The Membrane Glycoprotein of Friend Spleen Focus-Forming Virus: Evidence that the Cell Surface Component Is Required for Pathogenesis and that It Binds to a Receptor

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The leukemogenic membrane glycoprotein of Friend spleen focus-forming virus (SFFV) has an apparent M_r of 55,000 (gp55), is encoded by a recombinant *env* gene, and occurs on cell surfaces and in intracellular organelles. There is evidence that the amino-terminal region of gp55 forms a dualtropic-specific domain that is connected to the remainder of the glycoprotein by a proline-rich linker (C. Machida, R. Bestwick, B. Boswell, and D. Kabat, *Virology* 144:158-172, 1985). Using the colinear form of a cloned polycythemic strain of SFFV proviral DNA, we constructed seven in-phase *env* mutants by insertion of linkers and by a deletion. The mutagenized SFFVs were transfected into fibroblasts and were rescued by superinfection with a helper murine leukemia virus. Four of the mutants cause erythroblastosis. These include one with a 6-base-pair (bp) insert in the ecotropic-related sequence near the 3' end of the gene, two with a 12- or 18-bp insert in the region that encodes the proline-rich linker, and one with a 6-bp insert in the dualtropic-specific region. The other mutants (RI, Sm1, and Sm2) are nonpathogenic and contain lesions in dualtropic-specific sequences that are highly conserved among strains of SFFV. A pathogenic revertant (RI-rev) was isolated from one mouse that developed erythroblastosis 3 weeks after infection with RI. RI-rev contains a second-site *env* mutation that affects the same domain as the primary mutation does and that increases the size of the encoded glycoprotein. All pathogenic SFFVs encode glycoproteins that are expressed on cell surfaces, whereas the nonpathogenic glycoproteins are exclusively intracellular. The pathogenic SFFVs also specifically cause a weak interference to superinfection by dualtropic MuLVs. These results are compatible with the multidomain model for the structure of gp55 and suggest that processing of gp55 to plasma membranes is required for pathogenesis. The amino-terminal region of gp55 binds to dualtropic murine leukemia virus receptors, and this interaction is preserved in the SFFV mutants that cause erythroblastosis.

Friend spleen focus-forming virus (SFFV) is a defective murine C-type retrovirus that causes progressive erythroleukemia in mice and erythroblast proliferation in bone marrow cultures (4, 14, 16, 17, 53). Moreover, the SFFV *env* gene encodes a membrane glycoprotein with an apparent M_r of 55,000 (gp55) that is located on cell surfaces and within rough endoplasmic reticula (10, 20, 41, 42, 44, 45) and that is structurally related to the recombinant-type glycoproteins encoded by the *env* genes of dualtropic murine leukemia viruses (dt-MuLVs; some dt-MuLVs cause foci in mink cells and are called mink cell focus-forming viruses (MCFs) (2, 3, 7, 41, 50, 54). Accordingly, the SFFV *env* gene is a hybrid, with a conserved 5' region that is dualtropic specific and a less conserved 3' region that is related to an ecotropic MuLV *env* gene (2, 3, 7, 54). Previously we described spontaneous SFFV mutants that are nonleukemogenic or weakly leukemogenic (28, 43). Because these mutants contain substantial lesions in nonoverlapping regions of their *env* genes, we concluded that the entire folded gp55 glycoprotein was involved in SFFV pathogenesis (23, 27-29, 43).

Recently we developed a structural model of gp55 (27). According to this model, the dualtropic-specific (amino-terminal) and ecotropic-related regions of gp55 fold into separate domains that are connected by a flexible proline-

rich linker, and the ecotropic domain has a carboxy-terminal hydrophobic sequence that is embedded in the membrane. This model is consistent with nucleotide sequences of SFFV (2, 3, 7, 50) and dt-MuLV (1, 21, 22) *env* genes. The proline-rich region is a sequence of 50 amino acids that contains approximately 18 prolines (36%) and occurs at the junction of the dualtropic-specific and ecotropic-related amino acid sequences (2, 3, 7, 51, 54). Comparisons between viruses have implied that its sequence is highly variable, although its high proline content is conserved (8, 22). Also, disulfide bonds occur within the proposed folded domains but not between them, and the putative unfolded linker region contains a glutamyl peptide bond that is exceptionally susceptible to *Staphylococcus aureus* V8 protease (27). The resulting cleavage splits gp55 into its dualtropic-specific and ecotropic-related fragments (27). Furthermore, mutations that affect either domain of gp55 do not alter the proteolytic susceptibility of the other (27, 28, 43). Studies of the pathogenesis of the spontaneous mutants with lesions in different parts of their *env* genes also suggested that the dualtropic-specific domain might have an active site for causing erythroblast proliferation and that the other regions might perform ancillary functions related to glycoprotein stabilization or to subcellular localization (29). To further analyze gp55 structure and function, we have constructed SFFV *env* mutants with small in-phase linkers or with an in-phase deletion. These mutations cause focal lesions at specific sites in gp55 and have different effects on glycoprotein localization and

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FIG. 1. Construction of plasmids for SFFV mutagenesis. The starting plasmid (A) is a circularly permuted clone of the Lilly-Steeves strain of SFFV (25). Subgenomic fragments were subcloned and mutagenized by linker insertion or by a deletion, and the fragments were then religated to form colinear plasmid clones (B). One of the subclones (C) was used for linker insertion in the region that encodes the proline-rich sequences. (Pro)_n, Vector-derived sequences. The locations of seven in-phase mutations in the *env* genes of the SFFV mutants are shown in panel D. The numbering of the SFFV *env* gene is that of Wolff et al. (54). The NH₂ terminus shown is that of the processed glycoprotein which is encoded beginning at nucleotide 325. The signal sequence is encoded beginning at nucleotide 229. X-E is the proposed dualtropic-ecotropic recombination site in the *env* gene of the Lilly-Steeves strain of SFFV (8, 42). Although this site is downstream from the region of the gene that encodes the proline-rich linker, the intervening amino acid sequence is common to the dualtropic- and ecotropic-related *env* glycoproteins. Therefore the dualtropic-specific amino acid sequences all occur on the amino-terminal side of the proline-rich region (22, 27). (Pro)_n is the proline-rich region. In panel D, the region to the right of the X-E junction contains the ecotropic-specific sequences. p15E, p15E-specific portion of the glycoprotein; ■, hydrophobic region at the carboxy terminus of gp55 (2, 7, 54). The Sm1 and Sm2 mutants both contain a 45-bp *Sma*I-*Sma*I deletion (see text). Sm2 contains a 6-bp *Hpa*I linker added at the site of this deletion. RI, Pvu, and Bal are mutations at the *Eco*RI, *Pvu*II, and *Bal*I sites, respectively. Mutant Pst contains a 12-bp insert with a *Pst*I site that was added at the *Hin*PI site in the *env* gene. Mutant XB contains a net 18-bp insert at the same *Hin*PI site. (See Materials and Methods for a complete description of these constructions.)

SFFV pathogenesis. We present evidence that the pathogenic glycoproteins are expressed on cell surfaces, where they bind to a plasma membrane receptor.

MATERIALS AND METHODS

Construction of SFFV genomic and subgenomic plasmids for in vitro mutagenesis. Figure 1 shows the system of plasmids used for in vitro mutagenesis for the reconstruction of colinear SFFV mutant proviruses. The starting plasmid (Fig. 1A) is the circularly permuted molecular clone of F-SFFV (Lilly-Steeves polycythemia strain) that was cloned into the vector pBR322 by Linemeyer et al. (25). A *Hind*III-*Pst*I fragment containing the entire *env* gene and one copy of the long terminal repeat was ligated into the vector pSP65 through *Hind*III-*Pst*I sites, and the resulting plasmid (or a subclone derived from it) was used for in vitro mutagenesis of the *env* gene. A second *Pvu*II-*Hind*III fragment containing one copy of the long terminal repeat and the defective *gag* and *pol* regions of the SFFV genome was ligated into the vector pSP64 so that the two inserts would have an opposite orientation relative to the *Hind*III site in the SP6 vectors. Since the vector portions of pSP64 and pSP65 are identical, this enabled us to use a unique *Xmn*I site in the ampicillin resistance gene (*Amp*^r) in the vector together with the *Hind*III site to join the two fragments of the SFFV genome to form a plasmid that contains the entire SFFV genomic

sequence colinear to the proviral genome (Fig. 1B). When plasmids with this form were cotransfected into NIH 3T3 cells with pSV2neo plasmids at a mass ratio 20:1, usually about 20 to 60% of the Neo^r colonies that formed 2 weeks after transfection were actively synthesizing gp55.

Construction of in-phase mutations in the *env* gene by site-directed mutagenesis. Construction of in-phase insertions at restriction sites was based on the method of Stone et al. (48). For mutagenesis of the 5' xenotropic-related region or the 3' ecotropic-related region of the *env* gene, we used the *Sma*I, *Eco*RI, and *Pvu*II sites or *Bal*I site, respectively (Fig. 1D). Thus plasmids containing subgenomic fragments encoding the SFFV *env* gene were first linearized by a complete digestion with the restriction enzymes *Sma*I, *Eco*RI, *Pvu*II, or *Bal*I, and the full-sized, linearized molecules with blunt ends (digested by *Pvu*II or *Bal*I) were ligated with a phosphorylated dimeric *Xho*I linker (5'-CTCGAGCTCGAG-3'). The resulting plasmids were then digested with *Xho*I and recircularized to give a monomeric 6-base-pair (bp) (5'-CTCGAG-3') insert. The 12-bp dimeric *Xho*I linker was a generous gift of T. Pawson, University of British Columbia, Vancouver. The plasmids that had been cut with *Eco*RI contained 4-nucleotide cohesive ends that were filled in with Klenow fragment and then ligated to an 8-bp (5'-CCTC GAGG-3') *Xho*I linker (Collaborative Research, Inc.) to get a net 12-bp insert (5'-AATTCCTCGAGGAATT-3'). The plasmids that had been cut to completion with *Sma*I formed

linear DNAs with blunt ends that lacked the small 45-bp *Sma*I-to-*Sma*I fragment from the *env* gene (Fig. 1D). A portion of this linear DNA was ligated to form the *Sm*1 mutant. Another portion was mixed with a 6-bp *Hpa*I (5'-GTTAAC-3') insert before religation. This formed the *Sm*2 mutant. The other mutant SFFVs are called RI, Pvu, or Bal, corresponding to the locations of the in-phase mutations at the *Eco*RI, *Pvu*II, and *Bal*I sites in the *env* gene, respectively (Fig. 1D).

To implant an insert into the proline-rich region, we used the *Hin*PI site in the *env* gene (Fig. 1C). Since there are four additional *Hin*PI sites in the 3' long terminal repeat region (Fig. 1C) and many in the vector, it is not feasible to isolate the full-size linearized plasmid molecules by a partial *Hin*PI digestion. To circumvent this problem, the *Bam*HI-*Hin*PI fragment containing the 5' portion of the *env* gene was cloned into the compatible *Bam*HI-*Acc*I site of M13mp8 and the *Hin*PI-*Hin*PI fragment that contained the 3' portion of the *env* gene and part of the long terminal repeat was cloned into the *Acc*I site of M13mp9 in such an orientation that these two fragments could then be ligated back together through a *Pst*I site in the M13 polylinker to form an M13 recombinant bacteriophage with a 12-bp insert in the *env* gene. The insertion was verified by DNA sequencing of the M13 clone (data not shown). Further modification of the mutant involved cleavage with *Pst*I, treatment with S1 nuclease (to remove 4 bp), addition of a 6-bp *Xho*I linker and religation, cleavage with *Xho*I, and treatment with the Klenow fragment (to add 4 bp). The net effect was to add an additional 6 bp. Colinear constructs of the mutant viruses with the 12- and 18-bp inserts at the *Hin*PI sites in their *env* genes and no other differences from the wild-type sequence are called Pst and XB, respectively. A summary map of all seven mutants is shown in Fig. 1D.

DNA transfection and isolation of SFFV-producing cell lines. Transfections were performed as described by Graham et al. (15). At 1 day before transfection, 10^6 NIH 3T3 cells were seeded per 100-mm dish (Becton-Dickinson Labware). DNA precipitates were formed by dropwise addition of 20 μ g of purified plasmid containing the SFFV genome colinear to the proviral DNA plus 0.5 to 1 μ g of pSV2neo DNA in 1 ml of 250 mM CaCl_2 to an equal volume of a solution containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 2 mM NaH_2PO_4 , and 250 mM NaCl (pH 7.0) with concomitant vortexing. At 24 h after addition of the precipitate to the cells, the drug G418 (GIBCO Laboratories) was added to the culture medium at a concentration of 200 to 400 μ g/ml. At 2 weeks later, about 20 to 50 drug-resistant colonies appeared in each of the dishes. Individual colonies were screened for the synthesis of glycoproteins related to gp55 by immunoprecipitation of L-[^{35}S]methionine-labeled cell extracts with a goat antiserum made to Rauscher (R-MuLV) gp70 (44) followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography as described previously (33). SFFVs were then rescued from the selected cell lines by superinfection with R-MuLV. Successful rescue of the mutants as transmissible SFFVs was demonstrated by infecting fresh NIH 3T3 fibroblasts and analyzing them for glycoprotein synthesis as described above.

Transfections of mixed cultures of ψ 2 (30) and PA12 (32) cells were done by seeding 5×10^5 cells of each line onto 100-mm dishes 1 day before transfection. Transfections were performed as described above, except that no selectable marker was cotransfected and no drug selection was performed on the transfected cells. Virus spread was allowed to

occur for at least 2 weeks, and the level of SFFV gene expression was determined by Western blot analysis (5) with antisera that react with gp55.

Analysis of leukemogenicity. SFFV pathogenesis was analyzed by previously described methods (27–29, 33, 38). The wild-type and mutant SFFVs, prepared as described above, were injected into the tail veins of 4-week-old female NIH Swiss mice in a dose that contained 2×10^5 to 4×10^5 R-MuLV virions. Control mice were either uninfected or infected with R-MuLV alone. Mice were examined after 14 and 21 days for splenic enlargement and erythroblastosis (29, 38) and for hematocrits. Spleen homogenates were also used to infect NIH 3T3 fibroblasts that were subsequently analyzed for SFFV-encoded glycoproteins.

Processing of SFFV glycoproteins. The plasma membrane form of the SFFV glycoprotein has an apparent M_r of 58,000 to 65,000 and is called gp55^p (10, 20, 40–42, 44, 45). Its increased size compared with intracellular gp55 is caused by oligosaccharide processing in the Golgi apparatus (10, 44). Although gp55^p constitutes only approximately 5% of the total SFFV glycoprotein, it can be readily detected as a labeled component after lactoperoxidase-catalyzed ^{125}I iodination of intact cells (20, 41), as a labeled component larger than gp55 after incorporation of [^3H]glucosamine into cells (10, 20, 40, 42, 44, 45), or by protein blotting (5). For protein blotting, we used either an antiserum made to R-MuLV gp70 (45) or the 7C-10 rat monoclonal antibody that reacts specifically with gp70s of dt-MuLVs (52). The latter was generously donated by S. Ruscetti, National Cancer Institute, Bethesda, Md. Blots treated with monoclonal antibody were incubated with 0.2% rabbit anti-rat immunoglobulin (Litton Bionetics) before being incubated with ^{125}I -protein A.

Clonal assays for MCF infection. MCF 247 (18) was harvested from the culture medium of infected CCL64 mink lung or NIH 3T3 fibroblasts. Friend MCF(IE)-infected *Mus dunni* fibroblasts were generously given by B. Chesebro, Rocky Mountain Laboratories, Hamilton, Mont. (6). The infected murine fibroblasts were also transfected with pMSVneo (9), and transfectants that released MCF(neo) virions were isolated by cell cloning from the G418-resistant cell populations. Similarly, pMSVneo virions with an ecotropic envelope were harvested from the culture medium of ψ 2 cells (4, 30, 53) that had been transfected with the same plasmid.

Focal assays were used for measuring MCF infectivity in NIH 3T3 fibroblasts. One assay involves the immunological detection of cell surface MCF gp70 by using a polyclonal antiserum made to BALB virus 2 gp70 (donated by the Virus Diagnostics Branch, National Cancer Institute, Bethesda, Md.). The cultures were infected at a low cell density and were grown for 6 days before adsorption of the antibody and rosetting by using protein A-coated sheep erythrocytes as described previously (12). Although SFFV-infected cells with gp55 on their surfaces rosette weakly (45), the foci of MCF infection were heavily rosetted. The second assay is an infectious-center assay that involves the use of mink S+ L– cells (PG-4) (34). In that case, the infected NIH 3T3 cultures were irradiated with UV light and were then overlaid with PG-4 cells as described previously (37, 39). The PG-4 cells that became infected by nearby NIH 3T3 cells generated visible plaques in the PG-4 monolayers that formed in the dishes. The third assay involved the selection of G418-resistant colonies after infection with the MCF(neo) virus (see above). For this assay, portions of frozen viral stocks of known titer were used at initial concentrations that were

expected to produce approximately 120 foci per culture dish when assayed on uninfected NIH 3T3 fibroblasts. Titers of this initial sample were measured on different cell lines that were growing under identical conditions in petri dishes.

Although all three assays were useful, the MCF(neo) method is advantageous because the foci are clearer and because the assay is relatively amenable to proper interference controls. For example, the numbers of G418-resistant colonies were similar after infection of mink CCL64 and murine NIH 3T3 fibroblasts, and the formation of these colonies was efficiently prevented by prior infection with MCFs. Control studies were done by using the neo virus with an ecotropic envelope. As expected, the ecotropic virus infected only murine cells.

Other methods. The method for determining MuLV titer was described previously (10). All restriction endonucleases, the nuclease BAL 31, and the Klenow fragment of *Escherichia coli* DNA polymerase I were obtained from New England BioLabs, Inc., and were used under the conditions recommended by the supplier. DNA sequencing of the region of the in-phase insertion in the proline-rich encoding sequence of the *env* gene was performed by the dideoxy nucleotide method by using the protocol of Messing (31) as described previously (3). Helper-free SFFV with an ecotropic host range was isolated as described previously (4) by transfection of the colinear wild-type SFFV proviral molecular clone (Fig. 1) into ψ 2 cells (4, 30, 53). Statistical analysis of viral interference assays was done by the paired-comparisons *t* test (47).

RESULTS

Isolation and characterization of mutant SFFVs. A map of the SFFV *env* gene with the seven in-phase mutations (Sm1, Sm2, RI, Pvu, Pst, XB, and Bal) is shown in Fig. 1D. Table 1 indicates the positions and the amino acid sequence changes caused by the mutations.

The colinear SFFV plasmids (see Materials and Methods) were transfected into NIH 3T3 fibroblasts. Transfectant cell lines with stably integrated SFFVs were identified by their synthesis of gp55-related glycoproteins. Figure 2A and B shows a representative electrophoretic analysis of the glycoproteins that were synthesized in several of these cell lines after they had been superinfected with a helper R-MuLV. Mutants RI, Pvu, XB, and Bal (Fig. 2A, lanes 4, 8, and 9, and Fig. 2B, lane 2, respectively) encode glycoproteins with electrophoretic mobilities nearly identical to that of wild-type gp55 (Fig. 2A, lanes 2 and 3), whereas mutant Pst (Fig. 2A, lanes 1 and 5) encodes a glycoprotein with a slightly slower mobility. In contrast, the Sm1 and Sm2 mutants encode glycoproteins that electrophorese slightly faster than gp55 (see below). We previously observed that amino acid substitutions could alter the electrophoretic mobility of gp55 in the same gel system (23, 27), and similar results have been observed with other proteins (11, 46, 49). The small mobility changes could be caused by the amino acid sequence differences and by secondary effects on oligosaccharide processing.

Before analyzing the pathogenic properties of these mutants, we harvested viruses from the media of transfectant cells that had been superinfected with the R-MuLV helper and used aliquots to infect fresh NIH 3T3 fibroblasts. All of these cultures synthesized the SFFV-encoded glycoproteins, indicating that the SFFV mutants are transmissible and were present in substantial quantities in the rescued virus preparations. Representative data for the Sm1 and Sm2 mutants are shown in Fig. 2C, lanes 4 and 6.

FIG. 2. Electrophoretic analysis of L-[³⁵S]methionine-labeled glycoproteins encoded by SFFV mutants. Panels A and B are extracts from NIH 3T3 cell lines that had been transfected with plasmid DNAs encoding these SFFVs and then superinfected with MuLV. The superinfection was done to cause infectious transfer of SFFV and a severalfold increase in SFFV gene expression in the cultures. The labeled cell extracts were precipitated with goat antiserum against R-MuLV gp70 before being electrophoresed in a 10 to 20% polyacrylamide gel containing sodium dodecyl sulfate. (A) Lanes: 1 and 5, mutant Pst-8 (mutant Pst, transfectant line 8); 2 and 3, two independent cell lines transfected with the wild-type colinear SFFV plasmid (Wt-1 and Wt-2; also see Table 1); 4, mutant RI-4; 6, blank; 7, cells infected with R-MuLV only; 8, mutant Pvu-1; 9, mutant XB-14. (B) Lanes: 1, cells infected with R-MuLV only; 2, mutant Bal-8. (C) Glycoproteins encoded by the Sm1, Sm2, and RI-rev SFFVs. The methods were the same as for panels A and B. The labeled extracts were immunoprecipitated with either the goat antiserum to R-MuLV gp70 (lanes 1 to 4 and 6) or a normal goat serum (lane 5). Lane 1, nonproducer normal rat kidney fibroblasts infected with the wild-type SFFV. Lane 2, NIH 3T3 fibroblasts infected with the RI-rev that was recovered from an erythroblastic spleen. This revertant encodes a gp60 glycoprotein (see text). Lane 3, virus from the normal-sized spleen of a mouse infected with the nonpathogenic Sm1 mutant was recovered in NIH 3T3 fibroblasts. No SFFV-encoded glycoprotein was recovered. Lane 4, NIH 3T3 fibroblasts transfected with the Sm1 mutant plasmid were subsequently superinfected with R-MuLV. A portion of the rescued virus was infected into NIH 3T3 fibroblasts, and the resulting cells were analyzed in this lane. The results indicate that the Sm1 SFFV is transmissible. Lane 5, a normal goat serum control for the sample in lane 6. Lane 6, similar to lane 4 except with the Sm2 mutant. The glycoproteins encoded by Sm1 and Sm2 are slightly smaller than gp55.

Aliquots of the virus preparations were then injected into 4- to 6-week-old female NIH Swiss mice, and pathogenesis was assessed 2 to 3 weeks later (Table 1). As indicated by erythroblastic splenomegaly, the Bal, XB, Pst, and Pvu mutants are highly pathogenic. On the contrary, Sm1, Sm2, and RI are relatively nonpathogenic.

To confirm that the mutant SFFVs were replicating in vivo, we homogenized the spleens of infected mice and used the homogenates to infect NIH 3T3 fibroblasts. These cultures were then analyzed for synthesis of virus-encoded glycoproteins. Viruses that encode gp55-related glycoproteins were recovered from all of the mice with erythroblastosis (Fig. 3). However, mice infected only with R-MuLV lacked both splenomegaly (Table 1) and any SFFV-like virus (Fig. 3A, lane 7). Similarly, the Sm1, Sm2, and RI mutants did not cause splenomegaly and were not recovered from the spleens (Fig. 3A, lane 3 and Fig. 2C, lane 3, for RI and Sm1, respectively). This is consistent with earlier studies of nonleukemogenic mutant SFFVs and of wild-type SFFVs in Fv-2 restricted hosts: replication-defective retroviruses that do not cause neoplasms are lost by dilution in vivo (28, 43). The Pst mutant recovered from diseased mice encodes a glycoprotein with a lower mobility than gp55 (Fig. 3A and B,

TABLE 1. Pathogenic properties of mutant SFFVs

SFFV	Locus ^a	Residue change		Virus-producer cell line ^b	Spleen wt (g) (hematocrit) ^b on day:	
		From	To		14	21
Sm1	513–558	-Thr-Pro-(aa) ₁₅ -Gly-His-	-Thr-Pro-Gly-His-	Sm1-1	0.15	0.11 (42%)
Sm2	513–558	-Thr-Pro-(aa) ₁₅ -Gly-His-	-Thr-Pro-(Val-Asn)-Gly-His-		0.13	0.25 (41%)
				Sm2-5	0.16	0.14 (44%)
				Sm2-11	0.12	0.17 (39%)
RI	785	-Leu-Glu-Phe-Thr-	-Leu-Glu-(Phe-Leu-Glu-Glu)-Phe-Thr-		0.15	0.14 (44%)
				RI-1	0.09	0.17 (45%)
					0.14	0.15
Pvu	813	-Ala-Ser-Trp-Asp-	-Ala-Ser-(Ser-Ser)-Trp-Asp-		0.15	0.20
				RI-4	0.20	
					0.17	0.15 (49%)
Pst	1017	-Pro-Gly-Ala-Ala-	-Pro-Gly-(Asp-Leu-Gln-Val)-Ala-Ala-		0.18	0.59 (48%)
				Pvu-1	0.74	1.34 (48%)
				Pvu-4	0.75	1.78 (60%)
XB	1017	-Pro-Gly-Ala-Ala-	-Pro-Gly-(Asp-Pro-Arg-Ser-Arg-Val)-Ala-Ala-		0.65	
				Pst-8	0.75	
					1.21	0.86 (47%)
Bal	1282	-MetAlaLysLeu-	-MetAla(ArgGly)LysLeu-		1.49	2.25 (54%)
				Pst-12	1.00	2.70 (71%)
					1.14	
RI-rev	–	– ^c	– ^c		0.46	1.26 (57%)
				XB-9	1.20	2.07 (65%)
				XB-14	1.40	
Wild-type F-SFFV	–	–	–		0.54	2.16 (65%)
				Bal-8	1.00	2.49 (31%)
					1.38	1.48 (65%)
R-MuLV	–	–	–		0.25	0.17 (43%)
				RI-rev	0.40	0.66 (49%)
				Wt-1	0.56	1.48 (63%)
Uninfected	–	–	–		0.59	
				Wt-2	0.44	0.81 (44%)
					1.11	1.70 (65%)
Uninfected	–	–	–		0.25	0.12 (47%)
				Rwt	0.25	
					0.15	0.09 (48%)
Uninfected	–	–	–		0.15	0.10 (40%)
					0.15	
					0.15	

^a The loci in the F-SFFV *env* gene, from Wolff et al. (54). Only mutant Pst was verified by sequencing. The other mutants were verified by restriction mapping. The locus for the second-site *env* mutation RI-rev is uncertain (see text).

^b Hematocrits were measured by standard methods. Although one mouse infected with the Bal mutant was anemic, as indicated by its low hematocrit, the other mice infected with this virus were clearly polycythemic. We have observed previously and with other viruses from this study (see data for Pvu-1, Pst, and Wt-2), that polycythemic SFFVs occasionally either induce anemia or fail to induce polycythemia. Presumably this could occur because of secondary infection or bone marrow failure or excessive filtration in the enlarged spleen. However, anemic strains of SFFV do not in our experience ever produce polycythemia. We conclude that the Pvu, Pst, XB, Bal, and wild-type SFFV constructs all cause polycythemia.

^c NIH 3T3 cell lines transfected with various SFFV-containing plasmids. Mutant and wild-type SFFVs were harvested from the culture supernatants of these cell lines after superinfection with R-MuLV. These harvests were then used for pathogenesis studies. RI-rev was harvested from the culture medium of NIH 3T3 fibroblasts that had been infected with virus from the spleen of a mouse that developed splenomegaly 3 to 4 weeks after infection with RI. The RI-rev SFFV contains a second-site *env* mutation, but its precise locus has not been determined. The results show that RI-rev causes mild splenomegaly (see text). Rwt is a line of NIH 3T3 fibroblasts infected with R-MuLV. The same R-MuLV was used to rescue the SFFVs from the transfected cell lines.

lanes 5), consistent with the results in Fig. 2. This suggests that the mutations can be retained during the rapid replications and pathogenic selection that occurs in vivo.

Note that one infected with the RI mutant had a mild splenomegaly after 3 weeks (Table 1). In a more extensive subsequent study, we found that approximately 20% of mice infected with RI developed erythroblastosis after a lag of 3 to 4 weeks (results not shown), and we recovered virus from one of these enlarged spleens. Previously we observed that nonpathogenic SFFV *env* mutants often form revertants several weeks after injection into newborns and that the revertants contain second-site *env* mutations (27). The virus recovered in this case encoded a glycoprotein with apparent M_r of 60,000 (gp60) (Fig. 3B, lane 4), substantially larger than the gp55 encoded by the RI mutant (Fig. 2A, lane 4). Moreover, the recovered virus caused a uniform splenomeg-

aly without any lag when injected into adult mice (Table 1). However, this disease was relatively mild. The splenomegaly was less than with the other pathogenic SFFV mutants, and some mice seemed to recover. In addition, polycythemia was not observed with this mutant.

These results suggest that the recovered virus is an RI revertant (RI-rev) with a second-site *env* mutation that partially restores pathogenic function to the encoded glycoprotein. Although the RI mutant gp55 glycoprotein reacts with the 7C-10 monoclonal antibody, the gp60 encoded by the RI-rev lacks this reactivity (data not shown). This monoclonal antibody reacts specifically with an epitope in the dualtropic-specific region of SFFV and dt-MuLV *env* glycoproteins (52). Therefore both the primary and secondary mutations in the RI-revertant affect the dualtropic-specific region.

FIG. 3. Electrophoretic analysis of glycoproteins encoded by viruses recovered from the spleens of infected mice. At 2 weeks after infection, the spleen homogenates were used to infect NIH 3T3 fibroblasts. The fibroblasts were later labeled by a 2-h incorporation of L-[³⁵S]methionine, and the cell extracts were immunoprecipitated with antiserum made to R-MuLV gp70. The spleens were from mice that had been infected with R-MuLV plus the indicated SFFV. (A) Lanes: 1, wild-type SFFV (Wt-1; see Table 1); 2, XB-14; 3, RI-4; 5, Pst-8; 6, Pvu-1; 7, R-MuLV only; 4, labeled extract from normal rat kidney fibroblasts nonproductively infected with wild-type SFFV in the absence of any helper. (B) Another study of recovered viruses: Lanes: 1, control uninfected cells; 2, cells infected with wild-type SFFV without helper (nonproducer clone 17); 3, recovered mutant XB; 4, mice were infected with the nonpathogenic RI mutant, but one mouse developed a mild splenomegaly from which this RI-rev virus was recovered (see also Fig. 2C, lane 2 and Table 1); 5, recovered mutant Pst; 6, recovered mutant Pvu; 7, recovered mutant Bal.

Processing of glycoproteins encoded by the SFFV mutants. The plasma membrane form of the SFFV glycoprotein (called gp55^P) has an apparent M_r 58,000 to 65,000. Its increased size compared with the gp55 component that remains primarily in the rough endoplasmic reticulum (13) is caused by processing of oligosaccharides in the Golgi apparatus (10, 20, 44). The gp55^P-type components encoded by the SFFV mutants were identified by lactoperoxidase-catalyzed ¹²⁵I iodination of cells and as distinct labeled electrophoretic components after [³H]glucosamine labeling of cells or protein blotting of cell extracts (see below). Each of these techniques was used in at least three experiments involving all of the SFFV mutants and both the polyclonal antiserum made to R-MuLV gp70 and the 7C-10 monoclonal antibody that is specific for dt-MuLV gp70s (52).

Figure 4 shows an example of the ¹²⁵I iodination results with the polyclonal antiserum. As expected, NIH 3T3 fibroblasts infected only with R-MuLV contain gp70 but no gp55^P (lane 11), whereas cells infected only with SFFV contain gp55^P but no gp70 (lane 5). Examination of these results clearly establishes that SFFV-encoded plasma membrane components occur on cells that contain the pathogenic wild-type (lanes 5 and 10), RI-rev (lane 1), XB (lane 7), Pst (lane 6), and Bal (lane 9) mutants but not to a detectable extent on cells that contain the nonpathogenic RI (lanes 2 and 3), Sm1 (lane 12), and Sm2 (lane 13) mutants. The RI-rev results are striking because pathogenic reversion was associated with a recovery in processing of the encoded glycoprotein (gp60^P) to plasma membranes. Although the Pvu results were borderline in this experiment, our other iodination results clearly showed the presence of this glycoprotein on the cell surfaces (see below). We emphasize that even relatively long exposures of the autoradiograms failed to indicate gp55^P-type components encoded by the nonleukemogenic mutants (see Discussion).

As mentioned above, gp55^P-type components could also be detected by protein blotting of cell extracts. Figure 5A and B show analyses of the same extracts by using the antiserum made to R-MuLV gp70 and the 7C-10 monoclonal antibody, respectively. The results demonstrate that the antiserum to R-MuLV gp70 cross-reacts with all of the glycoproteins, whereas the 7C-10 monoclonal antibody reacts only with the gp55- and gp55^P-type components. The 7C-10 monoclonal antibody results demonstrate the utility of this method for visualization of the gp55^P components.

A typical protein-blotting analysis involving the use of the 7C-10 monoclonal antibody to detect the gp55 components in the cell lines that contained the SFFV mutants is shown in Fig. 6. The results of different experiments indicated clearly that larger processed gp55^P-type components were synthesized in cells that contained the wild type and the XB, Pst, Pvu, and Bal mutants, but not in cells that contained the RI, Sm1, and Sm2 mutants. However, the proportion of the more processed glycoprotein component appeared reproducibly relatively lower for the Pvu and Bal mutants (lanes 2 and 4, respectively) than for the other pathogenic mutants, XB and Pst (lanes 1 and 3, respectively). Results obtained with the [³H]glucosamine-labeled glycoproteins were concordant with the ¹²⁵I iodination and protein-blotting results.

Although the above results were informative, we were somewhat concerned about the relatively small amounts of glycoproteins synthesized in the cells that had been transfected with the Sm1, Sm2, and RI nonpathogenic SFFV mutant plasmids (see Discussion). Moreover, gp55^P constitutes only approximately 5% of the glycoprotein encoded by the wild-type SFFV. Therefore we developed another strategy to amplify the expression of these mutant DNAs. This involved transfection of the mutant SFFV DNAs into a

FIG. 4. Analysis of viral glycoproteins on the surface membranes of NIH 3T3 fibroblasts, as indicated by lactoperoxidase-catalyzed ¹²⁵I iodination. The fibroblast monolayers were labeled with ¹²⁵I as described in Materials and Methods, and the cell lysates were subsequently analyzed for radioactive viral glycoproteins by immunoprecipitation with a polyclonal antiserum made to R-MuLV gp70 and by electrophoresis followed by autoradiography of the dried gel. Except for those in lanes 4 and 5, all of the cells contained an R-MuLV helper (for the reasons described in the legend to Fig. 2), and therefore contained cell surface gp70. The SFFVs in the cells were as follows: lane 1, RI-rev (note the gp60^P component); lane 2, cells transfected with the RI mutant (lane RI-1); lane 3, transfected with RI (lane RI-4); lane 4, uninfected cells; lane 5, a nonproducer line infected only with wild-type SFFV (note the gp55^P component); lane 6, cells infected with the Pst mutant; lane 7, cells infected with mutant XB; lane 8, cells infected with mutant Pvu; lane 9, cells infected with mutant Bal; lane 10, cells infected with wild-type Friend virus complex; lane 11, cells infected only with the R-MuLV helper (these cells lack any labeled components in the gp55^P region of the gel); lane 12, cells transfected with mutant Sm1; lane 13, cells transfected with mutant Sm2. SFFV-encoded plasma membrane glycoprotein components are clearly evident in lanes 1, 5, 6, 7, 9, and 10.

FIG. 5. Characterization of the protein (Western) blotting technique for detection of *env* glycoproteins encoded by wild-type ecotropic MuLV and by SFFV. Unlabeled extracts from purified MuLV or from cells were analyzed by electrophoresis followed by protein transfer onto nitrocellulose membranes. The glycoproteins were visualized by reaction with either the antiserum to R-MuLV gp70 (A) or the rat 7C-10 monoclonal antibody (B and C). After reaction with the 7C-10 monoclonal antibody, a secondary reaction was performed with rabbit anti-rat immunoglobulin. Then the blots were reacted with ^{125}I -labeled protein A. The extracts in panels A and B were both as follows: lane 1, purified ecotropic AKR MuLV virions; lane 2, NIH 3T3 cells infected with R-MuLV; lane 3, normal rat kidney cells nonproductively infected with SFFV (NRK clone 1 cells); lane 4, nonproducer NIH 3T3 cells infected with wild-type f-SFFV (clone 17); lane 5, nonproducer SFFV-infected NIH 3T3 cell line CS-5; lane 7, NIH 3T3 cells infected with a mixture of R-MuLV and SFFV (R7 NIH/A cell line). (C) Different SFFV-infected nonproducer NIH 3T3 cell clones prepared with molecularly cloned helper-free SFFV (see text). The extracts were from the following cell clones: lane 1, CS-1; lane 2, CS-2; lane 3, CS-4; lane 4, CS-5. The four cell clones express widely different quantities of gp55 and gp55^P. This blot was developed with the 7C-10 monoclonal antibody.

mixed culture of the ecotropic-packaging cell line ψ 2 (30) and of the amphotropic-packaging cell line PA12 (32) (see Materials and Methods). The helper-free SFFV released from either cell type infects the other, and the virus then spreads and amplifies indefinitely by a process of back-and-forth

FIG. 6. Protein blotting of glycoproteins encoded by the wild-type and mutant SFFVs. Extracts from NIH 3T3 fibroblasts were analyzed as described for Fig. 5, with the 7C-10 monoclonal antibody. Lanes: 1, cells infected with mutant XB; 2, cells infected with mutant Bal; 3, cells infected with mutant Pst; 4, cells infected with mutant Pvu; 5 and 6, cells transfected with mutant RI (line RI-4); 7, cells transfected with mutant Sm1; 8, cells transfected with mutant Sm2. Larger gp55^P-type components were present in lanes 1 to 4, although the proportion of this component in lane 2 (mutant Bal) was lower than in the adjacent lanes. No corresponding larger components were seen in lanes 5 to 8, even after a threefold-longer exposure. These and other results suggest that the glycoproteins encoded by the nonpathogenic mutants are not processed to the plasma membranes (see text). The RI-rev mutant was not included in the experiment because it does not react with the 7C-10 monoclonal antibody (see text).

FIG. 7. Detection of Western blotting of the *env* glycoproteins encoded by mutant and wild-type SFFVs. The blot was prepared as described for Fig. 5. Protein extracts were prepared from cocultivated ψ 2 and PA12 cells that had been transfected at least 2 weeks previously with 10 μg of colinear plasmid DNAs encoding these SFFVs. The resulting expression of SFFVs was higher (ca. 50-fold) than for the NIH 3T3 transfectants (Fig. 2). The 7C-10 monoclonal antibody was used to detect gp55 and gp55^P. Lanes: 1, wild type; 2, mutant Pst; 3, mutant RI; 4, mutant Sm1; 5, mutant Sm2. This blot was exposed to film for 3 days at -80°C with an intensifying screen. Longer exposures failed to detect larger components in lanes 3 to 5. The larger pair of bands present most prominently in lane 3 are components from PA12 cells and can be detected in protein extracts from untransfected PA12 cells. These results establish that the nonpathogenic mutants (lanes 3 to 5) do not encode gp55 that is processed to the plasma membranes.

infection between the two cell types. Although this technique is still in a developmental phase, we have used it to successfully recover wild-type SFFV and the Pst, Sm1, Sm2, and RI mutants. However, we have not obtained virus from these mixed cultures in the absence of helper virus contamination. Consequently, the mixed cell cultures have not yet been applicable to the interference analyses described below. Nevertheless, these mixed cultures have provided exceptionally clear protein-blotting results with the 7C-10 monoclonal antibody (Fig. 7). These data strongly support the conclusion that all of the nonpathogenic SFFV *env* mutants (i.e., Sm1, Sm2, and RI) fail to encode gp55^P-type glycoprotein components.

Interference to MCF superinfection caused by SFFV. The evidence described above suggests that pathogenesis by SFFV may require the processing of gp55^P to plasma membranes and, in agreement with previous evidence (29), that the dualtropic-specific domain may contain an active site. Because this domain is nearly identical in sequence to the corresponding region of dt-MuLV gp70s (2, 3, 7, 22, 51, 54); see Discussion) and because the latter region forms a site that binds to dt-MuLV cell surface receptors (19, 22, 36, 37, 39), we hypothesized that gp55 might also bind to these receptors. Indeed, after this work was initiated, Chesebro and Wehrly reported that a polycythemia strain of SFFV strongly interferes with dt-MuLV superinfection (i.e., by 3 orders of magnitude) (6). Their conclusion was based on analysis of one nonproducer Sc-1 cell line by using Friend MCF(IE) as the superinfecting dualtropic virus.

Our initial studies involved the use of MCF 247 as the superinfecting virus and several different NIH 3T3 fibroblast lines that had been previously infected at a limiting dilution with the wild-type polycythemia-inducing Lilly-Steeves

TABLE 2. Relationship between the quantity of SFFV gene expression and interference to infection by MCFs

Cell line ^a	Cellular amt of gp55 ^b	MCF(neo) titers (foci/dish) ^c in:	
		Expt 1	Expt 2
CS-3 (uninfected)	None	82, 88	104, 96
CS-1	Small	60, 55	20, 26
CS-4	Small	53, 47	30, 26
CS-2	Large	7, 13	2, 7
CS-5	Large	10, 23	1, 0
MCF-infected	None		0, 1

^a The CS lines were cloned from a culture of NIH 3T3 fibroblasts after infection with helper-free SFFV. These cell clones were either uninfected (CS-3) or infected with the SFFV (see text). The MCF-infected NIH 3T3 cells contain MCF 247.

^b Screening for the quantity of gp55 was done by protein blotting with the 7C-10 monoclonal antibody (Fig. 5). A nearby lane on the electrophoresis gel contained an extract of NRK clone 1 cells (20, 43, 45), a standard high-expressor, nonproducer cell line infected with the wild-type Lilly-Steeves polycythemia-inducing SFFV, which was the source for the SFFV molecular clone (25). Approximately 0.1% of the protein in these cells is gp55 (unpublished estimate). The relative quantities of gp55 in the CS lines was visually estimated in comparison with the NRK clone 1 cells. CS-5 and CS-2 synthesize gp55 in amounts similar to NRK clone 1. CS-1 and CS-4 contain approximately 10 and 20% this amount of gp55, respectively. In our experience, high expressors such as CS-5 are very rare in clonally infected NIH 3T3 fibroblasts. The SFFV nonproducers (10) analyzed in our initial studies (see text) are similar to CS-1 and CS-4 in level of viral gene expression (e.g., Fig. 5A).

^c MCF(neo) titers (in foci per dish) were estimated by a titration series (see Materials and Methods). Each recorded number is the titer for a separate titration series. Experiments 1 and 2 were done with MCF(neo) from NIH 3T3 fibroblasts that were infected with MCF 247 and Friend MCF(IE), respectively.

strain of SFFV (10, 43). Using an immunological erythrocyte rosette assay to detect foci of MCF superinfection (12), we observed an interference of only 2- to 10-fold in different nonproducer cell lines (results not shown). This interference was much weaker than that reported previously (6). Subsequently we observed a similarly weak interference by using the infectious-centers assay and the MCF(neo) assay (see below). Control experiments involving the use of an Eco(neo) virus indicated that SFFV does not interfere with superinfection by ecotropic MuLVs. Moreover, infection by MCF(neo) was efficiently blocked by prior infection with MCFs (see below). Recloning of the SFFV-infected cell lines also established that the weakness of their interference to MCFs was not caused by shutoff of SFFV gene expression in a proportion of the cells.

Thus our initial studies suggested that SFFV causes only a weak 2- to 10-fold interference to MCF 247 superinfection in NIH 3T3 fibroblasts. Moreover, in another study we observed a similarly weak interference in Sc-1 cells (results not shown). To independently verify this interference and to analyze the cause for the variability among different SFFV-infected cell clones, we prepared new SFFV-infected nonproducer cell clones by infecting them with a helper-free SFFV from ψ 2 cells that had been transfected with the wild-type colinear SFFV proviral DNA (see Materials and Methods). After infection at a multiplicity of approximately 0.7, the NIH 3T3 cells were cloned, and several cell clones (CS1-5) were immediately analyzed for gp55 by protein blotting and for their susceptibility to MCF(neo) superinfection (Table 2). The protein-blotting results are shown in Fig. 5C. As reported previously, clonally infected cells synthesize widely different quantities of retroviral proteins (10). The relative quantities of gp55 in the clones increased in the order clone 1 (lowest), clone 4, and clones 2 and 5 (highest).

Clone 3 was uninfected. The degree of interference to MCF(neo) superinfection increased correspondingly (Table 2). Similar results were obtained by using the pMSVneo virus coated with MCF 247 or with Friend MCF(IE) (experiments 1 and 2, respectively). These results suggest that SFFV weakly interferes with MCF superinfection in NIH 3T3 fibroblasts and that the extent of interference may be related to the quantity of gp55 in the cells. We infer that the interference is incomplete because the MCF receptors are not saturated with gp55.

Interference by the SFFV mutants to MCF(neo). The SFFV *env* mutants that were pathogenic replicated extensively in mice and were recovered as high expressors in NIH 3T3 fibroblasts (Fig. 3). Because these cell lines produced the mutant glycoproteins in large amounts, it was possible to analyze them for their susceptibilities to superinfection by MCF(neo) (Table 3). The results suggest that the pathogenic SFFV mutants XB, Pst, Pvu, and Bal all cause a weak but significant interference to MCF(neo) ($P < 0.05$; Table 3). Although the results with RI-rev are not significant, it is important to realize that this virus is also only mildly pathogenic (Table 1).

DISCUSSION

The in-phase SFFV *env* mutants. This paper describes the properties of seven SFFV mutants that were constructed either by implanting small in-phase inserts into different sites in their *env* genes or by performing a small in-phase deletion. These mutations differ from those previously described (23, 24, 26, 28, 29, 43) in that they cause small focal lesions at specific positions in gp55, rather than multiple alterations (23), deletions and frameshifts (24, 29), or premature terminations (23). Moreover, four of the in-phase mutants are highly pathogenic and three are nonpathogenic (Table 1). In addition, we describe the weakly pathogenic RI revertant that formed spontaneously in a mouse infected with RI.

TABLE 3. Interference to MCF(neo) caused by SFFV mutants in NIH 3T3 fibroblasts

Virus present ^a	MCF(neo) titers (foci/dish) ^b in:			
	Expt 1	Expt 2	Expt 3 ^c	Expt 4 ^c
MuLV only	128	123	105, 99	86, 87
MuLV + wild-type SFFV	34	24	19, 18	18, 12
Wild-type SFFV	79	39	19, 35	9, 11
MuLV + XB	15	16	23, 15	8, 2
MuLV + Pst	43	57	36, 24	16, 16
MuLV + Pvu	81	70	50, 67	28, 44
MuLV + Bal	84	45	63, 48	68, 78
MuLV + RI-rev	168	101	63, 87	68, 56

^a NIH 3T3 fibroblasts infected with the viruses indicated. The helper is R-MuLV. The cell line containing MuLV plus wild-type SFFV is R7NIH/A. The cell line with only wild-type SFFV is clone 17 (10). The other lines are the high-expressors containing viruses recovered from spleens of infected mice (Fig. 4 and 5).

^b MCF(neo) titers (in foci per dish) were estimated by a titration series (see Materials and Methods). Each recorded number is the result of a separate titration. Experiments 1, 2, and 3 were done with MCF(neo) from cells with MCF 247. Experiment 4 was done with virus from cells with Friend MCF(IE). Statistical analysis of these results was done by the paired-comparisons *t* test (47). The data for XB, Pst, Pvu, and Bal indicate a highly significant interference to MCFs ($P < 0.05$). The results with RI-rev are not significant.

^c Experiments 3 and 4 in this table were done in combination with experiments 1 and 2 in Table 3, respectively, and the same MCF(neo) virus preparations were used. Therefore the titers observed in these experiments are directly comparable. These comparisons support other evidence that ecotropic MuLV alone does not interfere with MCF superinfection. An uninfected NIH 3T3 cell control experiment was also performed in experiment 2 of this table, and the titer obtained was 96 foci per dish.

A previous report described the deletion-insertion mutagenesis of a circularly permuted molecular clone of SFFV (Fig. 1A) (24). The mutagenized permuted DNAs were cotransfected into fibroblasts with molecularly cloned helper MuLV DNA, and pathogenic recombinant SFFVs that were released from the cells were identified by their ability to cause erythroblastosis in mice. One large deletion that was located entirely within the *env* gene region of mutant 22-23 eliminated the ability of the DNA to form pathogenic SFFV recombinants, whereas an *env* insertion in the 22-62 mutant DNA gave anomalous results (24). As discussed previously (43), the results in the earlier report (24) could be explained if the 22-23 deletion simply interfered with the process of recombination, if the deletion interfered with replication or intercellular transmission of the viral genome, or if, as suggested previously (24), the mutation was in an oncogene. The results for the 22-62 mutant do not clarify this issue, although they demonstrate that the assay system is complex. It is accepted that the effects of mutations can be conclusively studied only with recovered mutants that have a demonstrably heritable phenotype. However, the phenotype of the putative 22-23 mutant was inferred only from the negative results. The colinear reconstruction and characterization procedures described here have enabled us to solve these problems and to recover site-directed SFFV mutants as transmissible viruses.

The Bal insert in the ecotropic-related region did not reduce pathogenesis (Table 1). Similarly, a complete elimination of this carboxy-terminal region in one spontaneous mutant did not eliminate virus-induced erythroblastosis (28, 29). It has been suggested that this Bal region of the *env* gene might be responsible for the differences between SFFVs that induce anemia and those that cause polycythemia (3, 42, 51). However, the Bal insert did not convert the SFFV to an anemia strain (Table 1).

The Pst and XB mutants contain inserts that alter the proline-rich region of gp55 (Fig. 1; Table 1). This region contains a stretch of 50 amino acids, of which approximately 18 (36%) are proline (2, 3, 21, 22, 54). Because the sequence of this region is hypervariable, although its high proline content is conserved (8, 22), and because its susceptibility to proteolytic digestion is consistent with the idea that it lacks a stable secondary structure (27), we have proposed that it forms a flexible tether that links the dualtropic-specific and ecotropic-related domains (27, 29). Our observation that the Pst and XB mutants are fully pathogenic is compatible with the proposed linker function of this region.

Previously we reported that nonpathogenic SFFV *env* mutants often form spontaneous revertants after a lag when injected into newborn NIH Swiss mice (27). These revertants contain secondary *env* mutations that affect the same putative domain as the primary mutation and that restore leukemogenic function to the glycoprotein (23, 27). In this study we found that the nonpathogenic RI mutant occasionally forms such revertants after injection into 4- to 6-week-old NIH Swiss mice; and from the enlarged spleen of such a mouse we isolated the RI revertant that encodes a gp60 (Fig. 3) and that causes a rapid but mild erythroblastosis (Table 1). In agreement with the hypothesis that the dualtropic-specific and ecotropic-related regions of gp55 form independently folded domains, our results suggest that both the primary RI mutation and its intragenic suppressor mutation affect the same dualtropic-specific region of the glycoprotein. The secondary mutation eliminated glycoprotein reactivity with the 7C-10 monoclonal antibody (see Results).

Evidence that the plasma membrane component of gp55 is

required for pathogenesis. It is striking that the pathogenic SFFV *env* mutants all encode glycoproteins that are processed through the Golgi apparatus to form larger gp55^P-type components that are located on cell surfaces, whereas the nonpathogenic glycoproteins did not appear to be processed to cell surfaces. For example, gp60 encoded by RI-rev is expressed on plasma membranes, whereas gp55 encoded by the nonpathogenic RI mutant appeared to be present only in intracellular organelles. These results strongly support earlier evidence (28, 43) that pathogenesis requires the glycoprotein component that is processed through the Golgi apparatus to the plasma membranes. Since the plasma membrane component is shed at least partially into the culture medium (35), it is possible that this shed derivative performs the critical pathogenic function.

Evidence for gp55 interaction with a plasma membrane receptor. The structure of gp55 differs from that of some other oncogenic membrane proteins because gp55 lacks a cytosolic domain but nevertheless must transduce a mitogenic signal into the cells. Our results suggest that the plasma membrane gp55^P component is essential for pathogenesis and that the completely extracellular dualtropic-specific domain of this glycoprotein may contain an active site (29). Moreover, this domain of gp55 is nearly identical in sequence to the region of dt-MuLV gp70s that forms the site for binding to cell surface MCF receptors (2, 3, 7, 22, 51, 54). Although different dt-MuLVs differ in the lengths of their dualtropic-specific *env* gene sequences, they always contain as a minimum this region between the amino terminus and the proline-rich sequences (22), in agreement with the hypothesis that this region can independently fold to form the receptor-binding site. These considerations all support the hypothesis that the dualtropic-specific domain of gp55 on its flexible proline-rich tether may bind to a plasma membrane receptor to cause pathogenesis. Conceivably, such a leukemogenic receptor could be related or identical to the MCF receptors (see below).

We analyzed this hypothesis by determining whether SFFVs cause interference to superinfection by MCFs (Tables 2 and 3). Although our results suggest that the interference to MCFs caused by wild-type SFFV is much weaker in NIH 3T3 fibroblasts than previously reported by Chesebro and Wehrly for Sc-1 cells (6), we basically agree that the interference is specific and significant. Moreover, protein-blotting studies established that none of our cell lines contained any MCFs or MCF glycoproteins. Therefore we infer that gp55 interacts specifically with these MCF receptors. Moreover, the extent of interference seems to correlate with the quantity of gp55^P synthesized in the cells (Fig. 5; Table 2). This implies that the MCF receptors (36, 37) are not saturated with gp55^P, either because of a receptor excess or because of a weak binding affinity.

In addition, our results suggest that the highly pathogenic SFFV mutants (i.e., Pvu, XB, Pst, and Bal) all cause this interference to MCF superinfection (Table 3). The RI-rev SFFV is only mildly pathogenic (Table 1), and much more extensive studies that include statistical analyses would be required to determine whether it might cause a weak interference to MCFs. Therefore our results are consistent with the hypothesis that the ability of gp55 to bind to plasma membrane MCF receptors is related to SFFV pathogenic activity.

Unfortunately, we have been unable to analyze MCF interference by the nonpathogenic SFFV mutants RI, Sm1, and Sm2. As described above, only the pathogenic SFFVs were amplified in vivo and recovered as high expressers in

fibroblasts (Fig. 5). The nonpathogenic mutants have been available only in transfectant cell lines that express the SFFVs relatively weakly or in recently prepared cell lines that also contain a complex mixture of helper viruses that are not yet completely characterized (Fig. 7). We are currently striving to overcome this problem. In any case, it seems unlikely that the RI, Sm1, and Sm2 glycoproteins, which are not processed to cell surfaces, could interfere with MCF superinfection.

A model for gp55 function. On the basis of these results, we propose that gp55^p contains an active site in its dual-tropic-specific domain that binds to a leukemogenic target receptor on erythroblast plasma membranes to cause mitogenesis. Because the pathogenically active SFFV mutants encode glycoproteins that interact with MCF receptors on NIH 3T3 fibroblasts, we propose that the leukemogenic receptors may be related or identical to MCF receptors. Conceivably the leukemogenic and MCF receptors could be related members of a polymorphic family of plasma membrane proteins. A corollary of this model is that the gp70s of nonleukemogenic MCF viruses either could fail to bind to the leukemogenic receptors (if these were related but not identical to MCF receptors) or could bind as antagonists or as neutral ligands rather than as agonists. Conversely, the leukemogenic MCFs might cause premalignant proliferation of hematopoietic stem cells by a mechanism similar to that of SFFVs.

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