Sequences Responsible for the Altered Erythropoietin Responsiveness in Spleen Focus-Forming Virus Strain SFFV$_P$-Infected Cells Are Localized to a 678-Base-Pair Region at the 3' End of the Envelope Gene

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Two different strains of Friend spleen focus-forming virus, SFFV$_P$ and SFFV$_A$, are known to cause a rapid erythroleukemia. The SFFV$_P$-infected cells can proliferate and differentiate maximally without the addition of the erythroid-specific hormone erythropoietin, whereas the SFFV$_A$-infected cells require erythropoietin for differentiation and for maximum proliferation. We previously reported that a recombinant virus containing sequences from the 3' half of the SFFV$_P$ envelope gene and the SFFV$_P$ long terminal repeat on an SFFV$_A$ background has all of the biological and biochemical characteristics of SFFV$_P$. We are now presenting data on a new recombinant virus to show that only the 3' half of the SFFV$_P$ envelope gene is responsible for the differences observed between the two strains.

Friend virus is known to contain two viral components, the replication-defective spleen focus-forming virus (SFFV) and a replication-competent murine ecotropic virus, Friend murine leukemia virus (MuLV) (for review, see reference 11). The SFFV$_P$ component of this complex has been shown to be responsible, even in the absence of helper virus, for the acute erythroleukemia that develops after infection of susceptible mice. The pathogenicity of SFFV has been localized to the envelope (*env*) gene (for review, see reference 11 and L. Wolff, S. W. Chung, and S. Ruscetti, *in Modern Trends in Virology*, in press). Established evidence suggests that the major target cell for SFFV is a relatively mature population of erythroid progenitor cells which are normally under the control of an erythroid-specific hormone, erythropoietin (EPO) (4). However, infection of these cells with SFFV is known to alter their responsiveness to this hormone (11). Two different strains of SFFV, the polycythemia-inducing strain (SFFV$_P$) and the anemia-inducing strain (SFFV$_A$), have been described (3, 8, 20). Both can induce an acute erythroleukemia after a short latency period of 1 to 3 weeks. The SFFV$_P$-infected cells appear to be EPO independent and are capable of proliferating and differentiating without added EPO. On the other hand, the majority of the SFFV$_A$-infected cells seem to still require EPO for their proliferation, and their differentiation is also EPO dependent. Detailed sequence comparisons between the two strains reveal major differences at the 3' half of the *env* gene and some minor differences in the long terminal repeat (LTR) region (22). Earlier, a recombinant virus, SFFV$_{AP}$-L, which contains sequences from the 3' half of the SFFV$_P$ *env* gene and 3' SFFV$_P$ LTR, was constructed on an SFFV$_A$ background (23) and was shown to retain all the biological as well as biochemical features of SFFV$_P$ (12). However, since SFFV$_P$ LTR sequences are included in this construct, the possible role of the SFFV$_P$ LTR cannot be excluded. We have, thus, constructed a new SFFV$_A$ recombinant in which the only

SFFV$_P$ sequences are those present in the 678-base-pair *Eco*RI-to-*Cla*I fragment at the 3' end of the *env* gene.

Plasmid DNA of SFFV$_{AP}$-L, which contains SFFV$_P$ sequences between the *Eco*RI and *Kpn*I sites, was double digested with *Xma*III and *Kpn*I (Fig. 1). This generated a 1.8-kilobase fragment that was further digested with *Bal*31 for different lengths of time, followed by filling in the ends with the Klenow fragment of DNA polymerase I. *Cla*I linkers were attached, and the fragments were double digested with *Eco*RI and *Cla*I to obtain the final ~0.9-kilobase *Eco*RI-to-*Cla*I fragment. This ~0.9-kilobase *Eco*RI-to-*Cla*I fragment was then used in a three-way ligation to generate a new recombinant virus, SFFV$_{A-RC}$, (Fig. 1), and subsequently used to transform bacteria. Bacterial clones containing recombinant plasmids were screened first by hybridization to a nick-translated *env* probe and then by restriction analysis. Nucleotide sequence analysis of a positive clone, pSFFV$_{A-RC}$, using the protocol of Maxam and Gilbert (10), indicated that the *Cla*I linker was inserted 2 base pairs after the termination codon (data not shown). Infectious virus was recovered by cotransfecting pSFFV$_{A-RC}$ plasmid DNA with pSV*neo* DNA into Moloney MuLV-producing cells (17). G418-resistant colonies were picked, and production of the SFFV envelope protein, gp52, was examined by [^{35}S]methionine labeling and immunoprecipitation with anti-Rauscher MuLV gp70 or anti-mink cell focus-inducing virus gp70 serum. Only those colonies expressing gp52 were expanded, and culture supernatant from these cells was collected as virus stock.

When this new recombinant virus was injected into susceptible adult animals, a typical erythroleukemia with splenomegaly and high hematocrits (52 to 78%) was apparent in all of the animals (12/12) within 20 to 32 days. Spleen cells from infected animals were removed after 2 to 3 weeks and tested for their response to EPO. A modified [^{3}H]thymidine incorporation assay (6, 13) was used to study the proliferation of infected spleen cells in response to different concentrations of EPO (Fig. 2). The molecularly cloned anemia-inducing strain (SFFV$_A$-L) and the previously
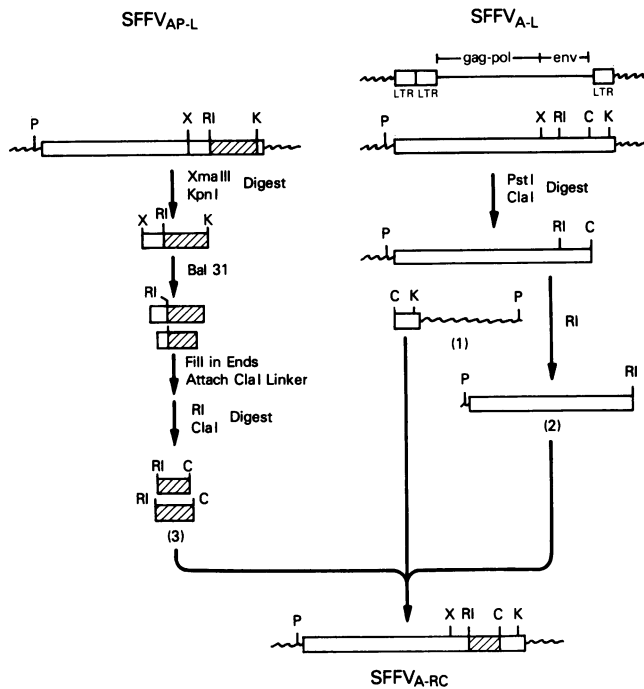
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SFFV_AP-L

SFFV_A-L

FIG. 1. Detailed protocol for constructing the new recombinant SFFV_A-RC (X, XmaIII; RI, EcoRI; K, KpnI; P, PstI; C, ClaI). Shaded area indicates sequences from SFFV_P.

FIG. 2. [³H]thymidine incorporation into spleen cells from mice infected with different SFFVs (●, SFFV_A-L; ○, SFFV_AP-L; ▲ and △, SFFV_A-RC) in response to different concentrations of EPO. See reference 13 for detailed procedures. Briefly, spleen cells from infected animals were cultured at 4×10^5 per well in the presence or absence of EPO in a 96-well plate for 24 h. Proliferation was then measured by [³H]thymidine incorporation by adding label during the last 2 h of incubation.

reported recombinant SFFV_AP-L were used as controls (12). SFFV_AP-L was shown earlier to have maintained all the biological activity of the polycythemia-inducing strain and was thus included as an SFFV_P control. The SFFV_A-L-infected spleen cells showed increased [³H]thymidine uptake with increasing concentrations of EPO. Like the SFFV_AP-L control, spleen cells from two animals infected with the new virus (SFFV_A-RC) proliferated at maximum levels without added EPO. To determine whether the virus-infected spleen cells require EPO for differentiation, they were analyzed for erythroid colony formation in a plasma clot colony assay (21). With spleen cells infected with SFFV_AP-L as well as SFFV_A-RC, almost equal numbers of erythroid colonies were observed 2 days after culture with and without EPO, whereas in the case of SFFV_A-L-infected spleen cells there was a significant drop to almost zero in the culture without EPO (Table 1). When SFFV_A-RC was used to infect bone marrow cells from phenylhydrazine-treated mice, hemoglobinized bursts (1, 2) were observed on day 5 to 6 even without added EPO (see Table 1), although the number and size of bursts were increased in the presence of 0.2 U of EPO per ml, a result typical of SFFV_P. In contrast, when cells were infected with the SFFV_A-L complex, hemoglobinized bursts were only observed in the presence of added EPO.

Both SFFV_P and SFFV_A are known to encode a 52-kilodalton (kDa) env-related glycoprotein (gp52) and a 45-kDa gag-related protein (p45). However, only in SFFV_P-infected cells can a 65-kDa env-related protein containing complex carbohydrates be detected (14, 18, 19). It has been postulated that this gp65, which can be detected on the cell surface, might be responsible for the biological differences between the two strains. Spleen cells from animals infected 3 weeks earlier with SFFV_A-RC were metabolically labeled with [³⁵S]methionine and immunoprecipitated with anti-gp70
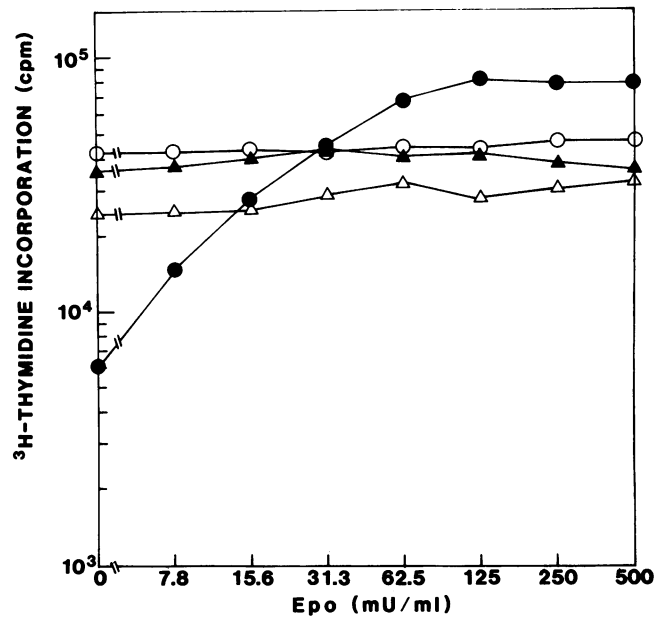
and anti-gag sera. It is clear that the infected cells made both gp52 as well as p45 (Fig. 3A). Further labeling experiments with [³H]galactose confirmed that this new virus, like the SFFV_AP-L control, produces a 65-kDa env-related glycoprotein (Fig. 3B, lanes 1 and 3), whereas no such protein was detected in SFFV_A-L-infected NIH 3T3 cells (Fig. 3B, lane 5). The dark, intensive band above gp65 in lane 1 (Fig. 3B) is derived from the helper virus, since a virus-producing line was used in this particular experiment whereas nonproducers were used for the controls (lanes 3 through 6). The helper

TABLE 1. Effect of different SFFV recombinants on erythroid colony formation

Virus	CFU-E/10⁵ cells[a]		vBFU-E/10⁶ cells[b]	
	+EPO	−EPO	+EPO	−EPO
SFFV_A-L	273	4	126	0
SFFV_AP-L	864	804	65	13
SFFV_A-RC	1,580	1,426	108	6
No virus	ND[c]	ND	0	0

[a] Spleen cells from animals infected with virus were cultured in plasma clots as previously described (21) with and without EPO. Two days later, clots were harvested and stained with benzidine-hematoxylin. Benzidine-positive colonies (CFU-E) were counted under a microscope.

[b] Assay was done according to previous procedures with some modifications (1, 2). Bone marrow cells from animals previously treated with phenylhydrazine were infected with different SFFV recombinants in vitro and then plated in methylcellulose at 5×10^5 cells per ml. Six days later, hemoglobinized bursts (vBFU-E) were counted in situ under an inverted microscope.
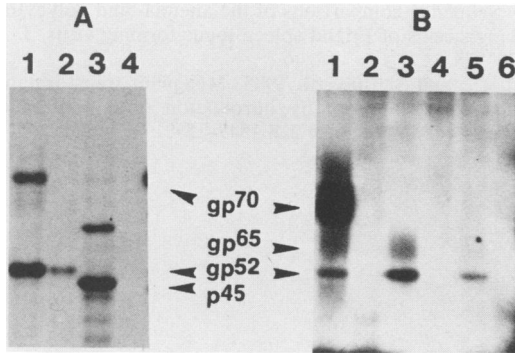
[c] ND, Not done.

FIG. 3. Immunoprecipitation of [^{35}S]methionine- and [^{3}H]galactose-labeled SFFV cytosol proteins. (A) SFFV$_{A\text{-}RC}$/Moloney MuLV-infected spleen cells were metabolically labeled with [^{35}S]methionine for 1 h, immune precipitated with anti-Rauscher MuLV gp70 serum (lane 1), anti-mink cell focus-inducing virus gp70 serum (lane 2), anti-*gag* serum (lane 3), and normal goat serum (lane 4), and electrophoresed on a 7% sodium dodecyl sulfate-polyacrylamide gel as described before (15). (B) SFFV$_{A\text{-}RC}$ producer cells (lanes 1 and 2), SFFV$_{AP}$-L nonproducer cells (lanes 3 and 4), or SFFV$_A$-L (lanes 5 and 6) nonproducer cells were labeled with [^{3}H]galactose for 2 h, immune precipitated with anti-Rauscher MuLV gp70 goat serum (lanes 1, 3, and 5) or normal goal serum (lanes 2, 4, and 6), and electrophoresed as described for panel A.

virus by itself did not synthesize any glycoprotein around 65 kDa (data not shown).

We can conclude from our studies that the sequences responsible for the biological and biochemical differences between SFFV$_P$ and SFFV$_A$ can be localized to a 678-base-pair region representing the 3' portion of the *env* gene and that any sequence differences in the LTR do not contribute to the different phenotypes of the viruses. Substitution of *env* sequence in SFFV$_A$ with the analogous *env* gene fragment from SFFV$_P$ resulted in a virus that was changed biologically in its requirement for EPO and biochemically in the posttranslational processing of its *env* gene product. This lends additional support to the hypothesis that differences in processing of the envelope protein to the cell surface may contribute to the biological differences between the two strains. The actual mechanism by which SFFV$_P$ alters the responsiveness of erythroid cells to EPO is still not known. Whether or not the cell surface form gp65 represents an activated EPO receptor will definitely need further verification. Although the human and murine EPO genes have been cloned and sequenced recently (7, 9, 16), not much is known about the EPO receptor. Using a [^{3}H]EPO binding assay, Krantz and Goldwasser (5) have shown some binding of EPO to SFFV$_A$-infected cells and estimated an average of 600 binding sites per cell. However, no data have been presented on SFFV$_P$-infected cells. We are currently doing experiments on EPO binding, using a modified binding assay. Studying the differences between SFFV$_P$ and SFFV$_A$ should shed more light on the regulation of this erythroid-specific hormone.

To localize more precisely which sequences within the 678-base-pair *env* gene fragment are responsible for the altered EPO responsiveness of SFFV$_P$-infected cells, we are further dissecting this region into two subregions. This will enable us to determine the significance of specific regions of the envelope glycoprotein to the phenotype of the virus produced.

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