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Envelope Gene Sequences Which Encode the gp52 Protein of Spleen Focus-Forming Virus Are Required for the Induction of Erythroid Cell Proliferation

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A series of insertion-deletion mutants was constructed in a molecularly cloned DNA copy of the Friend strain of spleen focus-forming virus (SFFV). The mutants were produced by inserting a synthetic oligonucleotide linker containing the recognition sequence of *Sal*I endonuclease into several different locations of the SFFV DNA. Three classes of mutants were isolated: insertion-deletion mutants in the 5′ half of the SFFV genome, in the long terminal repeat of the SFFV genome, and in the *env* gene of the SFFV genome. The *env* gene mutant has a deletion of sequences shared in common between the *env* gene of SFFV and the *env* genes of mink cell focus-inducing murine leukemia viruses. From analyses of the biological activity of the various mutants and a biologically active subgenomic SFFV DNA fragment described herein, we can deduce that the coding sequence encompassing the *env* gene of SFFV is required for the biological activity. This region, required for the pathogenic phenotype, cannot be larger than 1.5 kilobase pairs, a size only slightly more than that sufficient to encode the nonglycosylated precursor of the gp52 *env* gene product.

The Lilly-Steeves strain of the replication-defective spleen focus-forming virus (SFFV) was isolated from the Friend virus complex and has been shown to be responsible for the induction of a rapid erythroproliferative disease when injected as a viral complex into susceptible mice (37). The disease produced by this virus is characterized by the development of splenic foci, splenomegaly, erythroblastosis, and polycythemia (22). Although the rapidity of the disease varies depending on the helper virus used, the erythroid pathology is independent of the type of helper virus used in the infectious viral complex. SFFV has been shown to promote the proliferation of erythroid precursor cells in culture as well, but does not cause the abnormal growth of epithelial cells or fibroblasts. The in vivo and in vitro proliferation of erythroid precursor cells is induced also by other replication-defective retroviruses such as the avian erythroblastosis virus (13), the Harvey and Kirsten sarcoma viruses (31), the myeloproliferative variant of Moloney sarcoma virus (24), and even the Abelson leukemia virus (43). Each of these latter viruses can also transform fibroblastic cells in culture (9, 13, 24, 31), unlike SFFV, which has a select target specificity for erythroid cells.

Molecular studies of the avian erythroblastosis virus, the Harvey and Kirsten sarcoma viruses, and other defective highly oncogenic retroviruses (10, 26, 33) have shown that these viruses contain recombinant genomes with helper-independent retroviral sequences and unique sequences of host cellular information. For each of these viruses a protein has been isolated which is encoded at least in part by the cellular sequences and is believed to be responsible for the oncogenic potential of the virus (2, 16, 34, 35, 44).

In contrast to the above viruses, the genome of SFFV has been shown to be a recombinant between sequences of the helper-independent Friend murine leukemia virus (F-MuLV) and sequences related to the envelope gene of murine xenotropic and mink cell focus-inducing type C viruses (38, 39). No unique cellular sequences have been detected in the genome of SFFV. Two proteins encoded by SFFV have been detected, a p45 *gag* gene-related protein (1) and the recombinant *env* gene-related protein gp52 (8, 28, 29). However, due to the replication-defective nature of SFFV coupled with its

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inability to transform fibroblastic cells, no mutants of SFFV have been described. Thus, there exists no genetic information which could identify the sequences or gene product(s) of SFFV involved in the erythroid disease.

Previously, we reported the molecular cloning of SFFV proviral DNA (19) and a subgenomic fragment of this DNA (20) to begin a genetic analysis of the virus. More recently, a second polycythemia-inducing strain of SFFV has been molecularly cloned (45). Using a two-stage cotransfection assay developed to measure the biological activity of SFFV (19), we showed that the sequences of SFFV responsible for the virus-induced disease were derived from the 3′ half of the genome. These results were in agreement with our hypothesis that gp52 is responsible for this disease. We now report the cloning of a second subgenomic fragment of SFFV proviral DNA and the production and cloning of nine mutant SFFV DNAs. The results from analyses of these cloned DNAs offer compelling evidence that the gp52 protein encoded by SFFV is required for the virus-induced proliferation of erythroid precursor cells.

MATERIALS AND METHODS

Cells and viruses. NIH 3T3 fibroblasts, the normal rat kidney cell nonproductively infected with SFFV, and a culture of Fisher rat embryo cells, designated EY10, which stably produce an excess of SFFV over F-MuLV as determined by analysis of RNA subunits (11) have been described (38–40). Clone 4-1a3, the 5.7-kilobase pair (kbp) molecular clone of the Lilly-Steeves (18) strain of SFFV, and the molecularly cloned *Hin*dIII-*Pst*I subgenomic fragment of SFFV DNA have been described (19, 20). Molecular clones of helper-independent ecotropic virus DNAs used in this study include a clone of ecotropic Friend virus (F-MuLV clone 57) (23), a clone of ecotropic Moloney MuLV (clone 1387), and a clone of wild mouse amphotropic virus 4070A (AMT clone 8) (4, 15).

Molecular cloning of the *Bam*HI-*Pst*I DNA fragment. The *Bam*HI-*Pst*I subgenomic SFFV DNA fragment was molecularly cloned, as described in the text, by using standard methods (5, 6, 30) under P2 containment conditions as outlined in the National Institutes of Health recombinant DNA research guidelines, part II.

In vitro mutagenesis of cloned DNA. Before mutagenesis, the SFFV DNA insert of clone 4-1a3 was transferred to a pBR322 vector lacking a *Sal*I recognition site as outlined below. The pBR322 vector DNA was cleaved with *Sal*I and *Bam*HI; the resulting 4.1-kbp fragment was separated by electrophoresis through an agarose gel and collected by electroelution. This DNA fragment (2 μg) was made flush ended by treatment with 2 U of DNA polymerase I Klenow fragment from *Escherichia coli* (New England BioLabs) in the presence of deoxyribonucleoside triphosphates, and the potentially blunt ends were recircularized by treatment with 4 U of T4 DNA ligase (Bethesda Research Labs). The ligated DNA was digested with *Sal*I restriction enzyme, and the DNA was then cloned by transformation of *E. coli* strain RR1 and selection on ampicillin. After we screened the clones for the presence of a *Sal*I restriction enzyme site, the plasmid DNA of one clone lacking a *Sal*I site (clone 7-21) was linearized by cleavage with *Hin*dIII. The 5.7-kbp insert of clone 4-1a3 was removed by *Hin*dIII digestion and ligated to the *Sal*I(−) vector DNA. This ligated material was transformed into *E. coli* strain RR1 to generate a clone of SFFV DNA (clone 16-22a1) lacking *Sal*I recognition sites.

In vitro mutagenesis of clone 16-22a1 was performed by a modification of the procedure published by Heffron et al. (17). Briefly, a limited digestion of 10 μg of the SFFV DNA clone was made with *Hae*III to produce linear flush-ended molecules. The preparation was extracted once with phenol and electrophoresed through a 1% agarose gel, and the DNA migrating in the region of the full-length recombinant plasmid (~9.8 kbp) was collected by electroelution. Using T4 DNA ligase, we ligated this DNA (2 μg) to 0.25 unit of *Sal*I molecular recombination linkers (double-stranded octamer G-G-T-C-G-A-C-C; Collaborative Research, Inc.) which had been labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (P-L Biochemicals). The resulting preparation was digested with *Sal*I to remove excess linkers and form the appropriate staggered ends and subsequently electrophoresed through a 1% agarose gel to separate the recombinant plasmid from the linker molecules. After electroelution, the plasmid DNA was circularized, using T4 DNA ligase, and 30 ng was then used to transform *E. coli* strain RR1, yielding 430 clones.

Oligonucleotide fingerprint analysis. ^{32}P-labeled SFFV genomic RNA prepared from the EY10 culture was hybridized to cloned SFFV-pBR322 DNAs after digestion of the recombinant plasmids with *Hin*dIII. The hybridization reactions were incubated at 50°C for 12 h and treated with RNase T_1. The RNase-resistant hybrids were isolated by gel filtration, denatured, and analyzed as described previously (11, 12).

Cotransfection assay for SFFV biological activity. The cotransfection of NIH 3T3 fibroblasts with calcium phosphate-precipitated SFFV and helper virus DNAs has been detailed (19). To determine the biological activity of the cotransfected SFFV DNA, we injected samples of cell-free supernatants from virus-producing transfected cells into NIH Swiss mice. Intravenous injections of 0.5-ml samples into 6- to 8-week-old mice were made when F-MuLV DNA was used in the cotransfection, and intraperitoneal injections of 0.2-ml samples into newborn mice were made when mouse amphotropic virus DNA was used in the assay.

Metabolic labeling of cells, immunoprecipitation, and polyacrylamide gel electrophoresis. A suspension of 2 $\times 10^7$ cells in 3 ml was prepared from the spleens of diseased animals and labeled for 1 h at 37°C with 600 μCi of [^{35}S]methionine. Extracts of the cells were prepared, and analyses of immune precipitates were performed as described (27, 28).

RESULTS

Cloning of the *Bam*HI-*Pst*I subgenomic fragment of SFFV DNA. We previously cloned a 3.0-kbp *Hin*dIII-to-*Pst*I fragment of the Lilly-

Steeves strain of SFFV DNA in pBR322 (clone 23-1a2) and demonstrated that it retained the biological activity of full-length SFFV DNA (20). To obtain a smaller piece of SFFV DNA, this clone was digested with *Bam*HI, generating 4.6- and 3.45-kbp fragments. The 3.45- kbp fragment included the 2.4-kbp *Bam*HI-to-*Pst*I region of SFFV DNA from the 3' end of the viral genome, which contains *env* gene and long terminal repeat (LTR) sequences, and the *Pst*I-to-*Bam*HI segment of pBR322 (Fig. 1). These digestion products were electrophoresed through an agarose gel, and the 3.45-kbp fragment was isolated by electroelution and ligated to *Bam*HI-digested pBR322 DNA. This ligation mixture was used to transform *E. coli* strain RR1. By using selection for ampicillin resistance and hybridization to a ^{32}P-labeled probe prepared by nick translation of the *Hin*dIII-*Pst*I fragment, clone 21-3a1 was isolated. Figure 1 shows an agarose gel analysis of this *Bam*HI-*Pst*I fragment of SFFV DNA. Lanes 1a and 1b show the *Bam*HI plus *Pst*I cleavage products of the *Hin*dIII-*Pst*I and *Bam*HI-*Pst*I fragment DNA clones, respectively. Both recombinant DNAs have the 2.4-kbp viral DNA fragment as well as 3.3- and 1.1-kbp fragments generated from the pBR322 DNA. The *Bam*HI-*Pst*I clone has two copies of the 1.1-kbp plasmid DNA band, whereas the *Hin*dIII-*Pst*I clone has only one copy with an additional band which migrated at a size of 1.3 kbp due to the *Hin*dIII-to-*Bam*HI region of viral DNA present in this clone; these two smaller bands are only slightly visible in this photograph (see the schematic diagrams of the cloned recombinant DNAs in the figure). When the cloned DNAs were cut with *Pst*I plus *Pvu*II (lanes 3a and 3b), both had the 1.7-kbp viral DNA fragment as well as the 2.9- and 1.5-kbp bands of DNA derived from the vector DNA. The *Bam*HI-*Pst*I clone yields another 1.7-kbp band containing viral and vector sequences, whereas the *Hin*dIII-*Pst*I clone yields a band which migrates at 1.9 kbp, again owing to the *Hin*dIII-to-*Bam*HI region of this larger viral DNA insert. In the *Bam*HI-*Pst*I clone, the viral insert and plasmid vector bands are not present in equal molar ratios; this is due to loss of the insert, probably by recombination through the *Pst*I-*Bam*I region of pBR322, which is directly repeated in this clone. One can deduce by these restriction analyses that the cloned *Bam*HI-*Pst*I fragment DNA derives from the 3' end of the viral genome and contains the LTR of the permuted SFFV molecular clone as already demonstrated with the *Hin*dIII-*Pst*I DNA clone (20). In addition, as discussed below, the sequences of the *Bam*HI-*Pst*I fragment are complementary to RNase T_1-resistant oligonucleotides which map to the 3' end of SFFV genomic DNA.

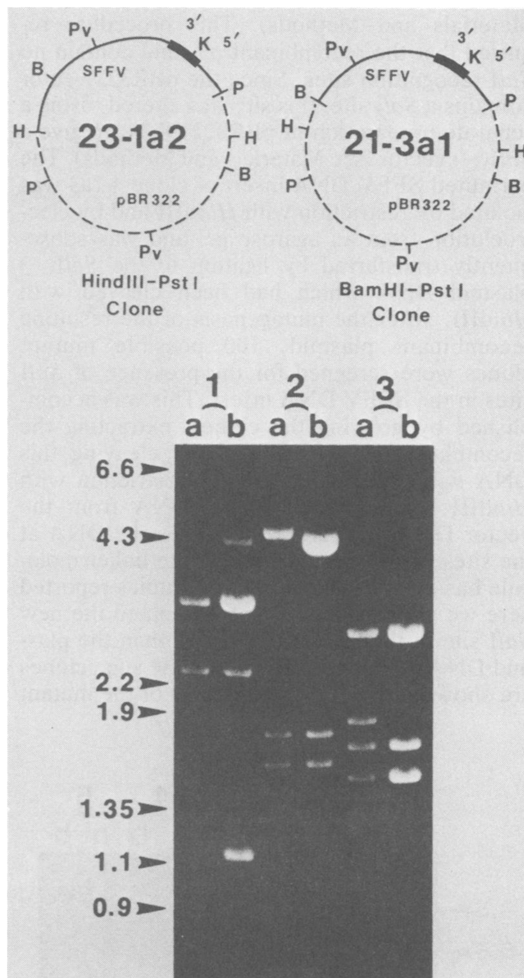

FIG. 1. Comparison of the two cloned SFFV DNA fragments by restriction enzyme analysis. The recombinant plasmid DNAs were isolated from the clone containing the *Hin*dIII-*Pst*I fragment (a lanes) and the clone containing the *Bam*HI-*Pst*I fragment (b lanes) of SFFV DNA. These recombinant DNAs were digested with *Bam*HI plus *Pst*I (lane 1), *Bam*HI plus *Kpn*I (lane 2), and *Pst*I plus *Puv*II (lane 3) and electrophoresed at 55 V for 18 h through a 1% agarose gel containing 0.5 μg of ethidium bromide per ml. The bands of DNA were visualized with UV light. The numbers at the left of the gel represent the location and size (in kilobase pairs) of *Hin*dIII-digested λ DNA fragments and *Hae*III-digested φX174 DNA fragments migrating in the same gel. Schematic circular restriction maps of the recombinant clones are shown for reference: H, *Hin*dIII; B, *Bam*HI; Pu, *Puv*II; K, *Kpn*I; and P, *Pst*I. The heavy line indicates the position of the viral LTR sequences; the relative location of the 3' and 5' areas of the SFFV genomic RNA are also indicated.

In vitro mutagenesis of full-length SFFV DNA. In vitro mutagenesis was performed on the cloned full-length SFFV DNA by using a modification of the procedure of Heffron et al. (17) (see

Materials and Methods). This procedure required that the recombinant plasmid contain no *Sal*I recognition sites. Since the pBR322 vector contains a *Sal*I site, this site was altered, using a separate preparation of pBR322 DNA, to give a *Sal*I(−) vector (see Materials and Methods). The permuted SFFV DNA insert of clone 4-1a3 was isolated by restriction with *Hin*dIII and by electroelution from an agarose gel and was subsequently transferred by ligation to the *Sal*I(−) plasmid DNA which had been cleaved with *Hin*dIII. After the mutagenesis of the resulting recombinant plasmid, 100 possible mutant clones were screened for the presence of *Sal*I sites in the SFFV DNA insert. This was accomplished by growing the clones, extracting the recombinant plasmid DNA, and cleaving this DNA with *Hin*dIII plus *Sal*I. Restriction with *Hin*dIII separates the SFFV DNA from the vector DNA, and the *Sal*I cleaves the DNA at the sites of mutagenesis where the linker molecule has been inserted. For the studies reported here we chose clones which contained the new *Sal*I site in the viral DNA rather than the plasmid DNA, and the results with nine such clones are shown in Fig. 2. The majority of the mutant clones were found to have deletions of from 0.15 to 2.0 kbp, although a few clones had little or no deleted DNA. The deletions presumably occurred by multiple cuts with *Hae*III during the mutagenesis procedure, yielding smaller than full-length molecules to which the *Sal*I linkers were attached. The precise locations of the *Sal*I sites and the deletions in the mutant DNA clones were determined by restriction analysis with other enzymes, for instance, *Hin*dIII plus *Sal*I plus *Kpn*I as shown in the b lanes of Fig. 2. The schematic maps of these sites are shown in Fig. 3. To insure the location of the new *Sal*I sites, we examined the orientations of the two viral *Hin*dIII-*Sal*I fragments of each mutant by Southern blot analysis; the fragments hybridized to a nick-translated probe prepared from the insert of the cloned 600-base pair (bp) *Bam*HI-*Eco*RI subgenomic fragment of SFFV DNA located in the *env* gene region (3). In each case this probe hybridized only to the *Hin*dIII-*Sal*I fragment which mapped to the left-hand side of the cloned permuted SFFV DNA, as would be expected (data not shown).

Biological activity of the *Bam*HI-*Pst*I fragment and mutant DNA clones. The biological activity

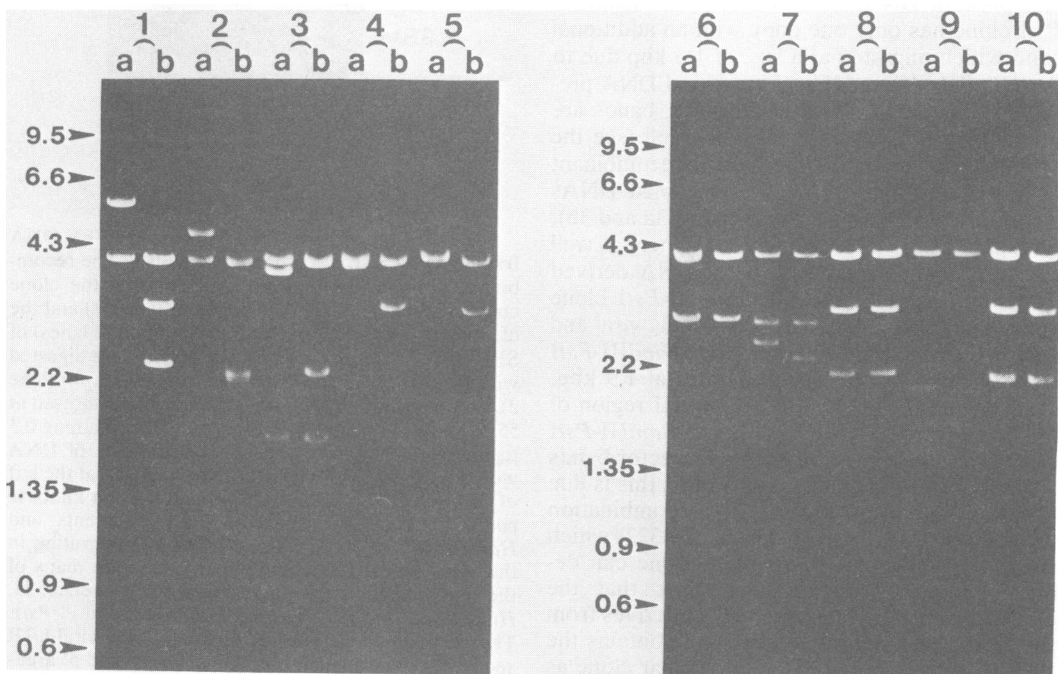

FIG. 2. Restriction enzyme analysis of various clones of in vitro-mutagenized SFFV DNA. Recombinant plasmid DNAs isolated from the clones of mutagenized SFFV DNA were digested with *Hin*dIII plus *Sal*I (a lanes) and *Hin*dIII plus *Sal*I plus *Kpn*I (b lanes). The DNA was electrophoresed and visualized as described in the legend to Fig. 1. The clones analyzed are: 16-22a1, the nonmutagenized SFFV DNA of clone 4-1a3 inserted into the *Sal*I (−) pBR322 plasmid (lane 1); 22-2 (lane 2); 22-12 (lane 3); 22-23 (lane 4); 22-62 (lane 5); 22-6 (lane 6); 22-8 (lane 7); 22-17 (lane 8); 22-25 (lane 9); and 22-35 (lane 10).

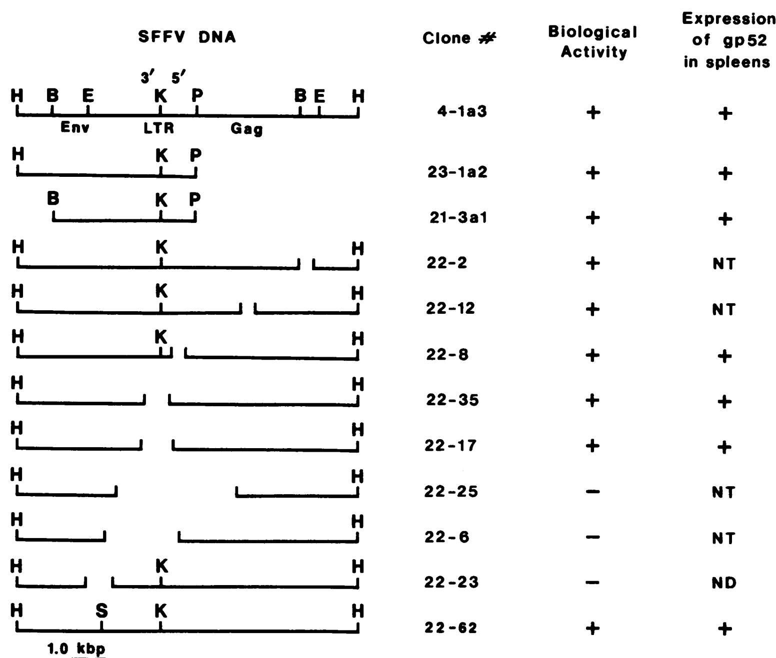

FIG. 3. Schematic map and summary of analyses of subgenomic fragments and mutant clones of SFFV DNA. The restriction maps of the designated clones of SFFV DNA are shown for the enzymes *Hin*dIII (H), *Bam*HI (B), *Eco*RI (E), *Kpn*I (K), *Pst*I (P), and *Sal*I (S). The maps of the deletion mutant DNAs are separated at the added *Sal*I site to indicate the area lost by the mutagenesis. The biological activity was measured by the two-stage cotransfection assay; a positive result indicates that splenomegaly, splenic foci, and polycythemia were induced in the infected mice (see Tables 1 and 2). The expression of gp52 was measured as in the legend to Fig. 5. ND, none detected; NT, not tested.

of the cloned SFFV DNA was tested by using the two-stage cotransfection assay reported previously (19). Briefly, the cloned fragment or mutant SFFV DNA was released from the vector DNA by cleavage with the appropriate restriction enzyme(s) and transfected into NIH 3T3 fibroblasts with infectious molecularly cloned helper virus DNA of F-MuLV or wild mouse amphotropic virus 4070A. The progeny virus resulting from the cotransfections with F-MuLV DNA was injected intravenously into 6- to 8-week-old NIH Swiss mice, and the mice were monitored for SFFV disease characteristics. The virus produced after cotransfection with wild mouse amphotropic virus DNA was similarly monitored for biological activity after intraperitoneal injection of newborn NIH Swiss mice. The results of these cotransfection studies are shown in Tables 1 and 2.

Cotransfections of the *Bam*HI-*Pst*I DNA fragment with the F-MuLV or the amphotropic virus DNA yielded virus progeny capable of inducing all of the characteristics of SFFV disease, including splenic foci, splenomegaly, erythroblastosis, and polycythemia. Injection of the virus produced by cells transfected with either helper virus DNA alone did not induce SFFV disease in the mice.

The SFFV disease was induced similarly in mice injected with the virus released from cells cotransfected with amphotropic helper virus DNA and any mutant DNA with deletions in the right-hand half of the circularly permuted cloned sequences (the 5′ portion of the SFFV genome) such as clones 22-2 and 22-12. This result was expected since these 5′ sequences are not present in either the *Bam*HI-*Pst*I or *Hin*dIII-*Pst*I (20) subgenomic fragment DNA, both of which demonstrate biological activity in the assay.

A virus capable of inducing the SFFV erythroid disease in mice is also produced by cells cotransfected with helper virus DNA and SFFV mutant DNA containing a deletion in the central area of the permuted cloned DNA. These mutant DNAs from such clones as 22-8, 22-17, and 22-35 contain deletions of the sequences from the very 3′ or 5′ ends (or both) of the viral genome; these deletions include sequences in the LTR. When amphotropic viral DNA was used as a helper, the latency period for disease in mice was rather prolonged, suggesting that the titer of biologically active virus was low

TABLE 1. Biological activity of various SFFV DNAs cotransfected with amphotropic viral DNA

Source of SFFV DNA	Time after injection (days)	Spleen wt (g)[a]	Hematocrit (%)[a]	Production of splenic foci
SFFV permuted clone (4-1a3)	20	0.21, 0.33	44, 45	+ +
	41	2.3 , 3.4	60, 72	TNTC[b]
Subgenomic SFFV fragments				
21-3a1	20	0.10, 0.10	43, 44	+ +
	41	1.2 , 3.2	63, 67	TNTC
23-1a2	29	1.9 , 0.46	58, 47	+ +
5′ Deletion mutants				
22-2	25	0.88, 0.75	57, 60	+ +
22-12	25	0.33, 0.96	43, 60	+ +
22-8	25	0.70, 0.95	52, 56	+ +
LTR deletion mutants				
22-35	85	4.5 , 0.17	88, 50	TNTC, −
22-17	52	1.3 , 0.10	74, 47	+ −
env Deletion mutants				
22-6	25	0.19, 0.13	43, 44	− −
	42	0.13, 0.08	47, 46	− −
22-25	29	0.11, 0.13	40, 42	− −
	85	0.15, 0.13	45, 43	− −
22-23	25	0.14, 0.11	45, 43	− −
	42	0.16, 0.14	44, 43	− −
env Insertion mutant (22-62)	52	2.2 , 0.39	69, 62	+ +
None	42	0.15, 0.15	43, 45	− −
	85	0.18, 0.13	46, 48	− −

[a] Values from individual mice. The normal spleen weight and hematocrit values are 0.1 to 0.3 g and 42 to 50%, respectively.
[b] TNTC, Too numerous to count.

(Table 1). However with F-MuLV helper DNA, a fairly rapid onset of disease was observed (Table 2).

Cotransfections of helper virus DNA and certain deletion mutant SFFV DNAs have never yielded a virus capable of inducing the SFFV disease in mice. These mutant DNAs include those from clones with deletions that extend leftward into the *env* gene sequences (clones 22-6 and 22-25) or with a deletion of *env* sequences alone (clone 22-23). One SFFV mutant DNA clone, designated 22-62, contained the insertion of a new *Sal*I site in the *env* gene region, with no deletion of sequences detectable by agarose gel analysis. The progeny virus of cells cotransfected with this insertion mutant DNA and helper virus DNA of F-MuLV or the amphotropic virus was capable of inducing all of the characteristics of the SFFV disease when injected into mice. To insure the purity of this clone, the mutant DNA insert was isolated by electroelution from an agarose gel, ligated into fresh pBR322 at the *Hin*dIII site, and recloned by transformation of *E. coli*. The recloned sequences still demonstrated the full biological activity of SFFV (data not shown).

RNase T_1-resistant oligonucleotide analysis. It was of interest to determine whether the sequences deleted in the mutant DNAs were unique to SFFV or related to the parental helper F-MuLV. Since the relatedness of the RNase T_1-resistant oligoribonucleotides of SFFV genomic RNA to F-MuLV has been studied (11, 12), we analyzed which of these oligonucleotides were homologous to the sequences retained in several of the cloned mutant DNAs. The cloned DNAs were hybridized to ^{32}P-labeled SFFV genomic RNA, and the pattern of oligonucleotides which hybridized to each DNA was determined. The resulting patterns obtained with four mutant DNAs are shown in Fig. 4A through 4D. The analysis was also performed with the *Bam*HI-*Pst*I subgenomic fragment clone (data not shown). A schematic summary of the results

TABLE 2. Biological activity of various SFFV DNAs cotransfected with F-MuLV viral DNA

Source of SFFV DNA	Time after injection (days)	Spleen wt (g)[a]	Hematocrit (%)[a]	Production of splenic foci
LTR deletion mutants				
22-35	22	2.1 , 0.59	58, 53	+ +
	43	2.8 , 2.9	78, 76	TNTC[b]
22-17	33	0.76, 0.18	48, 47	+ +
	69	2.8 , 1.8	70, 48	TNTC
env Deletion mutants				
22-25	22	0.21, 0.14	45, 49	– –
	43	0.19, 0.14	45, 48	– –
22-23	33	0.28, 0.19	46, 43	– –
	69	0.23, 0.15	43, 48	– –
env Insertion mutant (22-62)	19	0.33, 0.22	44, 37	+ +
	33	1.2 , 1.1	52, 56	TNTC
None	69	0.32, 0.13	42, 47	– –

[a] Values from individual mice. The normal spleen weight and hematocrit values are 0.1 to 0.3 g and 42 to 50%, respectively.
[b] TNTC, Too numerous to count.

found is shown in Fig. 4E. The oligonucleotides are presented in the nonpermuted orientation of the genomic RNA. As expected, clone 22-62 hybridizes to all of the oligonucleotides identifiable by this technique as does the full-length clone of SFFV DNA, clone 4-1a3 (see reference 12). The *Bam*HI-*Pst*I fragment clone contains the sequences of the 3' oligonucleotides from 12 to the right, including the terminally redundant oligonucleotide 13. The biologically active clone 22-35 and clone 22-17, which give the identical pattern (data not shown), lack only sequences homologous to oligonucleotides 18, 15, and 13, which are located at the ends of the genome. Clone 22-23, which demonstrates no biological activity in the two-stage assay, is missing the sequences of the SFFV-specific oligonucleotide 6 as well as oligonucleotides 11 and 8. The sequences of a number of oligonucleotides located at both ends of the genome are missing in clone 22-25, which is also negative for SFFV biological activity. These data are consistent with the restriction maps presented in Fig. 3.

Expression of gp52 in spleens of infected mice. Since all of the biologically active clones of SFFV DNA had the *env* gene sequences, it was of interest to determine whether *env* gene product gp52 was expressed in cells containing these DNAs. The efficiency of cotransfection and rescue of the SFFV sequences is generally too low to allow detection of the SFFV-specified proteins in the total population of cotransfected fibroblasts. Thus, [^{35}S]methionine was used to pulse-label cells from the enlarged spleens of mice injected with viruses produced by the cotransfected fibroblasts. Cellular extracts were then prepared and analyzed for gp52 by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 5). The gp52 was detected in the spleens of mice injected with the virus progeny of cells cotransfected with helper virus DNA and either the *Bam*HI-*Pst*I fragment of SFFV DNA (lane B), the mutant clone 22-17 DNA (lane C), the mutant clone 22-62 DNA (lane D), or the mutant 22-35 DNA (lane E). In all cases the gp52 demonstrated the same antigenic properties and gel migration as the gp52 expressed in normal rat kidney cells nonproductively infected with the Lilly-Steeves strain of SFFV (lane A). The expression of gp52 was never detected in spleen cells from mice infected with virus produced by fibroblasts transfected with any of the helper virus DNAs alone (data not shown).

DISCUSSION

In this paper we report the molecular cloning of a second subgenomic fragment from the *env* gene region of SFFV proviral DNA as well as the cloning of nine mutant DNAs prepared from cloned full-length SFFV proviral DNA. The subgenomic fragment extends from a *Bam*HI recognition site to the *Pst*I site of the previously reported *Hin*dIII-*Pst*I subgenomic fragment of circularly permuted SFFV DNA (20). This *Bam*HI-*Pst*I fragment is 2.4 kbp in size, lacking 0.6 kbp from the left-hand end of the *Hin*dIII-*Pst*I DNA fragment. Like the *Hin*dIII-*Pst*I fragment, the *Bam*HI-*Pst*I DNA fragment contains

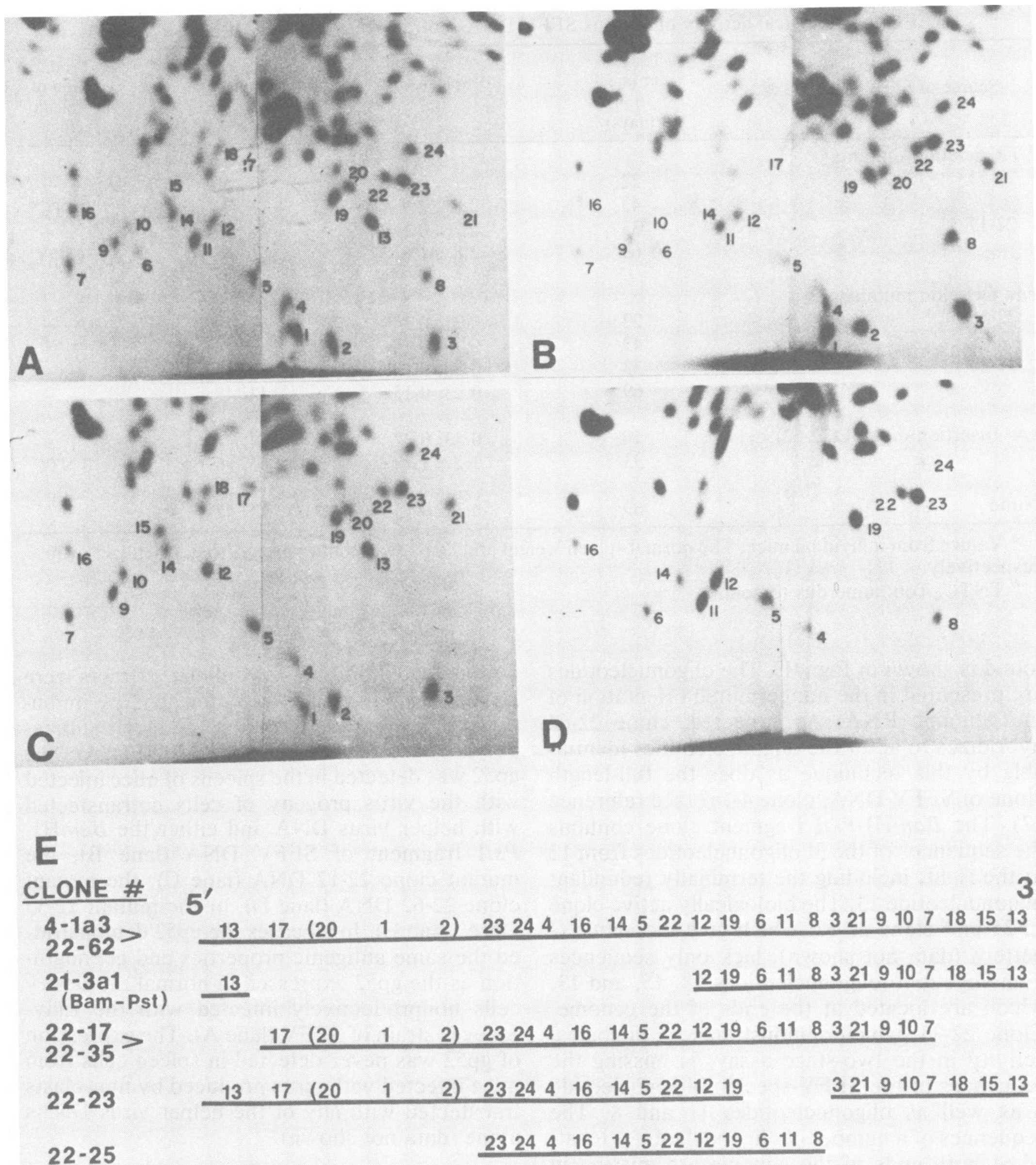

FIG. 4. RNase T_1-resistant oligonucleotides of SFFV genomic RNA complementary to the cloned fragment and mutant DNAs of SFFV. ^{32}P-Labeled SFFV RNA (5×10^5 cpm) was hybridized to 2 μg of plasmid DNA isolated from the SFFV mutant DNA clones 22-62 (A), 22-35 (B), 22-23 (C), and 22-25 (D). The hybridized RNA was then isolated and separated in two dimensions by electrophoresis and homochromatography. (E) Schematic maps of the oligonucleotides homologous to the indicated clones of SFFV DNA are shown. The relative positions of the oligonucleotides on the genomic map of SFFV RNA were published previously (11).

the 0.6-kbp LTR and 0.4 kbp from the 5′ portion of the proviral SFFV genome, but contains only 1.4 kbp, rather than 2.0 kbp, of the *env* gene region from the 3′ portion of the proviral genome. Since the *Hin*dIII-*Pst*I DNA fragment clone was used as the starting material for the molecular cloning of the *Bam*HI-*Pst*I fragment, the above structure was easily derived by restriction enzyme analyses, and the structure was confirmed by examining the RNase T_1-resistant oligonucleotides of genomic SFFV RNA which formed hybrids with the fragment DNA. It was significant that the cloned *Bam*HI-*Pst*I fragment was found to retain the full biological activity of SFFV when analyzed by the two-stage cotransfection assay. Thus, this narrows

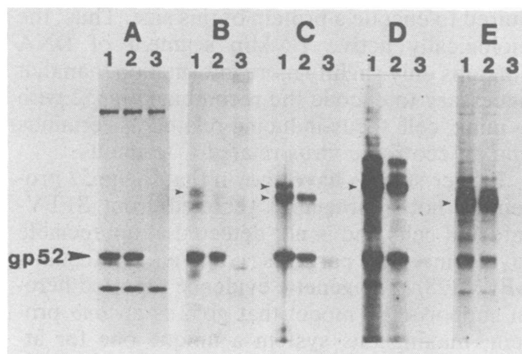

FIG. 5. Immunoprecipitation of proteins expressed in spleens of mice infected with the virus produced after cotransfections. Labeled spleen cell extracts were prepared as described in the text and precipitated with goat anti-Rauscher MuLV gp70 antiserum (lanes 1), goat anti-Moloney mink cell focus-inducing virus gp70 antiserum absorbed with Moloney MuLV (lanes 2), or normal goat serum (lanes 3). The spleens were from mice infected with virus produced from NIH 3T3 cells cotransfected with the *Bam*HI-*Pst*I fragment and Moloney MuLV DNA (lane B), mutant clone 22-17 and F-MuLV DNA (lane C), mutant clone 22-62 and amphotropic viral DNA (lane D), and mutant clone 22-35 and amphotropic viral DNA (lane E). Normal rat kidney cells nonproductively infected with SFFV were also labeled and immune precipitated (lane A). The small arrows denote the helper virus envelope precursor proteins.

the amount of SFFV-specified information required for the induction of disease by 600 bp from that previously reported with the *Hin*dIII-*Pst*I DNA fragment.

The ability to produce mutations at essentially random sites in the genome by the method used in this paper allows one to examine areas which cannot be conveniently cloned into subgenomic fragments due to the lack of appropriate restriction enzyme sites. We report here the studies of DNA from nine molecular clones containing such random mutations. DNA from clones such as 22-2 or 22-12 which have a deletion of sequences from the 5′ region of the genome, the right half of the circularly permuted cloned DNA, retain the full biological activity of SFFV, as expected, considering the positive results with the subgenomic fragments of DNA containing 3′ genomic information. In addition to the LTR and *env* gene sequences, these cloned subgenomic fragments also contain some information from the very 5′ end of the genome. However, when sequences in this 5′ end region are deleted, as in clone 22-8, the DNA is still biologically active in the two-stage cotransfection assay, demonstrating the insignificance of these 5′ SFFV sequences in disease induction.

It is of interest that DNA clones with deletions of sequences at the 3′-5′ junction of the genome, the LTR region, are also positive for biological activity. The LTR has been implicated to be a necessity for the disease induced by the avian leukosis viruses (41) by providing a promoter for transcription of host sequences adjacent to the integrated proviral DNA. Similarly, a promoter-like sequence has been found in the LTRs of Moloney MuLV and Moloney sarcoma virus (7, 25, 36, 42) and is located about 60 bp to the left of the *Kpn*I recognition site, which is present in the LTRs of other murine retroviruses (14, 21, 23). It is assumed that the LTR of SFFV contains a promoter sequence. However, the sequence of DNA deleted in clones 22-17 and 22-35 reported here includes at least the right-hand half of the SFFV LTR. Assuming that the *Kpn*I site and the possible promoter sequence are located in the same place in the SFFV LTR as that found for the LTR of the Moloney viruses, the possible promoter sequence would also be deleted in SFFV clones 22-17 and 22-35. Since these two mutant DNA clones retain the complete biological phenotype of SFFV, the SFFV LTR is probably not critical to SFFV-induced disease. Of course an analysis of the nucleotide sequence will be required to determine how much of the SFFV LTR actually remains in the DNA of clones 22-17 and 22-35 and whether the presumed promoter in this LTR is deleted. It should also be pointed out that the biologically active virus rescued from the cotransfections is probably formed after recombination of the SFFV sequences with those of the cotransfected helper virus sequences. Thus, the biologically active form would probably contain the helper virus LTR with its promoter. Obviously, the LTR is required for certain functions of the virus, most likely including its rescue. However, since the transfections of helper virus DNA alone do not generate virus preparations which induce disease in the mouse assay, the rapid proliferative phenotype must be determined by the *env* gene of SFFV, possibly in conjunction with LTR sequences, rather than by either a purely SFFV LTR or by downstream promotion by the helper viral LTR of some cellular *onc* gene.

Molecular clones of mutated SFFV DNA, with deletions extending leftward into the *env* gene sequences, such as clones 22-6 and 22-25, are negative for the biological activity. It is significant that clone 22-23, which lacks sequences only from the *env* gene region, is also negative for biological activity of SFFV in the cotransfection assay described here. Therefore, this is the first definitive proof of the necessity of the *env* sequences of SFFV for the initiation of the virus-induced erythroproliferative disease.

The only protein known to be encoded by the

env sequences of SFFV is the gp52 protein. Indeed the expression of gp52 is always correlated with the disease, and all results to this date are consistent with the hypothesis that this protein is required in the disease process. Nucleotide sequence data will be necessary to determine whether open reading frames for other proteins exist in the *env* gene region. The only confusing observation comes from results with mutant clone 22-62. The DNA of this clone contains a *Sal*I linker inserted in the *env* sequences, yet it still demonstrates SFFV biological activity in the described assay. However, critical to the hypothesis, the biologically active virus recovered still leads to the expression of gp52. The DNA of this clone 22-62 does not involve the deletion of enough sequences to be detected in the agarose gels, but certainly contains an insertion of the *Sal*I recognition sequence. It is possible that this change occurs in a region of nonessential sequences which are removed by splicing during a processing step or that the change is a minor one and does not alter the reading frame or function of the translated protein product, although a simple insertion of the 8-bp linker should give a reading frame shift. Alternatively, this small region of the *env* gene may not be unique to SFFV and could be repaired by recombination with the helper virus. Again, a study of the DNA sequence of this clone and of the recovered virus should help clarify the dilemma.

When the results obtained with the subgenomic DNA fragments are compared with those from the mutant DNA studies, one can define a 1.5-kbp segment of SFFV DNA which contains the only unique SFFV information essential for induction of the disease. This segment spans the region from the *Bam*HI site of the *Bam*HI-*Pst*I fragment to the *Sal*I site of the mutant clone 22-17 and encodes the gp52 protein. As further proof, this 1.5-kbp subgenomic DNA fragment was cloned from *Bam*HI plus *Sal*I double-digested clone 22-17 DNA in a plasmid vector containing the amphotropic virus LTR sequences. When analyzed by the two-stage assay after cotransfection with amphotropic viral DNA, this subgenomic DNA fragment clone demonstrated full SFFV biological activity in the mouse (data not shown). The fragment begins on the left, with the area containing the SFFV-specific oligonucleotides 12, 19, and 6, which are related to the recombinant *env* sequences of mink cell focus-inducing viruses (12), and continues through the area of oligonucleotides 11, 8, 3, 21, 9, 10, and 7, which are more related to the parental ecotropic F-MuLV (11, 12). Studies of nonglycosylated gp52 demonstrate an unglycosylated apoprotein of about 46,000 daltons (32). Approximately 1.4 kbp of DNA would be required to encode a protein of this size. Thus, the biologically active 1.5-kbp segment of DNA contains only slightly more information than that necessary to encode the recombinant gp52 with a mink cell focus-inducing-related N-terminus and an ecotropic virus-related C-terminus.

Earlier studies have shown that the gp52 protein is not appreciably secreted from SFFV-infected cells and is not detected at appreciable levels in viral particles containing infectious SFFV (28). The genetic evidence reported herein supports the model that gp52 is an *onc* protein, making this system a unique one for attempting to unravel how a glyprotein can induce unrestrained proliferation of erythroid cells.

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