ANEMIA- AND POLYCYTHEMIA-INDUCING ISOLATES OF FRIEND SPLEEN FOCUS-FORMING VIRUS

Biological and Molecular Evidence for Two Distinct Viral Genomes*

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Friend leukemia virus (1) is a type C murine retrovirus that has rapid effects on erythropoiesis. The original isolate of Friend leukemia virus complex (FV-A)[1] induces splenomegaly, the proliferation of mononuclear cells, and anemia (2). Subsequently, a variant of the anemia-inducing strain of Friend virus was isolated from stocks of FV-A by Mirand (3). This isolate of Friend leukemia virus complex (FV-P) also induces splenomegaly and the rapid proliferation of mononuclear cells in susceptible mice. However, in contrast to the leukemia induced by FV-A, the disease associated with FV-P is characterized by polycythemia and the rapid appearance of large numbers of cells capable of erythroid differentiation both in vivo (4) and in cell culture (5, 6) in the absence of added erythropoietin (EPO).

Preparations of FV-P contain both a replication-defective transforming virus, designated spleen focus-forming virus ($SFFV_P$) (7), and a helper murine leukemia virus, $F\text{-}MuLV_P$, that provides functions necessary for $SFFV_P$ to complete its replicative cycle (8–10). Although an SFFV has been reported in some stocks of FV-A (11), its relationship to $SFFV_P$, and possible role in the pathogenesis of the erythroleukemia induced by FV-A, is not clear.

In this and the accompanying study (12), the erythroleukemic potential of clonal isolates of both the replication-defective viruses, $SFFV_A$ and $SFFV_P$; and the replication-competent viruses, $F\text{-}MuLV_A$ and $F\text{-}MuLV_P$ present in stocks of FV-A and FV-P, have been analyzed. We reasoned that the availability of related isolates of

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[1] *Abbreviations used in this paper:* B6, C57BL/6; EPO, erythropoietin; FFU, focus-forming unit; $F\text{-}MuLV_A$, replication-competent virus present in FV-A; $F\text{-}MuLV_P$, replication-competent virus present in FV-P; FV-A, anemia-inducing isolate of Friend leukemia virus complex; FV-P, polycythemia-inducing isolate of Friend leukemia virus complex; PBSTDS, 10 mM sodium phosphate, pH 7.2, 0.9% NaCl, 1% Triton X-100, 5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate; PFU, plaque-forming unit(s); SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SFFV, spleen focus-forming virus; $SFFV_A$, replication-defective transforming virus contained in preparations of FV-A; $SFFV_P$, replication-defective transforming virus contained in preparations of FV-P; *Sl, Steel.*

Friend leukemia virus that apparently induce different disease states may be useful in understanding the role of viral functions in leukemic hemopoiesis. The results presented in this study demonstrate that at least two related, but biologically and molecularly distinguishable, forms of SFFV can be obtained after cloning in rat fibroblasts.

Materials and Methods

Mice. Female mice of strain DBA/2J, aged 6–10 wk, and female F_1 hybrids produced by crossing mice of strains WC/Re-*Steel* (*Sl*)/+ and C57BL/6J-+/Sl^d (WCB6F1) or WB/Re-W/+ and C57BL/6J-+/W^v (WBB6F1) were obtained from The Jackson Laboratory, Bar Harbor, Maine. Each of these crosses yields mice of four different genotypes: +/+, Sl/+, +/Sl^d, and Sl/Sl^d or +/+, W/+, +/W^v and W/W^v. Mice of the Sl/Sl^d and W/W^v genotypes have severe macrocytic anemia; mice of the other genotypes have normal (+/+) or near-normal (Sl/+, +/Sl^d, W/+, +/W^v) phenotypes. Mice congenic at the *Fv-2* locus, B6.S, and C57BL/6 (B6) mice, were obtained by serial intercrosses and backcrosses with the Friend leukemia virus-sensitive SIM strain (13). B6 mice are $Fv\text{-}2^{rr}$, whereas B6.S mice are $Fv\text{-}2^{ss}$. These mice were bred at the Division of Laboratory Animal Science, University of Toronto, Toronto, Canada, and were obtained from Dr. A. Axelrad, University of Toronto.

Culture Conditions. NIH/3T3 cells (14) and RAT-1 cells (15), obtained from Dr. B. Ozanne, University of Toronto, were maintained in minimum essential medium (α-MEM) (16), without nucleosides, that was supplemented with 10% fetal calf serum (KC Biological, Inc., Lenexa, Kans.). Cells were routinely passaged by trypsinization every 3–4 d.

Virus and Virus Assays. NB-tropic FV-A, passaged in vivo and prepared from the enlarged spleens of Swiss mice, was originally obtained from Dr. C. Friend. The spleen extract was passaged in DBA/2J mice, and a spleen extract from these mice was then used to infect NIH/3T3 cells as described previously (9). These cells were cloned immediately after infection in microtiter wells, passaged in vitro for several months, cloned again, and a stable clone that released into the cell culture fluids at least 2×10^3 focus-forming units (FFU)/ml of $SFFV_A$ was selected. $SFFV_A$ and $SFFV_P$ titers, in FFU/ml, were determined in DBA/2J mice by the spleen focus assay method (7). The isolation of NIH/3T3 cells productively infected with NB-tropic FV-P has been previously described (9). Clonal isolates of $F\text{-}MuLV_A$ and $F\text{-}MuLV_P$ were obtained after end point dilution of FV-A and FV-P, respectively and infection of NIH/3T3 cells, as described in the accompanying study (12). Titers of $F\text{-}MuLV_A$ and $F\text{-}MuLV_P$ in plaque-forming units (PFU)/ml were determined by the XC plaque assay as described previously (17). Clones of RAT-1 cells nonproductively infected with $SFFV_A$ or $SFFV_P$ were isolated as described previously (9). Briefly, RAT-1 fibroblast cells were infected with FV-A at a multiplicity of 1 PFU and 0.025 FFU per cell or with FV-P at 0.24 PFU and 2.4 FFU per cell. After 3 h, the cells were cloned at 0.25 cells/well in microtiter wells. Nonproducer clones were identified by superinfection with F-MuLV as described previously (9). Virus was rescued from RAT-1 nonproducer clones by superinfection with F-MuLV at a multiplicity of 0.1 PFU/cell. Culture fluids containing rescued virus were harvested after the cells had been passaged four times.

Culture of Erythroid Colonies. Cells were cultured in 0.8% methylcellulose-containing Iscove's modified Dulbecco's medium (lot No. 430-2200; Grand Island Biological Co., Grand Island, N. Y.). Each 1.0-ml culture contained 1×10^5 nucleated bone marrow or spleen cells, 7.5×10^{-5} M 2-mercaptoethanol, and 10% fetal calf serum (lot No. 4055899; Flow Laboratories, Inc., Rockville, Md.) (18, 19). EPO (step III, lot No. 3025-1; Connaught Medical Research Laboratories, Willowdale, Ontario, Canada) was added at the outset of culture where indicated. After 2 d of incubation, colonies of eight or more erythroid cells were scored using a light microscope (magnification: × 100). At least two cultures were prepared for each experimental point in each assay.

Competition Immunoassays. Competition immunoassays were performed by analysis of unlabeled antigens, at serial twofold dilutions, for ability to compete with ^{125}I-labeled Rauscher-murine leukemia virus structural proteins, p30, p15, p10, p12, and gp70, for binding limiting amounts of goat antiserum directed against detergent-disrupted FV-P isolated from chronically

infected NIH/3T3 cells (9). Proteins were purified and ^{125}I-labeled according to previously described procedures (20). Competition immunoassay reaction mixtures contained 0.01 M Tris-HCl, pH 7.8, 1.0 mM EDTA, 0.4% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.), 1% bovine serum albumin, and 0.2 M NaCl in a total vol of 0.2 ml. Antiserum and unlabeled competing antigen were incubated at 37°C for 1 h followed by the addition of 10,000 cpm ^{125}I-labeled antigen. After further incubation for 3 h at 37°C and 18 h at 4°C, antigen-antibody complexes were immunoprecipitated as previously described (20).

Immunoprecipitation and Sodium Dodecyl Sulphate (SDS)-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Exponentially growing cells were labeled with [^{35}S]methionine (1,075 Ci/mmol, Amersham Corp., Arlington Heights, Ill.) as previously described (21). After labeling, cell monolayers were washed twice with serum-free medium and disrupted at 4°C in 10 mM sodium phosphate, pH 7.2, 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS (PBSTDS) lysis buffer. Cell extracts (1 ml) were incubated at 4°C for 18 h after addition of RNase A (200 μg/ml), RNase T_2 (0.1 μg/ml), DNase I (4.0 μg/ml), normal goat serum (5 μl), protein-A Sepharose Cl-4B (1.0 ml of 10% wt:vol suspension) (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.), and clarified by centrifugation for 1 h at 100,000 *g*. Viral-specific proteins were immunoprecipitated by incubation for 18 h at 4°C in the presence of 5 μl of specific antisera, addition of 50 μl of 10% (vol/vol) protein-A Sepharose Cl-4B suspension, and incubation for a further 2 h at 4°C. Immunocomplexes were collected by centrifugation at 2,000 *g* for 10 min, washed three times in PBSTDS and analyzed by SDS-PAGE on 5–20% polyacrylamide gradient slab gels (21).

Two-Dimensional Tryptic Peptide Analysis. Viral-specific proteins were purified by immunoprecipitation and SDS-PAGE and subjected to tryptic peptide analysis as previously described (21). Samples resuspended in electrophoresis buffer (acetic acid:formic acid:water, 15:5:80) were spotted on 10- × 10-cm cellulose-coated thin-layer chromatography glass plates (EM Laboratories, Inc., Elmsford, N. Y.). Electrophoresis was performed for 30 min at 500 V, and chromatography was conducted in the second dimension in buffer that contained butanol, pyridine, acetic acid, and water (32:25:5:20). Radiolabeled tryptic peptides were visualized by autoradiography using Kodak X-Omat XR2 film (Eastman Kodak Co., Rochester, N. Y.) as previously described (21).

Results

Infection of Mice with FV-A Derived from NIH/3T3 Fibroblasts. To facilitate the comparative studies to be described, clones of NIH/3T3 cells chronically infected with FV-A or FV-P were established as described in Materials and Methods. To determine whether the FV-A produced by fibroblasts elicits anemic transformation in vivo as described previously, DBA/2J mice 6 wk of age were injected with supernatant fluid from these fibroblast cultures and hematocrits, spleen weight, and the number of spleen foci were assayed on day 0, 7–10, 14–16, and 25–35. DBA/2J mice were also injected with FV-P produced by chronically infected NIH/3T3 fibroblasts (9) to provide a comparison of the leukemogenic, focus-forming ability and hematologic effects of these two strains of Friend leukemia virus. As shown in Table I, stocks of FV-A passaged in DBA/2J mice contained low titers of a spleen focus-forming activity, $SFFV_A$. The morphology of the spleen foci induced by FV-A were different from those induced by FV-P and exhibited a more flattened and diffuse morphology. Individual spleen foci produced by either FV-A or FV-P were obscured late in infection by large increases in spleen weight (Table I). The data in Table I also indicate that the cell-culture-derived FV-A induced progressive anemia, whereas the cell-culture-derived FV-P induced polycythemia, similar to animal-passaged preparations of FV-A and FV-P.

To examine further the ability of FV-A harvested from NIH/3T3 cells to induce spleen focus formation, 6-wk-old DBA/2J mice were injected with various dilutions of

TABLE I

*Properties of Cell Culture-derived FV-A and FV-P**

Time after infection	Virus	Hematocrit	Spleen weight	Number of foci/spleen
d		%	*g*	
7–10	Mock‡	50	0.1	0
	FV-P	52	0.6	80
	FV-A (in vivo)	50	0.4	38
	FV-A (cell culture)	50	0.4	50
14–16	Mock	49	0.1	0
	FV-P	65	2.6	Confluent§
	FV-A (in vivo)	44	1.4	Confluent
	FV-A (cell culture)	43	1.9	Confluent
25–35	Mock	51	0.1	0
	FV-P	89	3.9	Confluent
	FV-A (in vivo)	39	2.6	Confluent
	FV-A (cell culture)	37	3.0	Confluent

* FV-A and FV-P were obtained from cloned NIH/3T3 fibroblasts that chronically produce these viruses or, where indicated, from spleen extracts from FV-A passaged only in vivo in DBA/2J mice as described in Materials and Methods. DBA/2J mice were injected intravenously on day 0. Hematocrits were determined using blood obtained from the tail vein. The virus titers were determined using the spleen focus assay (7).

‡ Mock-infected mice received similar amounts of culture fluid from uninfected NIH/3T3 cells.

§ Confluent spleens were too enlarged to count individual foci.

FV-A. Infected mice were killed at day 9 and their spleens examined for macroscopic foci. As shown in Fig. 1 c, the spleens of mice that received low dilutions of FV-A were greatly enlarged, whereas those receiving higher dilutions exhibited discrete foci (Fig. 1 b). Furthermore, as shown in Fig. 2, the number of spleen foci was linearly related to the dilution of FV-A.

Erythroid Progenitor Cells in FV-A- and FV-P-infected DBA/2J Mice. Polycythemic transformation by FV-P is characterized by EPO-independent erythropoiesis in vivo (4), and by the appearance of cells in the marrows and spleens of FV-P-infected mice that are capable of forming erythroid colonies in semisolid medium in the absence of added EPO (5, 6). To determine whether infection with FV-A also alters the sensitivity of erythroid progenitor cells to EPO, bone marrow and spleen cells were plated in methylcellulose culture in the presence or absence of added EPO at various times after 6-wk-old DBA/2J mice had been infected with FV-A. As shown in Fig. 3, the number of EPO-dependent erythroid colony-forming cells in the marrow of FV-A-infected mice continued to decrease during infection and colony formation in the absence of added EPO was not observed. In contrast, erythroid colony-forming cells were detected both in the presence and in the absence of added EPO in FV-P-infected marrow at day 8 and their numbers continued to increase with time after infection. The number of EPO-dependent erythroid colony-forming cells in the spleens of FV-A or FV-P-infected mice increased early after infection (days 8–10) and continued to rise to very high numbers. Infection with FV-P rapidly resulted in the appearance of EPO-independent erythroid colonies, whereas only low numbers of EPO-independent colony-forming cells were observed in FV-A infected spleens very late after infection (days 25–35).

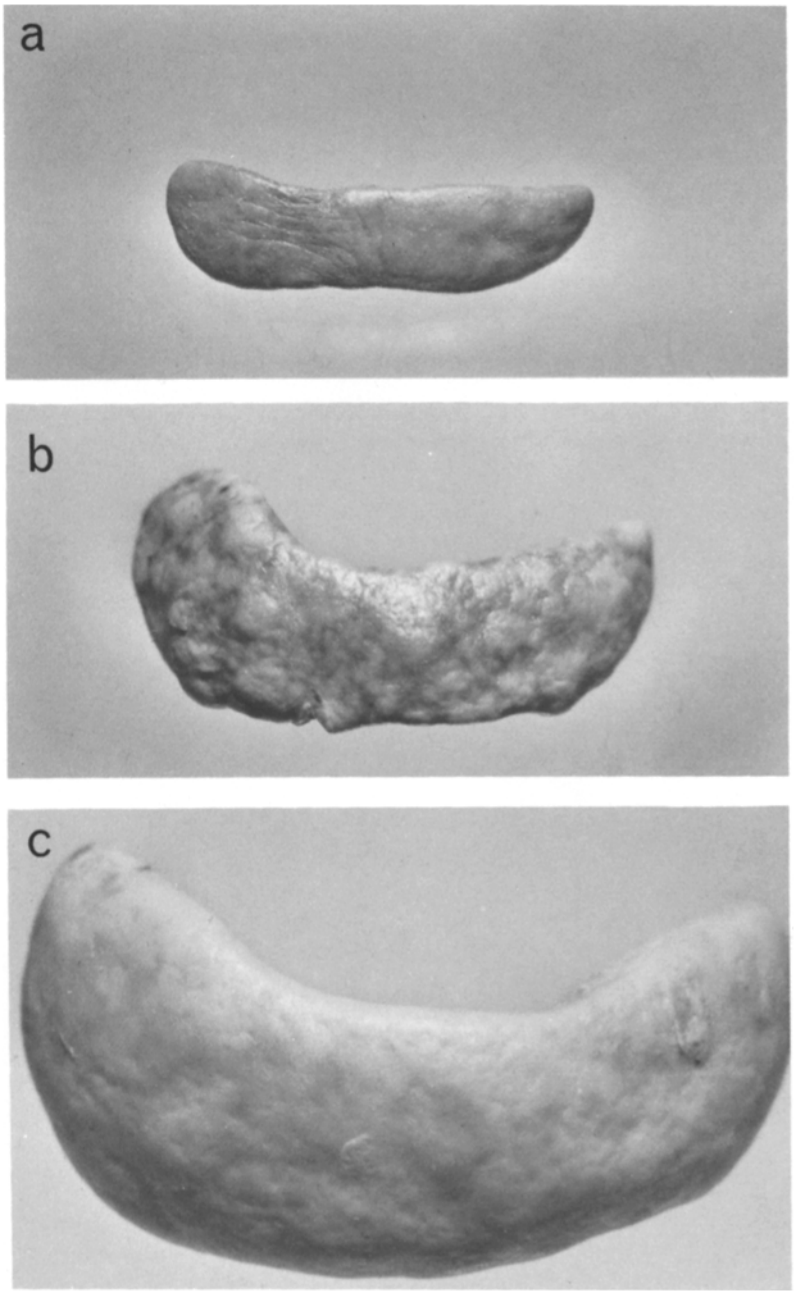

FIG. 1. Spleens of mock-infected and FV-A infected DBA/2J Mice. Spleens from DBA/2J mice-injected intravenously 9 d previously with (a) undiluted supernate from NIH/3T3 cells (mock-infected), (b) 10^{-3} dilution of supernate from NIH/3T3 fibroblasts chronically infected with FV-A, and (c) 10^{-1} dilution of supernate from NIH/3T3 fibroblasts chronically infected with FV-A.

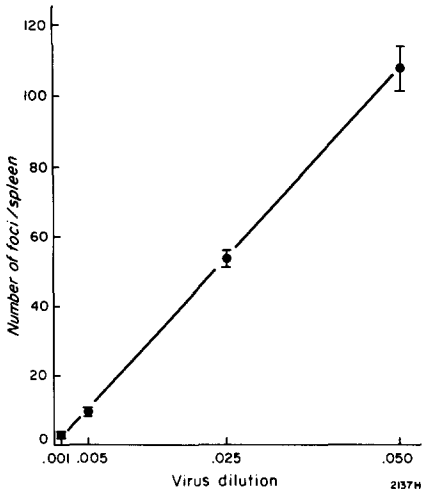

FIG. 2. Titration of tissue culture-derived FV-A by the spleen focus assay. Tissue culture medium harvested from NIH/3T3 cells chronically infected with FV-A was assayed in 6-wk-old DBA/2J mice for spleen focus-forming activity. Groups of six mice were injected intravenously with 0.25 ml of dilutions of culture medium. Mice were killed at day 9, the spleens fixed in Bouin's solution and the number of macroscopic foci counted.

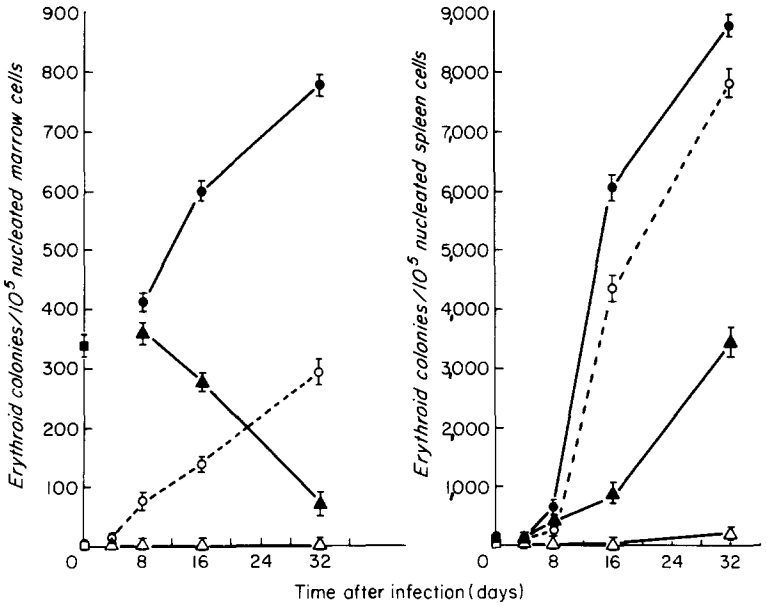

FIG. 3. Effects of FV-A and FV-P on bone marrow and splenic erythroid colony formation with time after infection. DBA/2J mice were injected intravenously with 5×10^3 FFU of FV-A (▲, △) or FV-P (●, ○) NIH/3T3 cell supernates at various times. The mice were sacrificed at the same time and bone marrow and spleens were taken and plated in 2-d methylcellulose culture without the addition of EPO (△, ○) or in the presence of 0.50 U/ml EPO (▲, ●). The symbols ■, □ indicate the number of erythroid colonies/10^5 nucleated cells obtained from the marrow or spleen of mice that were sacrificed immediately after virus was injected as determined with 0.50 U/ml of EPO (■) or without EPO (□).

Isolation and Characterization of Cell Clones Nonproductivity Infected with SFFV$_A$. The observations that the NIH/3T3 cell clones productively infected with either FV-A or FV-P contained SFFV (SFFV$_A$ and SFFV$_P$), and that FV-A and FV-P induce different effects on erythroid progenitor cells, suggested that SFFV$_A$ and SFFV$_P$ may be biologically distinguishable and responsible for the two forms of Friend disease observed in adult mice. To test this possibility, RAT-1 fibroblast cell clones nonproductively infected with either SFFV$_A$ or SFFV$_P$ were isolated as described in Materials and Methods. The defective SFFV genome present in these clones was rescued by superinfection with F-MuLV$_A$ or F-MuLV$_P$ and the rescued virus was injected into 6-wk-old DBA/2J mice. As shown in Table II, infection of adult DBA/2J mice with SFFV$_P$ rescued from a RAT-1 cell clone nonproductively infected with SFFV$_P$ (RAT-1P-5) with either F-MuLV$_A$ or F-MuLV$_P$, resulted in polycythemia and a marked increase in the number of both EPO-dependent and EPO-independent erythroid colony-forming cells. In contrast, infection of adult DBA/2J mice with SFFV$_A$ rescued from a RAT-1 cell clone nonproductively infected with SFFV$_A$ (RAT-1A-1) rescued with either F-MuLV$_A$ or F-MuLV$_P$, resulted in anemia and an increase only in the number of EPO-dependent colony-forming cells. As shown in the accompanying study (12), infection of adult mice with either F-MuLV$_A$ or F-MuLV$_P$ alone did not result in any change in either the number, or EPO-dependence, of these erythroid progenitor cells, nor were changes in hematocrit or spleen weight detected.

Comparison of Translational Products of the Anemia and Polycythemia Strains of SFFV. To characterize these two isolates of SFFV further and identify possible differences in

TABLE II

*Rescue of SFFV$_A$ and SFFV$_P$ from RAT-1 Nonproducer Clones**

Rescuing virus	Cell	Virus titer‡	Spleen weight	Hemato-crit	Number of erythroid colonies per 10^5 spleen cells; EPO (U/ml)	
					0	0.5
		FFU/ml	*g*	%		
Mock§	RAT-1	0	0.10	49	0	32
F-MuLV$_A$	RAT-1	0	0.09	47	0	29
F-MuLV$_P$	RAT-1	0	0.11	49	0	54
Mock	RAT-1A-1	0	0.13	48	0	31
F-MuLV$_A$	RAT-1A-1	6.0×10^3	1.37	38	55	1,693
F-MuLV$_P$	RAT-1A-1	1.6×10^3	0.88	40	12	1,053
Mock	RAT-1P-5	0	0.10	47	0	36
F-MuLV$_A$	RAT-1P-5	2.3×10^3	1.53	68	2,520	4,864
F-MuLV$_P$	RAT-1P-5	5.2×10^3	2.61	76	3,983	6,841

* DBA/2J mice were injected intravenously with a 1:5 dilution of culture fluid from RAT-1 nonproducer cell clones that had been superinfected four cell passages earlier with F-MuLV$_A$ or F-MuLV$_P$ as described in Materials and Methods. Spleen weight, hematocrit, and spleen erythroid colony formation were evaluated 25 d after infection as described in Materials and Methods.

‡ DBA/2J mice were injected with various dilutions of the same culture fluid from superinfected RAT-1 nonproducer clones that was used to determine hematocrit spleen weight and erythroid colony formation. Spleens were removed at day 9 and examined for foci.

§ Mock-infected mice received similar amounts of culture fluid from uninfected NIH/3T3 cells.

their genomic structure which might account for their biologic properties, the translational products of $SFFV_A$ and $SFFV_P$ were studied. RAT-1 cells nonproductively infected with each strain were analyzed by competition immunoassay. As summarized in Table III, the $SFFV_A$ nonproducer cell clone, RAT-1A-1, expressed high levels of the *gag*- and *env*-gene coded proteins p15, p12, p30, and gp70. In contrast, the $SFFV_P$ nonproducer cell clone, RAT-1P-5, was only reactive in the p15 and gp70 assays. The specificity of the reactivity observed is established by the lack of detectable competition by uninfected RAT-1 cell extracts in any of the five assays and by the high level of expression of all four *gag* proteins, and gp70, in NIH/3T3 cells productively infected with FV-P (9).

SDS-PAGE and Tryptic Peptide Analysis of SFFV env-encoded Proteins. The demonstration of gp70 immunologic cross-reactivity in the RAT-1P-5 cell line is consistent with previous reports of a 55,000-mol wt glycoprotein encoded by $SFFV_P$ (22, 23). To determine whether the $SFFV_A$ genome also encodes a 55,000-mol wt *env*-related protein, RAT-1 cell lines nonproductively infected by the two isolates of SFFV were analyzed by [^{35}S]methionine labeling followed by immunoprecipitation and SDS-PAGE. As shown in Figure 4, both RAT-1A-1 and RAT-1P-5 cells contained a protein of ~55,000 daltons that was immunoprecipitable by goat anti-Rauscher gp70 but not normal goat serum. The demonstration of a 55,000-mol wt protein in both SFFV nonproductively infected cell lines, but not in uninfected RAT-1 cells, indicated that $SFFV_A$, as well as $SFFV_P$, encoded a 55,000-mol wt glycoprotein.

The possibility that the *env*-related gp55 glycoprotein encoded by $SFFV_P$ is involved in the leukemogenic properties associated with this virus has been suggested (22, 24). It was therefore of interest to compare the [^{35}S]methionine-containing tryptic peptide composition of the 55,000-mol wt proteins encoded by the anemia- and polycythemia-inducing strains of SFFV. As shown in Fig. 5A, $SFFV_P$-encoded gp55 contains at least eight well-resolved [^{35}S]methionine-labeled tryptic peptides. In contrast, under similar conditions, $SFFV_A$ gp55 exhibited three labeled peptides (Fig. 5B), each of

TABLE III

Type C Viral Antigen Expression in Rat-1 Cells Nonproductively Infected by $SFFV_A$ or $SFFV_P$

Cell	Viral antigen expression				
	gag-gene coded				*env*-gene coded
	p15	p12	p30	p10	gp70
	ng/mg cellular protein				
RAT-1	<20	<5	<2	<10	<20
RAT-1A-1	510	380	430	<10	120
RAT-1P-5	320	<5	<2	<10	80
NIH/3T3 (FV-P)	2,200	1,800	3,100	1,300	2,600

Cell extracts, prepared as described in Materials and Methods, were tested at serial twofold dilution in competition immunoassays using goat anti-Rauscher MuLV for precipitation of ^{125}I-labeled structural proteins purified from the Rauscher strain of MuLV. Results are based on the extent of displacement of binding relative to purified FV-P structural proteins and represent mean values from duplicate determinations at each dilution tested.

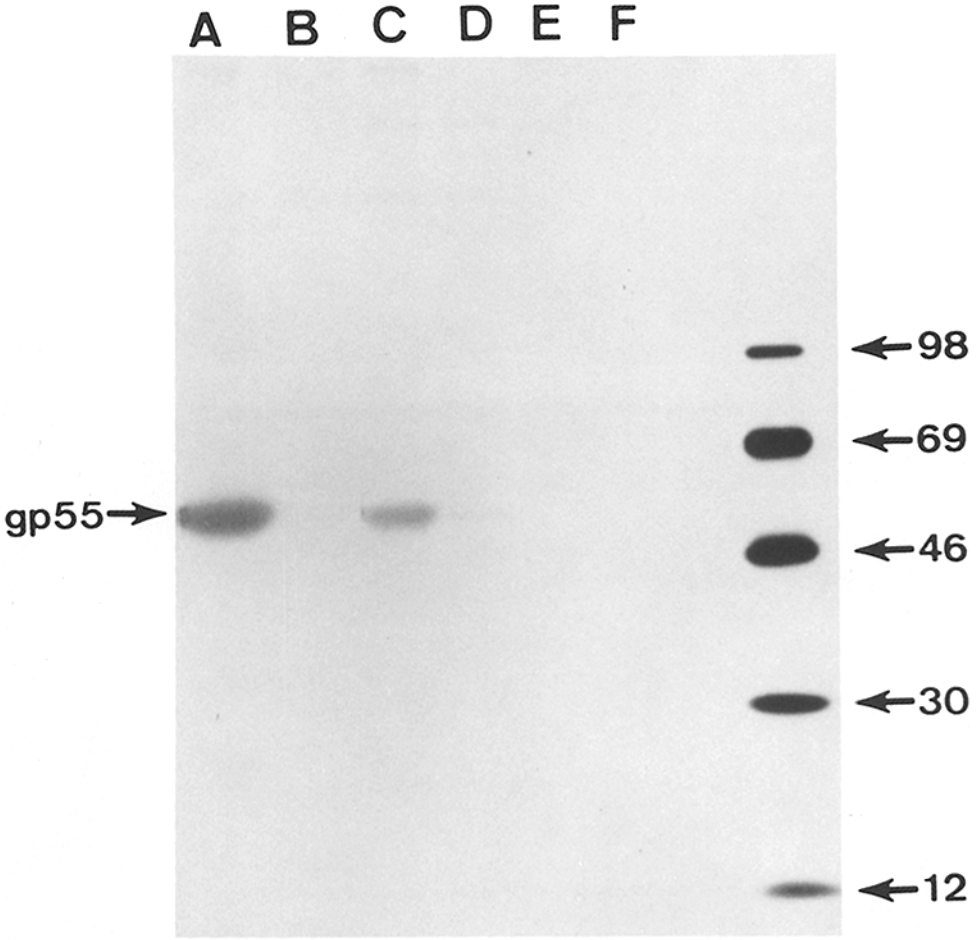

FIG. 4. Immunoprecipitation and SDS-PAGE analysis of envelope glycoproteins expressed in RAT-1 cells nonproductively-infected by the anemia and polycythemia strains of SFFV. Cell lines (A, B) RAT-1A-1; (C, D) RAT-1P-5; and (E, F) control RAT-1 (cells) were pulse-labeled in medium containing [^{35}S]methionine (250 μCi/ml) for 30 min, immunoprecipitated by goat anti-Rauscher MuLV gp70 (A, C, E) and normal goat serum (B, D, F), and analyzed by SDS-PAGE. Molecular weight standards include ^{14}C-labeled phosphorylase B (98,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and cytochrome C (12,000).

which appears to correspond in mobility to tryptic peptides represented in SFFV$_P$ gp55. To demonstrate further the relatedness of the SFFV$_A$- and SFFV$_P$-specific [^{35}S]methionine-labeled peptides, tryptic digests were mixed before analysis. As shown in Fig. 5C, the three major peptides specific to SFFV$_A$ were not resolved from the corresponding SFFV$_P$ peptides.

Host Genetic Control of Spleen Focus Formation and Anemic Transformation by FV-A. A number of host genes, including *W*, *Sl*, and *Fv-2*, are known to govern susceptibility to FV-P (25–28). Mice carrying two mutant alleles at either the *W* or the *Sl* locus (W/W^v and Sl/Sl^d) are severely anemic (29–31) and exhibit partial resistance to SFFV$_P$-associated erythroleukemia and spleen focus formation. To determine whether these loci also influence susceptibility to FV-A, normal +/+ and their genetically

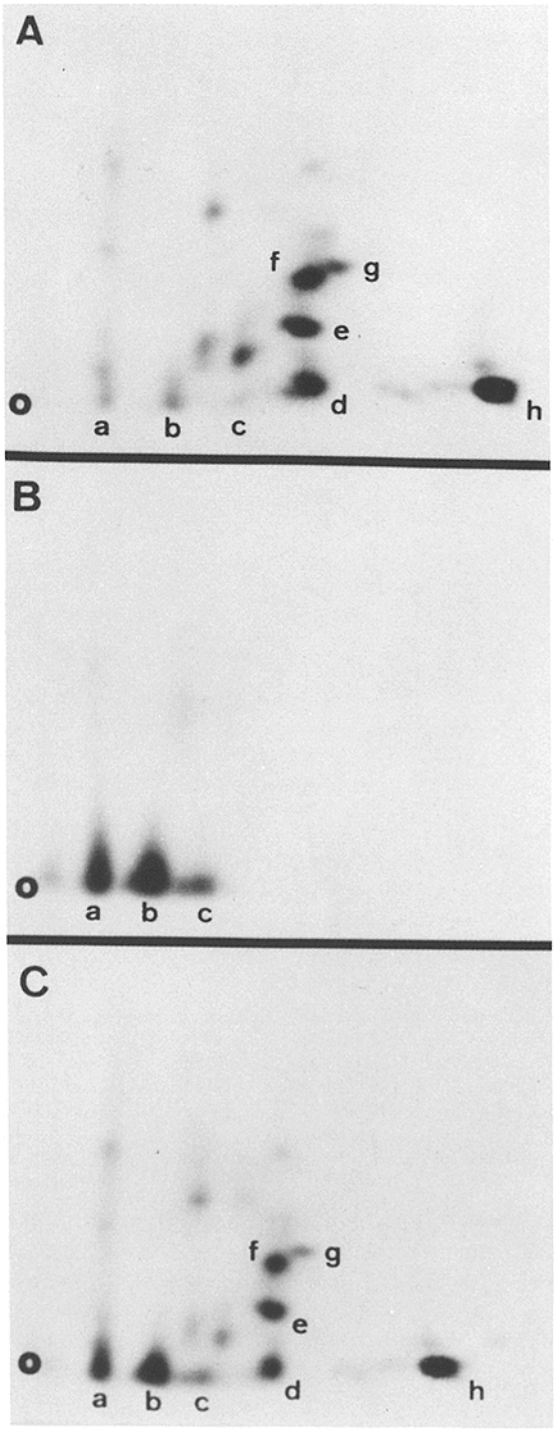

FIG. 5. [^{35}S]Methionine tryptic peptide analysis of 55,000-mol wt glycoproteins encoded by the anemia and polycythemia strains of SFFV. [^{35}S]Methionine-labeled proteins were isolated by immunoprecipitation and SDS-PAGE as described in the legend to Fig. 4. Proteins were digested with TPCK-trypsin as described previously (21). Tryptic digests (6,000–10,000 cpm) were resuspended in electrophoresis buffer and spotted at the origin (O) on cellulose thin-layer glass plates. Separation in the first dimension was by electrophoresis and in the second by ascending chromatography. Samples included the 55,000-mol wt glycoproteins isolated from (A) RAT-1P-5; (B) RAT-1A-1; and (C) the two proteins mixed at a ratio of 1:1. Individual peptides are designated a through h.

anemic W/W^v and Sl/Sl^d littermates were injected with FV-A or FV-P and examined after 9 d. The data summarized in Table IV demonstrate that these host loci conferred incomplete resistance to FV-A and FV-P, as determined by spleen weight and spleen focus formation.

Fv-2 is another major host locus that regulates susceptibility to erythroleukemia induction and spleen focus formation by FV-P (27). $Fv\text{-}2^{rr}$ mice have been shown to be highly resistant to the induction of splenomegaly and spleen foci (27), polycythemia, and the appearance of EPO-independent erythroid progenitor cells (32). To determine whether the *Fv-2* gene locus also controls susceptibility to FV-A, congenic B6 ($Fv\text{-}2^{rr}$) and B6.S ($Fv\text{-}2^{ss}$) mice were injected with either FV-A or FV-P. The results shown in Table V demonstrate that B6.S ($Fv\text{-}2^{ss}$) mice were fully susceptible to both FV-A and FV-P. In contrast, infection of congenic B6 ($Fv\text{-}2^{rr}$) mice with either FV-A or FV-P did not result in the appearance of spleen foci, splenomegaly, or changes in hematocrit; nor were any alterations observed in the number of erythroid colony-forming cells. Thus, the anemia- and polycythemia-inducing strains of Friend SFFV are both subject to regulation at the *W*, *Sl*, and *Fv-2* gene loci.

Discussion

The results presented in this study indicate that the two SFFV activities present in stocks of FV-A and FV-P, $SFFV_A$ and $SFFV_P$, represent distinct, but related, viral

TABLE IV

*Susceptibility of Normal and Genetically Anemic Mice to Induction of Spleen Focus Formation and Erythroleukemia by FV-A and FV-P**

Genotype	Virus	Virus titer‡	Spleen weight	Hematocrit
		FFU/ml	*g*	%
+/+§	Mock‖	0	0.10	52
	FV-P	1.2×10^4	1.70	84
	FV-A	9.0×10^3	2.52	39
Sl/Sl^d	Mock	0	0.12	34
	FV-P	2×10^2	0.32	57
	FV-A	4.8×10^2	0.98	10
W/W^v	Mock	0	0.11	36
	FV-P	3.5×10^2	0.53	61
	FV-A	6×10^2	1.10	13

* Mice were injected intravenously with FV-A and FV-P harvested from cloned chronically infected NIH/3T3 fibroblasts. Spleen weight and hematocrit were determined as described in Materials and Methods 16 d after infection.

‡ Mice were injected with various dilutions of the same cell-culture-derived preparations of FV-A and FV-P that were used to determine hematocrit and spleen weight. Spleens were removed and placed in Bouin's solution and counted for spleen foci 9 d after infection.

§ As the data obtained for the +/+ littermates of Sl/Sl^d and W/W^v mice were not significantly different, only the data obtained with the +/+ littermates of Sl/Sl^d mice is shown.

‖ Mock-infected mice were injected with medium harvested from uninfected NIH/3T3 cells.

TABLE V

*Susceptibility of Fv-2 Congenic Mice to FV-A and FV-P**

Mouse genotype	Virus	Virus titer‡	Hemato-crit	Number of erythroid colonies per 10^5 cells			
				Bone marrow cells		Spleen cells	
				EPO (U/ml)			
				0	0.5	0	0.5
		FFU/ml	%				
Fv-2^{ss} (B6.S)	Mock§	0	46	0	248	0	31
	FV-P	1.0×10^4	78	270	863	3,180	4,029
	FV-A	4.0×10^3	38	0	136	22	860
Fv-2^{rr} (B6)	Mock	0	45	0	269	0	36
	FV-P	0	46	0	255	0	25
	FV-A	0	45	0	273	0	43

* Mice were injected intravenously with FV-A and FV-P harvested from cloned chronically infected NIH/3T3 fibroblasts. Hematocrit and erythroid colony formation were evaluated 25 d after infection as described in Materials and Methods.

‡ Mice were injected with various dilutions of FV-A and FV-P derived from chronically infected NIH/3T3 fibroblasts. The titers were determined 9 d after infection using the spleen focus assay (7).

§ Mock infected mice received the same volume of culture fluid from uninfected NIH/3T3 cells.

isolates. Furthermore, these two isolates of SFFV appear to be the major determinants of at least the early stages of Friend disease: $SFFV_P$ induces polycythemia, whereas the disease associated with $SFFV_A$ is accompanied by anemia. Experiments in which SFFV nonproducer cell clones were rescued by different helper viruses demonstrated that the type of erythroleukemia induced by $SFFV_A$ and $SFFV_P$ was independent of the source of the Friend helper virus. This conclusion is consistent with the finding, presented in the accompanying study (12), that neither $F\text{-}MuLV_A$ nor $F\text{-}MuLV_P$, the helper viruses used in this study, induced disease in adult DBA/2J mice.

The present studies also define further the cellular differences between the anemic and polycythemic forms of Friend disease. Infection of susceptible mice with FV-P resulted in the appearance of large numbers of EPO-independent erythroid colony-forming cells in the marrow and spleen, in agreement with previous findings (5, 6). In contrast, infection of susceptible mice with FV-A produced a reduction in the number of EPO-dependent erythroid colony-forming cells in marrow and an increase in EPO-dependent erythroid colony-forming cells in spleen. The observation that erythroid progenitor cells from FV-A- but not FV-P-infected hemopoietic tissue retain EPO dependence has also been observed in studies using FV-A ([33]; B. Fagg and W. Ostertag. Personal communication.) and the anemia-inducing Rauscher MuLV virus (34) obtained from extracts of the spleens of infected mice.

The conclusion that FV-A and FV-P induce related diseases is further supported by the finding that a number of host genes that had previously been shown to control susceptibility to FV-P also govern susceptibility to FV-A. Anemic W/W^v mice have defective hematopoietic stem cells which are incapable of extensive proliferation or erythroid differentiation (29, 30). In contrast, genetically anemic Sl/Sl^d mice have been shown previously to have a defective hematopoietic environment which prevents

extensive erythroid differentiation of normal hematopoietic stem cells (31) and also confers resistance to FV-P (25, 28). The finding that genetic defects in normal hemopoiesis result in a decrease in susceptibility to both FV-A and FV-P suggests that these two isolates of Friend virus are subject to similar host regulatory mechanisms.

The *Fv-2* locus is another host genetic determinant that governs susceptibility to erythroleukemia induction by FV-P (27). As shown in our study, the *Fv-2* gene locus also affects susceptibility to FV-A. All the in vivo and in vitro parameters of leukemic transformation induced by FV-A and FV-P that were tested—anemia or polycythemia induction, splenomegaly, spleen focus formation, and changes in the number and EPO responsiveness of erythroid progenitor colony-forming cells—were affected. The finding that the *Fv-2* locus controls both spleen focus formation and these various hematological parameters suggests that the distinct SFFV activities present in FV-A and FV-P are responsible for the cellular changes observed in Friend virus-infected animals.

Insight into the mechanism of resistance to viral replication and transformation mediated by *Fv-2* may be derived from the observation that *Fv-2* regulates levels of $SFFV_P$-specific RNA sequences in both uninfected and infected mice (35). This observation thus raises the question of whether the sequences specific to $SFFV_P$ are genetically related to or distinct from those specific to $SFFV_A$.

Further evidence that $SFFV_P$ and $SFFV_A$ represent genetically related, but distinct, transforming viruses derives from an analysis of their translational products. In confirmation of our previous results (9), the *gag* region of $SFFV_P$ genome is shown to encode an amino-terminal type C viral *gag*-gene protein, p15. In contrast, *gag*-gene expression in $SFFV_A$ nonproducer cells is even more extensive, including p15, p12, and p30 antigenic reactivities. These findings, in combination with previous observations that the *gag* gene of a second clone of $SFFV_P$ encodes p15 and p12 (36), suggests that the extent of *gag*-gene expression is highly variable between different SFFV isolates and indicates that the intracistronic arrangement of the Friend SFFV *gag* genes, 5′-p15-p12-p30-p10-3′, is analogous to that of other MuLV isolates (37, 38).

This study also demonstrates that both $SFFV_P$ and $SFFV_A$ encode 55,000-mol wt *env*-gene products, designated gp55. The gp55 of $SFFV_P$ includes at least eight methionine-containing peptides, only three of which are represented in $SFFV_A$ gp55. This finding is consistent with previous suggestions that the *env* region of the SFFV genome may include sequences involved in erythroleukemia induction (22, 24). Historically, $SFFV_P$ appears to be derived from $SFFV_A$. Thus, if the *env* gene does include transforming sequences, the transition from anemia to polycythemia induction may be associated with differences in *env*-gene products of the two SFFV isolates as described in this study.

Summary

Two distinct clones of Friend spleen focus-forming virus (SFFV), differing in their erythroleukemic potential, are described. These isolates have been cloned free of their associated helper viruses and shown to be replication-defective. Both SFFV isolates have been rescued from rat fibroblast nonproducer cell clones with cloned replication-competent viruses, $F\text{-}MuLV_A$ and $F\text{-}MuLV_P$, obtained from the anemia- or polycythemia-inducing isolates of Friend virus complex, respectively. These rescued viruses

induce a rapid proliferative disease associated with the appearance of macroscopic spleen foci and splenomegaly. In addition, each is subject to regulation by the *W*, *Steel* (*Sl*), and *Fv-2* host gene loci. These two isolates of SFFV can, however, be distinguished by both biological and molecular criteria. Friend $SFFV_P$ induces a rapid polycythemia associated with the appearance of large numbers of erythropoietin (EPO)-independent erythroid colony-forming cells in the marrow and spleen. In contrast, $SFFV_A$ induces a rapid anemia associated with a progressive decrease in the number of EPO-dependent erythroid colony-forming cells in marrow, and a rapid increase in the number of EPO-dependent erythroid colony-forming cells in spleen. Furthermore, the nature of the disease induced by the two isolates of SFFV is independent of the Friend helper virus: $SFFV_P$, rescued from a nonproducer cell clone with either $F\text{-}MuLV_A$ or $F\text{-}MuLV_P$, induced a polycythemic transformation, whereas $SFFV_A$, rescued with either $F\text{-}MuLV_A$ or $F\text{-}MuLV_P$, induced an anemic transformation. The two Friend SFFV isolates can also be discriminated on the basis of translational products encoded by their *gag* and *env* genes: $SFFV_P$ encodes the amino-terminal *gag*-gene protein p15, whereas $SFFV_A$ encodes the *gag*-gene proteins p15, p12, and p30. In addition, the SFFV isolates encode nonidentical 55,000-mol wt *env* gene-related proteins that can be distinguished by analysis of their methionine-containing tryptic peptides.

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