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Biological activity of the spleen focus-forming virus is encoded by a molecularly cloned subgenomic fragment of spleen focus-forming virus DNA

(DNA transfection/Friend virus/restriction endonuclease/erythroproliferative disease)

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ABSTRACT **A biologically active subgenomic DNA fragment of a polycythemia-inducing strain of the replication-defective spleen focus-forming virus (SFFV) has been molecularly cloned. The SFFV DNA fragment includes 2.0 kilobase pairs (kbp) from the 3′ end of SFFV, the long terminal repeat sequences of SFFV, and 0.4 kbp from the 5′ end of SFFV. The fragment contains the previously described *env*-related gene of SFFV. All the properties associated with SFFV can be assigned to this SFFV DNA fragment by using a two-stage DNA transfection assay with infectious helper virus DNA. The virus recovered from the transfection assays can induce erythroblastosis, splenic foci, and polycythemia in infected mice. Fibroblast cultures transfected with the SFFV DNA fragment synthesize gp52, the known intracellular product of the *env*-related gene of SFFV. gp52 can also be detected in spleens from diseased mice infected with the virus recovered in the two-stage transfection. The results are consistent with the hypothesis that the *env*-related gene sequences of SFFV and their product gp52 are required for the initiation of SFFV-induced disease.**

A diverse group of retroviruses can induce the *in vivo* or *in vitro* proliferation of erythroid precursor cells. Avian erythroblastosis virus can induce malignant erythroid precursors in chickens (1), Harvey and Kirsten sarcoma viruses cause erythroleukemia in susceptible mice (2), and a variant of Moloney sarcoma virus has recently been shown to be responsible for an erythroproliferative disease in adult mice (3). Each of these viruses can also transform fibroblastic cells in culture (1–4). Another group of retroviruses which can cause the abnormal growth of erythroid hemopoietic cells are those present in stocks of Friend virus complex: the helper-independent Friend murine leukemia virus (F-MuLV) and the replication-defective spleen focus-forming virus (SFFV) (4–6). Although SFFV causes abnormal growth of erythroid precursor cells both in cell culture and in susceptible mice, infection of fibroblastic or epithelial cells with SFFV does not lead to abnormal growth of these cells. Because of the distinguishable biological properties of these five viruses, we assume that the viral genes and viral gene products responsible for the erythroproliferative phenotype induced by each virus will be different. In order to test this assumption, genetic analysis of each virus is necessary. However, only a small amount of data are available on the viral genes responsible for erythroproliferative diseases (7–11).

Molecular analyses of SFFV by hybridization and oligonucleotide pattern ("fingerprinting") methods revealed that the viral RNA contains sequences related to the *env* genes of both ecotropic and xenotropic murine leukemia viruses (12–15). The recombinant *env*-related gene of SFFV encodes a 52,000-dalton glycoprotein, termed gp52 (16–18). Because gp52 is the only gene product that we could detect as common to all strains of SFFV, we postulated that gp52 is required for SFFV-induced erythroblastosis (18). Recently, we described a method for molecularly cloning a complete copy of the Lilly–Steeves strain of SFFV (19). The molecular clones of SFFV were shown to be biologically active in a two-stage transfection assay and, most importantly, the SFFV phenotype could be recovered from molecularly cloned SFFV-pBR322 circular DNA by cotransfection with infectious type C retroviral DNA (19).

We have now extended these studies by molecularly cloning a subgenomic fragment of SFFV DNA which retains all the pathogenic properties of the originally described SFFV. This fragment is derived from the 3′ half of the SFFV genome and contains the gene that encodes the *env*-related gp52 protein.

MATERIALS AND METHODS

Cells and Viruses. NIH 3T3 fibroblasts, Fisher rat embryo cells (FRE clone 2), and normal rat kidney cells (NRK) have been described (12, 13). The strain of SFFV used in this study, $SFFV_{LS}$, was described by Lilly and Steeves (20). The 5.7-kilobase pair (kbp) molecular clone of SFFV (clone 4-1a3) used in this study has recently been described (19). EY10, a FRE cell that stably produces an excess of SFFV over the helper F-MuLV (21), has also been described (12). A helper-independent ecotropic Friend virus, F-MuLV clone 57, was obtained after transfection of NIH 3T3 cells with molecularly cloned F-MuLV 201 DNA (22). Other molecular clones used include a molecular clone of ecotropic Moloney virus (Mo-MuLV clone 1387) and a molecular clone of wild mouse amphotropic virus 4070A (23) (AMT clone 32).

Molecular Cloning of the SFFV DNA Fragment. The subgenomic SFFV DNA fragment was molecularly cloned as described in the text, by using standard methods (24–26) under P2 containment conditions as outlined in the National Institutes of Health recombinant DNA research guidelines, part II.

Oligonucleotide Pattern Analysis. ^{32}P-Labeled SFFV genomic RNA was hybridized to cloned SFFV fragment-pBR322 DNA after cleavage of the circular recombinant plasmid with *Hin*dIII endonuclease. The hybridization reaction was incubated for 12 hr at 50°C and then treated with RNase T1, and

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Abbreviations: SFFV, spleen focus-forming virus; F-MuLV, Friend murine leukemia virus; Mo-MuLV, Moloney murine leukemia virus; kbp, kilobase pair; LTR, long terminal repeat sequence.

the RNase-resistant hybrid was isolated by gel filtration, denatured, and analyzed as detailed (14, 21).

Cotransfection Assay for SFFV Biological Activity. The calcium phosphate precipitation cotransfection of NIH 3T3 cells with SFFV DNA and helper virus DNA has been described in detail (19). A 0.5-ml sample of cell-free supernatants from virus-producing transfected cultures was injected intravenously into 6- to 8-week-old NIH Swiss mice to determine the biological activity of the cotransfected SFFV DNA.

Metabolic Labeling of Cells, Immunoprecipitation, and Polyacrylamide Gel Electrophoresis. The metabolic labeling of cells and analyses of immune precipitates were performed as described (17, 27).

RESULTS

Molecular Cloning of SFFV Subgenomic Fragment. Using the *Hin*dIII site in pBR322, we had previously cloned SFFV in a permuted form at a unique *Hin*dIII site in the $SFFV_{LS}$ genome (19). The orientation of the SFFV DNA insert in the pBR322 vector and the location of the two *Pst* I restriction sites in the SFFV portion of the circular SFFV-pBR322 molecule are shown in Fig. 1, along with the *Pst* I site in the pBR322 DNA. This clone, like the original pBR322, contains the gene required for resistance to ampicillin (19). When the DNA of this clone is cleaved at the *Pst* I site, the gene required for resistance to ampicillin is inactivated and three fragments, termed A, B, and C, are generated.

To clone fragment A, which is homologous to the 3′ portion of the SFFV genome, we ligated *Pst* I-cut pBR322 DNA to the mixture of fragments derived from the *Pst* I cleavage of the recombinant SFFV-pBR322 DNA. We used this mixture of ligated fragments to transform *Escherichia coli* and selected for ampicillin-resistant colonies (25). By hybridizing the ampicillin-resistant colonies to an appropriate ^{32}P-labeled cDNA, we could identify the colonies containing the fragment of SFFV DNA among the total population of ampicillin-resistant colonies. After obtaining this transformant, the pBR322-A fragment cloned DNA was digested with *Pst* I and the A fragment was electrophoresed on agarose gels and recovered by electroelution (26). The gel-purified fragment was then recloned a second time by ligating it again to fresh *Pst* I-cleaved pBR322. All studies reported below were performed with the twice molecularly cloned fragment of SFFV DNA (clone 23-1a2).

Authenticity of SFFV Fragment. Fig. 2 shows agarose gel analysis of the A fragment of SFFV DNA. Lanes 1a and 1b show the complete SFFV DNA and the SFFV DNA fragment after cleavage of the recombinant pBR322 molecules with *Hin*dIII. The fragment migrated at a size of 3.7 kbp compared to the 5.7-kbp complete SFFV proviral DNA. The *Hin*dIII-released SFFV DNA and SFFV fragment DNA were then digested with *Pst* I (lanes 2a and 2b), *Eco*RI (lanes 3a and 3b), or *Kpn*I (lanes 4a and 4b). From the previously derived restriction map of SFFV DNA (19) one can deduce that the cloned fragment derives from the 3′ half of the genome and includes the long terminal repeat sequence (LTR) of the permuted SFFV molecular clone. This is most clearly seen from the *Hin*dIII/*Kpn* I digests in lanes 4a and 4b. The complete SFFV DNA yielded two fragments, one at 3.3 kbp and one at 2.4 kbp. The digest of the subgenomic DNA fragment yielded an identical 2.4-kbp fragment but no 3.3-kbp product. Instead, a small fragment (1.4 kbp) was apparent in lane 4b, representing the *Kpn* I–*Pst* I fragment in the middle of the permuted molecule attached to a small piece of pBR322 DNA (*Pst* I to *Hin*dIII in Fig. 1). In both lanes 4a and 4b the released pBR322 DNA also was seen at 4.3 kbp.

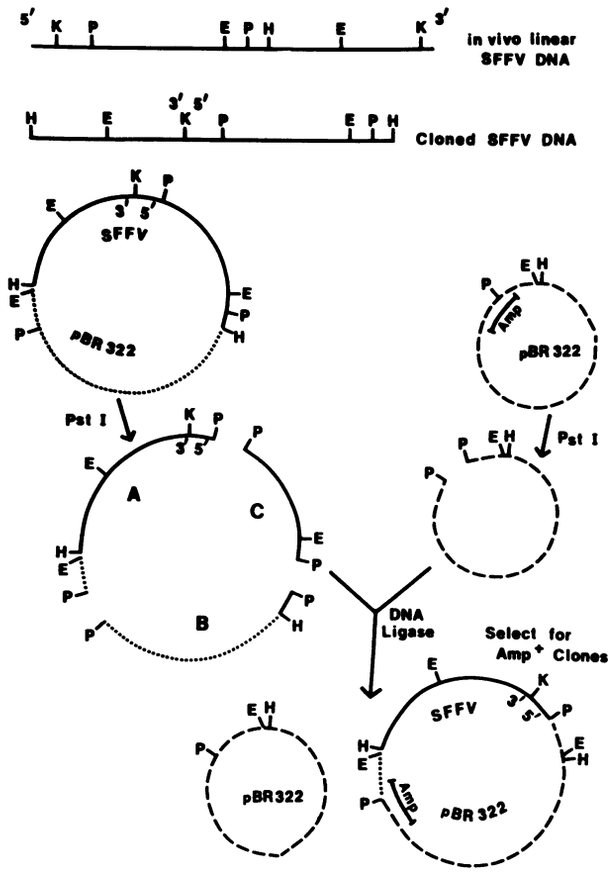

FIG. 1. Scheme for molecular cloning of the *Hin*dIII/*Pst* I fragment of SFFV DNA. $SFFV_{LS}$ DNA had been previously cloned in pBR322 at the unique *Hin*dIII site, yielding a circularly permuted clone of SFFV DNA with only one copy of the terminal repeat sequences (top diagram). The closed circular recombinant SFFV-pBR322 DNA was digested with *Pst* I, generating three fragments of DNA, designated A, B, and C. This mixture of fragments was ligated to *Pst* I-digested pBR322 DNA and used to transform *Escherichia coli*. Because *Pst* I cleavage of pBR322 inactivates the ampicillin-resistance gene, the growth of the transformed *E. coli* in the presence of ampicillin selects for clones containing either wild-type pBR322 or a plasmid that is a recombinant between pBR322 and the A fragment of the SFFV-pBR322 DNA. The latter clones were then identified by hybridization to ^{32}P-labeled EY10 cDNA which has regions homologous to SFFV. The relative positions of the recognition sites for the enzymes *Eco*RI (E), *Hin*dIII (H), *Kpn* I (K), and *Pst* I (P) are shown as are the areas homologous to the 3′ and 5′ ends of the SFFV genomic RNA.

After digestion with *Hin*dIII and *Eco*RI (lanes 3a and 3b), complete SFFV DNA yielded three fragments, a 3.8-kbp *Eco*RI/*Eco*RI piece, a 1.2-kbp *Hin*dIII/*Eco*RI piece, and a 0.7-kbp *Eco*RI/*Hin*dIII piece. The cloned subgenomic fragment yielded the same 1.2-kbp *Hin*dIII/*Eco*RI fragment along with a 2.5-kbp fragment again representing the *Eco*RI/*Kpn* I/*Pst* I fragment of SFFV with a small piece of pBR322 DNA terminating at the *Hin*dIII site of pBR322 shown in Fig. 1. Southern blot analysis (28) of the gel-electrophoresed SFFV DNA fragments [hybridization to ^{32}P-labeled SFFV(F-MuLV) cDNA derived from virus released by the EY10 rat cell line] verified this analysis of ethidium bromide staining.

To confirm the portion of the SFFV genome cloned, we hybridized the entire SFFV DNA clone or its A fragment DNA clone (23-1a2) to ^{32}P-labeled SFFV genomic RNA and determined the pattern of the oligoribonucleotides hybridized to each DNA. If the cloned *Hin*dIII/*Pst* I fragment corresponds

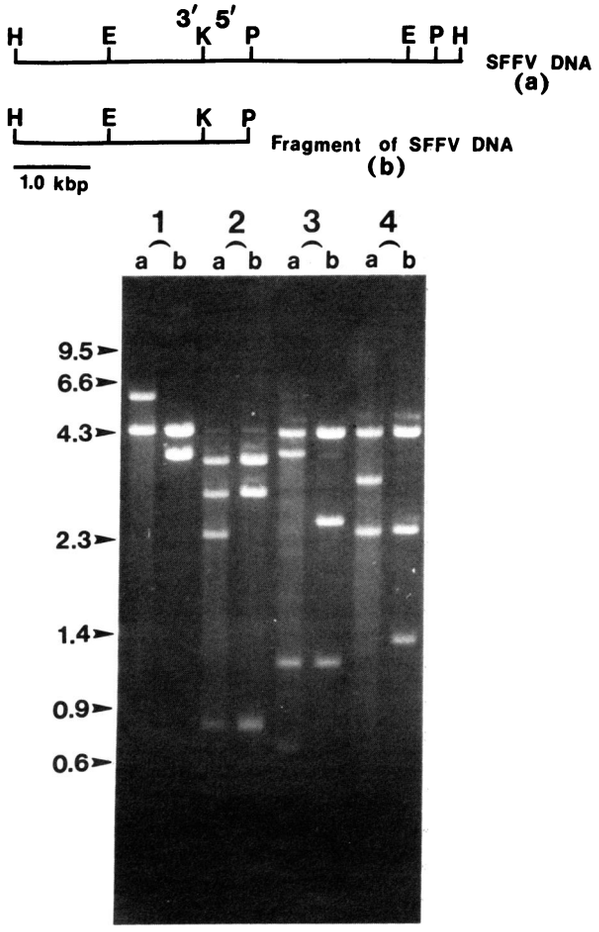

FIG. 2. Comparison of cloned SFFV DNA and the cloned SFFV DNA fragment by restriction enzyme analyses. The recombinant plasmid DNA was isolated from the clones containing the entire SFFV DNA (a) and clones containing the SFFV DNA fragment (b). These recombinant DNAs were digested with *Hin*dIII (lanes 1), *Hin*dIII plus *Pst* I (lanes 2), *Hin*dIII plus *Eco*RI (lanes 3), and *Hin*dIII plus *Kpn* I (lanes 4) and electrophoresed at 55 V for 18 hr through a 1% agarose gel containing 0.5 μg of ethidium bromide per ml. The bands of DNA were visualized with ultraviolet light. The numbers at the left of the gel represent the location and size (in kbp) of *Hin*dIII-digested wild-type λ DNA fragments migrating on the same gel. In the maps of restriction enzyme recognition sites on cloned SFFV DNA (a) and the cloned SFFV DNA fragment (b) at the top of the figure for reference: H, *Hin*dIII; E, *Eco*RI; K, *Kpn* I; and P, *Pst* I. The relative location of 3′ and 5′ areas of the SFFV genomic RNA are also indicated.

to a region encompassing 2.0 kb of the 3′ end of the SFFV genome and the 0.6-kbp LTR, it should include all of the SFFV dualtropic *env* gene sequences (14, 21). As shown in Fig. 3*B*, 15 of 24 large SFFV RNase T1 oligonucleotides were retained in such a hybrid. These oligonucleotides corresponded to a contiguous segment of the SFFV RNase T1 oligonucleotide map which extends from the 3′ poly(A) terminus to approximately 3 kb. These oligonucleotides included no. 6, 19, and 12, which are unrelated to the helper F-MuLV but have identical or closely related counterparts in several dualtropic MuLVs (14, 21, 29, 30).

The results indicate that the cloned subgenomic fragment of SFFV DNA represents the 3′ half of the SFFV RNA genome plus the LTR of the SFFV proviral DNA.

Biological Activity of the 3′ Subgenomic Fragment of SFFV DNA. In order to test the biological activity of the subgenomic fragment of SFFV DNA, we cotransfected the fragment into NIH 3T3 fibroblasts with infectious molecularly cloned Mo-MuLV DNA, F-MuLV DNA, or wild mouse amphotropic viral DNA. The transfections were performed with the fragment still attached to pBR322 DNA or released from the plasmid vector with *Hin*dIII. The progeny virus obtained from each transfection was injected intravenously into 6- to 8-week-old NIH Swiss mice. The results of transfections using the SFFV DNA fragment released from the plasmid are shown in Table 1.

Adult mice injected with the virus progeny of the cells cotransfected with the SFFV DNA fragment and F-MuLV DNA developed splenic foci, erythroblastosis, enlarged spleens, and, in the later stages of the disease, polycythemia. Generally higher SFFV activity was recovered in the cotransfections when the SFFV DNA first was released from the circular pBR322-A fragment DNA by digestion with *Hin*dIII. Transfections of Mo-MuLV DNA alone, F-MuLV DNA alone, or amphotropic virus DNA alone did not yield viruses that caused SFFV-like disease in adult Swiss mice.

Presence of gp52. Cells from a culture of NIH 3T3 fibroblasts cotransfected with Mo-MuLV DNA and the SFFV DNA fragment were cloned in Falcon microtest II plates. Twenty-three

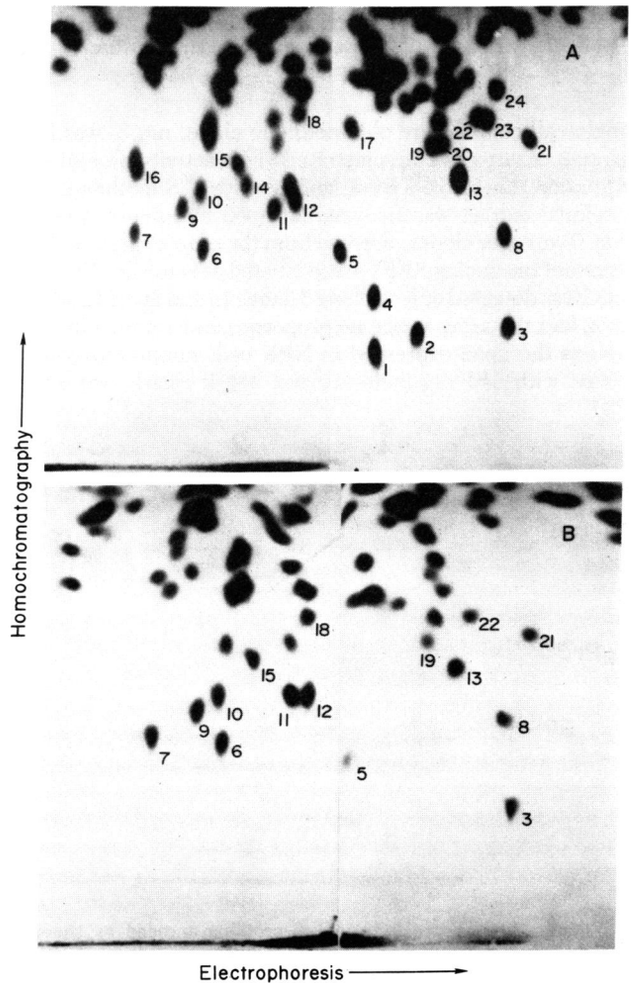

FIG 3. RNase T1-resistant oligonucleotides of SFFV genomic RNA (*A*) and of SFFV RNA complementary to the *Hin*dIII/*Pst* I fragment (*B*). ^{32}P-Labeled SFFV DNA (5×10^5 cpm) was "fingerprinted" by two-dimensional electrophoresis and homochromatography (*A*) or hybridized to 2 μg of plasmid DNA containing the inserted *Hin*dIII/*Pst* I SFFV DNA fragment. The hybridized DNA was subsequently isolated and fingerprinted (*B*).

Table 1. Biological properties of virus produced from cells transfected with cloned SFFV DNA released from the plasmid by *Hin*dIII digestion*

Exp.	Transfection of *Hin*dIII-digested cloned SFFV DNA	Rescue of SFFV activity by cotransfection with DNA	Time after injection, days	Spleen wt,† g	Hematocrit,† %	Splenic foci production
I	5.7-kbp clone	F-MuLV clone 57	15	0.71, 0.95	46	+
			28	*2.2, 4.9*	*62,69*	
	None	F-MuLV clone 57	48	0.20, 0.31	47	−
	5.7-kbp clone	Mo-MuLV clone 1387	15	0.23, 2.2	50	+
			28	*2.0, 5.6*	*45,60*	
	None	Mo-MuLV clone 1387	48	0.18, 0.23	46,48	−
II	3.0-kbp fragment	F-MuLV clone 57	14	0.66, 0.71	NT	+
			21	*2.6, 2.2*	*63,67*	
	None	F-MuLV clone 57	21	0.28, 0.24	47,46	−
	3.0-kbp fragment	Mo-MuLV clone 1387	14	1.7, 1.4	NT	+
			21	*2.2, 4.9*	*66,55*	
	None	Mo-MuLV clone 1387	21	0.22, 0.24	47,48	−
III	3.0-kbp fragment	AMT clone 32‡	14	0.33, 0.36	NT	+
			35	*2.0, 2.8*	*53,70*	
	None	AMT clone 32‡	35	0.23, 0.14	50,51	−

* The characteristics of disease were monitored in intravenously injected 6- to 8-week-old NIH Swiss mice.
† Values from individual mice. NT, not tested.
‡ Molecularly cloned wild mouse amphotropic virus 4070A.

single cell clones were obtained; one clone, no. 3, was found to produce virus that demonstrated SFFV activity after injection into mice. These cells were labeled with [^{35}S]methionine and a cellular extract was analyzed for gp52 by immunoprecipitation. Two other clones, derived from the same experiment, that were not producing SFFV were labeled as controls. The SFFV gp52 was detected only in clone 3 (lanes 1b and 2b) (Fig. 4). This gp52 had the same antigenic properties and gel migration pattern as the gp52 expressed in NRK cells nonproductively infected with $SFFV_{LS}$ (lanes 1e and 2e); it clearly was not expressed in cells transfected with the helper Mo-MuLV DNA alone (lanes 1d and 2d).

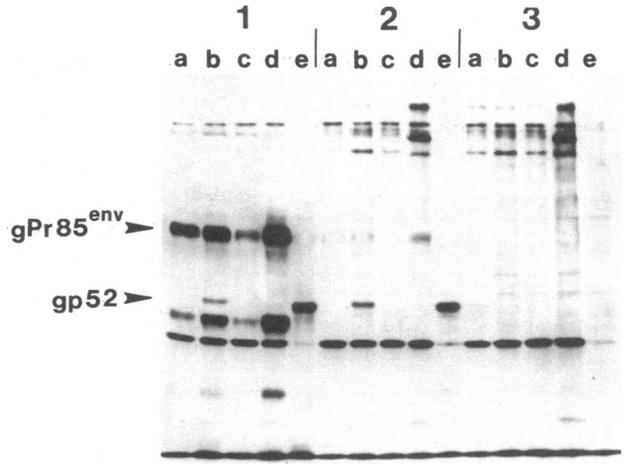

FIG. 4. Immunoprecipitation of proteins encoded by the SFFV DNA fragment. Single cell clones obtained from a culture of NIH 3T3 cells cotransfected with Mo-MuLV DNA and the SFFV DNA fragment were prepared for immunoprecipitation analyses. The labeled extracts were precipitated with goat anti-Rauscher MuLV gp70 antiserum (lanes 1 a–e), goat anti-Moloney MCF virus gp70 antiserum absorbed with Mo-MuLV (lanes 2 a–e), or normal goat serum (lanes 3 a–e). Three single-cell clones, clone 2 (lanes a), clone 3 (lanes b), and clone 4 (lanes c) were analyzed as were NIH 3T3 cells transfected with Mo-MuLV DNA alone (lanes d) and NRK cells nonproductively infected with SFFV (lanes e).

In other results (not shown), gp52 was also present in spleens of diseased mice that had been infected with the virus progeny of cells cotransfected with the SFFV DNA fragment and F-MuLV DNA or amphotropic virus DNA.

DISCUSSION

In this paper we report the molecular cloning of a subgenomic proviral DNA fragment of the Lilly–Steeves strain of SFFV. This fragment is 3.0 kbp in size and stretches from a *Hin*dIII recognition site to a *Pst* I recognition site of a previously reported circularly permuted molecular clone of SFFV (19). The fragment represents 2.0 kbp of the 3′ portion of the proviral SFFV genome, a single copy of the 0.6-kbp LTR, and 0.4 kbp from the 5′ portion of the genome. The evidence for this structure comes from: (*i*) comparison of the restriction enzyme cleavage pattern of the fragment and of the full-length SFFV DNA, and (*ii*) the RNase T1-resistant oligonucleotides of genomic SFFV RNA that hybridize to the fragment.

This SFFV DNA fragment encodes the information necessary for the biological activity of the whole virus and therefore is a subgenomic fragment of a replication-defective acute leukemia virus that is biologically active. The fragment contains *env* gene sequences as shown by: (*i*) its hybridization to SFFV oligonucleotides 6, 19, and 12, which are related to the recombinant *env* sequences of MCF viruses (14, 21), and (*ii*) the ability to detect, by immunoprecipitation, the expression of the SFFV-specified gp52 in NIH 3T3 cells cotransfected with the fragment and in spleens of mice with disease induced by the virus recovered from the cotransfection. Thus, the results are consistent with the hypothesis that the *env*-related sequences of SFFV and their product, gp52, are required for initiation of erythroblastosis induced by SFFV (13, 18).

It should be noted that the cloned 3′ SFFV DNA fragment also contains the LTR. The LTR has been postulated to be involved in certain diseases induced by other leukemia viruses (31). However, in all these cases the induction of the disease

required a long latency and was unlike the acute disease induced by SFFV. For this reason, we think that the LTR itself may not be responsible for the rapidly observed SFFV-induced erythroproliferative disease. On the other hand, the LTR may well play an indirect role in promoting the expression of high levels of the gp52.

It should also be noted that the information encoded in the SFFV DNA fragment described here can be implicated only in the initiation of the erythroblastosis. Until temperature-sensitive mutants of SFFV are available, we cannot determine if the presence of SFFV-specified products are required for the maintenance of the erythroproliferative disease.

The two-stage assay used herein poses two limitations on the current understanding of SFFV-induced disease. First, we cannot rule out that a complementary or cooperative effect of both SFFV and the helper-independent virus is involved. However, it is worth emphasizing that two helper-independent viruses used to rescue the SFFV DNA fragment in this assay, Mo-MuLV and amphotropic virus 4070A, are not associated with erythroproliferative diseases in mice. Second, as a result of the requirement for the helper-independent virus, characterization of the type of transformation induced by SFFV has been limited. In the case of other replication-defective oncogenic viruses, transformed nonproducer cells have been isolated immediately after virus infection and the malignant nature of these cells has been demonstrated by transplantation of the nonproducer cells in appropriate hosts (32–34). Whether SFFV-induced erythroblasts are malignant is not known because nonproducer erythroid bursts have never been isolated, propagated in long-term cultures, and then transplanted into a syngeneic host. Thus, although it is clear that SFFV causes a rapid hyperplastic proliferation of erythroid precursor cells, it is possible that this erythroblastosis is not a transplantable disease. In order to clarify some of these issues remaining about the nature of the disease induced by the SFFV DNA fragment and the role of gp52 which it encodes, it will be necessary to introduce the molecularly cloned, biologically active, SFFV DNA into appropriate hemopoietic precursors in the absence of helper-independent virus DNA.

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