$ and $\times 857$, respectively. (G and H) Peripheral blood smears stained with new methylene blue to detect reticulocytes (magnification, $\times 857$) from a helper virus-injected control mouse (G) and an infected mouse showing a striking increase in reticulocytes (H). (I) Cytocentrifuged cell suspension from an infected mouse spleen showing an erythroid island composed of macrophages attached to erythroid precursors; magnification, $\times 857$. A benzidine stain confirmed the presence of hemoglobin in the immature erythroid cells.

passed virus. Furthermore, PCR amplifications using total RNA and genomic DNAs from these diseased spleens did not indicate virus-encoded nucleic acids with EpoR sequences.

We then used an antiserum that is broadly reactive with Env glycoproteins of MuLVs to search for virus-encoded proteins in the original spleen samples from infected mice (Fig. 1). The small spleen from a mouse injected only with helper virus contained a negligible amount of Env glycoproteins (lane 1). In contrast, the spleen from the original 67-day mouse with transmissible disease (Table 1) contained a relatively large concentration of MuLV gPr90 and gp70 glycoproteins plus traces of

smaller components, including one with an apparent M_r of 60,000 (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 (Fig. 2B and C). Microscopic examination of fragments from the enlarged spleens revealed the presence of structures typical

FIG. 3. Proliferation assay of spleen cells from mice demonstrating amplified Epo-independent growth. Spleen cells from control mice and two animals infected with first-passaged virus were incubated in serial dilutions of Epo for 22 h followed by addition of [3 H]thymidine for 2 h. The cells were then assayed for [3 H]thymidine incorporation (see Materials and Methods). (A) Proliferation of spleen cells from infected mice (solid symbols) compared to that of control spleen cells (open symbols). (B) Expanded plot of Epo-dependent proliferation of spleen cells used as controls (i.e., cells from mice injected with nonpathogenic helper virus and spleen cells from phenylhydrazine-pretreated and normal mice).

of erythroid islands (Fig. 2I). Benzidine staining confirmed the presence of hemoglobin in the erythroid cells. The livers of these mice also appeared to contain erythropoietic islands (compare Fig. 2D to Fig. 2E and F), and blood from these polycythemic mice contained relatively large proportions of reticulocytes (compare Fig. 2G and H).

Although erythropoiesis in normal adult mice is substantially confined to bone marrows, anemia induces some erythroblast migration to the spleens and livers (20). As shown in Fig. 3B, mice with phenylhydrazine-induced anemia had a slight elevation of Epo-dependent cell proliferation in cultured spleen cells. In contrast, cells from the grossly enlarged spleens of

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 ^a	14, 13

^a Possible burst.

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

Mouse strain	No. of mice with splenomegaly/3 tested						
	813 virus prep ^a on day:				1118 virus prep ^a on day:		
	21	29	35	42	14	20	27
DBA/2	0	2	3	3	0	3	3
D2.R	0	0	0	0	0	0	0

^a Second-passaged virus.

mice infected with the new virus proliferated at a greatly amplified rate in an Epo-independent manner (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 3 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 to 3 weeks, whereas the D2.R mice reproducibly did not (Table 3). Thus, like the classical strains of SFFV, the new viruses were unable to overcome *Fv-2* resistance.

Effects of the new virus in LTBM cultures. LTBM cultures are generally made in stages by first establishing an adherent layer consisting principally of stromal cells and later adding fresh marrow onto this stromal support (12, 36). 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 (Fig. 4). This did not occur when we used only bone marrow cells from diseased mice, presumably because the erythroblasts in these mice had already differentiated and 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 IL-3-dependent hematopoietic cells, whereas BER is a derivative that expresses EpoR and can grow in either IL-3 or Epo (31). The newly formed pathogenic virus (Table 1) and its progeny that had been passaged in secondary recipient mice were able to convert BER 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.

Figure 5 shows a protein immunoblot analysis of Env-related glycoproteins that were encoded by these pathogenic viruses. In agreement with the results in Fig. 1, the spleen from the 67-day mouse that originally developed the disease (Table 1) contained helper virus-encoded glycoproteins plus a low-abundance M_r -60,000 component (Fig. 5, lane 1). The population of factor-independent cells that formed when BER cells were infected with passaged virus 1218 contained the M_r -60,000 component plus a minor proportion of a component that comigrated with gp55 (lane 3 in comparison to the gp55 standard in

FIG. 4. Cytospin preparation of nonadherent cells in LTBM cultures of normal NIH/Swiss mice after addition of infected bone marrow. Normal LTBM cultures were recharged with normal bone marrow or with bone marrow from mice infected with the new virus. First- and second-passage viruses were examined and found to be comparable. Nonadherent cells were collected from the medium each week and examined. The samples shown are cytospin preparations taken from the cultures 4 weeks after recharge and stained with Wright-Giemsa stain (magnification, $\times 428$). (A) Normal LTBM culture recharged with bone marrow from an infected mouse; (B) normal LTBM culture recharged with normal bone marrow. Benzidine staining for hemoglobin confirmed that the cells surrounding the central macrophages were erythroid.

lane 4). Moreover, after additional *in vivo* passages (passaged virus 429), the virus encoding the gp55 component became predominant and the M_r -60,000 component was no longer evident (lane 2).

Sequence analysis of the novel SFFV. To determine the sequence of the ultimate virus that encodes an M_r -55,000 protein, an enlarged spleen was obtained from a mouse injected with a highly passaged preparation of the novel virus. Total RNA was extracted from the spleen as described in Materials and Methods, and RT-PCR was performed with the U5-PV3

primer pair. The single amplified band of approximately 2 kb was TA cloned, and the sequence of the DNA was determined.

The *env* gene sequence of DE410 was highly homologous to the *env* sequences of previously studied Friend and Rauscher SFFVs and contained the 585-base deletion, a 6-base duplication, and a single-base insertion that are characteristic of SFFVs. Figure 6 shows the deduced amino acid sequence of DE410 in comparison to the sequences of previously studied gp55s. An interesting feature in the new protein sequence is the insertion of an alanine at position 167, which results in a length of 410 amino acids. Although absent from gp55s of Friend virus strains, this same insertion occurs in gp55s of Rauscher SFFV (5) and in endogenously inherited retroviral sequences (26). Features unique to DE410 include R47K, I103T, and A353T substitutions (Fig. 6). No changes in cysteine residues or potential glycosylation sites were observed.

DISCUSSION

Origin and evolution of a new lineage of SFFV-related erythroleukemia viruses. This report 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 non-pathogenic, biologically cloned ecotropic helper MuLV plus a replication-defective virus that encodes wild-type EpoR. Our

FIG. 5. Env glycoproteins in growth factor-independent BER cells infected with passaged virus. Protein immunoblot methods were as described for Fig. 1. Lane 1, original spleen lysate from the infected day 67 mouse; lane 2, BER/passaged virus 429 (second passage); lane 3, BER/passaged virus 1218 (first passage); lane 4, BER/SFFV wild type. BaF3 cells express an M_r -85,000 protein (16, 28).

FIG. 6. Sequence comparison of the novel SFFV DE410 and several other SFFVs. In the Clustal W alignment, the sequence of the novel SFFV, DE410, is compared to the sequences reported by Wolff et al. (50), Amanuma et al. (2), and Clark and Mak (10) (GenBank accession no. V01552, J02193, and K00021, respectively). Amino acid identities are shaded, conservative substitutions are boxed, and nonconservative differences are shown without shading. Deletions are shown by dashes. Classical landmarks of SFFVs are indicated as follows: 1, proline-rich region (approximately amino acids 235 to 280); 2, the site of the 585-base in-frame deletion that causes the gp70/p15E cleavage site to be absent in SFFVs; 3, insertion of two leucines caused by a 6-base duplication; 4, site of the single-base insertion causing a downstream frameshift and early termination.

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 (33). On the contrary, our results suggest that the virus-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 recombinant that was able to activate EpoR to stimulate proliferation and differentiation of infected erythroblasts. When sacrificed at 67 days, the original mouse contained virus that encodes a gp60 (Fig. 1, lane 2; Fig. 5, lane 1). After passage of this virus into

a secondary recipient, a virus that encodes a gp55 formed. This gp55-encoding SFFV then rapidly overgrew its progenitors during subsequent in vivo passages (Fig. 5).

These results have several important implications for our understanding of murine retroviral leukemogenesis. First, our results strongly support the idea that highly pathogenic SFFVs can derive from less pathogenic progenitors by a process of in vivo selection. Second, our evidence that intermediates in this derivation are able to activate EpoR expressed in BER cells also strongly suggests that the progenitor viruses have a degree

of mitogenic activity. If these evolutionary intermediates were nonpathogenic, they would also not have become amplified *in vivo* and their detection and isolation would have been much more difficult. Therefore, our results suggest that *env* genes able to activate EpoR can readily form during ecotropic MuLV replication in mice and that the resulting viruses can evolve to form SFFVs by a stepwise process of mutation, recombination, and pathogenic selection. In this process, successive intermediates are presumably increasingly pathogenic. Third, the fact that we can readily isolate viruses that activate EpoR by using BER cells has enabled us to isolate intermediates in this evolutionary pathway. 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 possible to use BER cells to isolate new lineages of viruses that activate EpoR or other hemopoietin receptors.

Although we initiated this study by using a retroviral vector that encodes EpoR, the EpoR sequences were rapidly deleted and it seems very unlikely that they were essential. Rather, we would anticipate that any vector that could recombine with endogenously inherited *env* sequences might suffice to initiate potential SFFV evolution. Indeed, we have recently found in collaboration with J. Portis and colleagues (Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Mont.) that our SFF viral vector without EpoR sequences also readily and reproducibly participates in generation of novel SFFVs after injection with a nonpathogenic helper virus into newborn mice (24). A manuscript describing these results in detail is in preparation. In this context, we wish to emphasize that the pSFF vector was initially derived from a 5.9-kb SFFV molecular clone that had large deletions in its *gag* and *pol* regions (6). The vector construction involved deletion of *pol*-related and polytropic *env* sequences and creation of a multicloning site. Additional studies will be required to learn how the sequences of this vector have participated in formation of the initial recombinants and whether other vectors would also function in this process.

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