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Structural and Functional Studies of the Friend Spleen Focus-Forming Virus: Structural Relationship of SFFV to Dualtropic Viruses and Molecular Cloning of a Biologically Active Subgenomic Fragment of SFFV DNA

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A. Introduction

The Friend virus induces an acute erythroproliferative disease in adult mice characterized by splenomegaly and pronounced erythroblastosis. In early stages of the disease, discrete splenic foci of proliferating erythroid cells are apparent, and thus the virus has been termed the spleen focus-forming virus (SFFV) (Axelrad and Steeves 1964). As is the case with nearly all retroviruses which cause acute proliferative diseases, SFFV is defective in replication and is associated in a complex with a helper virus, termed the Friend murine leukemia virus (F-MuLV), which provides replicative functions (Dawson et al. 1968; Rawson and Parr 1970; Steeves et al. 1971; Troxler et al. 1977c). Several substrains of SFFV have been derived from the original isolate, some of which have pathologic properties distinguishable from others (Troxler et al. 1980). The present report deals with the structure and function of a polycythemia-inducing strain of SFFV originally obtained by Lilly and Steeves ($SFFV_{LS}$) (Lilly and Steeves 1973). We describe structural analyses which indicate that $SFFV_{LS}$ is a deletion mutant of a dualtropic Friend mink cell focus-inducing virus. In addition, we describe the derivation of molecularly cloned $SFFV_{LS}$ as well as a subgenomic fragment of this clone, both of which contain the information necessary to encode all pathogenic properties of the intact virus.

B. "Specific" Sequences of SFFV: Characterization and Location in the SFFV Genome

I. Specific Sequences Defined by Hybridization Studies

In all known replication-defective retroviruses which induce acute proliferative diseases, two classes of RNA sequences have been identified. One class is closely related to RNA sequences of associated helper viruses, and a second class is unrelated to helper virus RNA and, therefore, specific to the defective retrovirus genome (Duesberg 1980). The specific sequences very likely correspond to the genes encoding the pathogenic functions of these viruses. Hybridization analyses of SFFV, using specific cDNA probes prepared from SFFV-F-MuLV complexes, have also identified sequences closely related to F-MuLV as well as F-MuLV-unrelated sequences (Bernstein et al. 1977; Mak et al. 1978; Pragnell et al 1978; Troxler et al. 1977a,b). In contrast to the initial findings of others studying different isolates of SFFV, our analyses indicated that the specific sequences of $SFFV_{LS}$ exhibited homology to the RNAs of helper-independent dualtropic (infecting murine and nonmurine cells) and xenotropic (infecting preferentially nonmurine cells) viruses. Subsequent work by us (Troxler et al. 1980) and others (Bernstein et al 1979) identified dualtropic sequences in all SFFV isolates tested. It is likely that the earlier discrepancies were the result of xenotropic or dualtropic sequences present in helper virus RNA preparations used for the construction of SFFV-specific probes.

II. Identification and Location of Specific Sequences in SFFV RNA by RNase T_1-oligonucleotide Fingerprinting Analyses

In the hybridization analyses discussed above, the SFFV genome had not been examined directly. Rather, SFFV RNA from nonproducer cells (Troxler et al. 1977c) or mixtures of SFFV and F-MuLV RNAs were utilized (Berstein et al. 1977; Mak et al. 1978; Pragnell et al. 1978). Thus, the location of specific and F-MuLV-related sequences in SFFV RNA had not been determined. Our approach to the structural analysis of Friend virus RNA components has been to identify the large RNase T_1-resistant fragments (T_1-oligonucleotides) of each RNA and to determine the order of the oligonucleotides along the RNA relative to the 3′ poly A terminus (Wang et al. 1975; Coffin and Billeter 1976). A direct comparison of the oligonucleotide maps in many cases will identify related and specific sequences of the RNAs.

The SFFV genome was identified as a 50S virion RNA consisting of monomers of approximately 6–7 kilobases (kb) by rescue of the SFFV component from nonproductively infected cells (Evans et al. 1979). This size is considerably smaller than the RNA monomers of F-MuLV (9 kb); thus, SFFV RNA could be isolated free of helper virus RNA sequences on the basis of size. The comparison of SFFV RNA to F-MuLV RNA by T_1-oligonucleotide fingerprinting was complicated by the existence of numerous minor sequence differences between related sequences of the two RNAs. Although SFFV RNA was 85%–90% homologous to F-MuLV by hybridization, only 8 of 24 SFFV oligonucleotides were found in F-MuLV RNA. This problem was circumvented by the identification of T_1-oligonucleotides in SFFV RNA-F-MuLV cDNA hybrids. Briefly, ^{32}P-labeled RNA was hybridized to F-MuLV cDNA, and the reaction mixture was digested with RNase to remove unhybridized RNA. The hybrid was then isolated, denatured, and fingerprinted to identify oligonucleotides from homologous sequences. This procedure identified 21 of the 24 SFFV T_1-oligonucleotides as F-MuLV related. The three specific SFFV oligonucleotides defined a region on the SFFV genome extending from approximately 2 to 2.5 kb from the 3′ terminus of SFFV. A comparison of the three specific SFFV oligonucleotides with oligonucleotides of dualtropic viruses indicated that they were sequence elements of dualtropic *env* genes (Evans et al. 1979, 1980). Thus, SFFV is comprised entirely of sequences closely related to a combination of different helper virus RNAs. SFFV is fundamentally different from other retroviruses which induce rapid proliferative disease in that SFFV does not contain specific sequences unrelated to helper virus RNA.

C. Structural relationship of SFFV to F-MuLV and to Dualtropic Viruses

I. Structure of the Dualtropic Friend Mink Cell Focus-Inducing Virus

Dualtropic viruses are generated by recombination of ecotropic (murine host range) MuLVs with *env* gene sequences of uncertain origin (Faller and Hopkins 1978; Rommelaere et al. 1978; Shih et al. 1978). Since SFFV was shown above to be comprised of sequences related to F-MuLV and dualtropic envelope sequences, it is plausible that a dualtropic variant of F-MuLV may contain all SFFV sequences. To test this possibility, we have compared the structure of a dualtropic variant of F-MuLV, termed the Friend mink cell focus-inducing virus (F-MCF) (Troxler 1978), to F-MuLV and subsequently to SFFV (see below). A direct comparison of the oligonucleotide fingerprints of F-MuLV and F-MCF indicated that approximately 75% of their oligonucleotides were shared and about 25% of the oligonucleotides in each virus were unique. A comparison of the oligonucleotide maps of F-MuLV and F-MCF indicated that the two viruses were virtually indentical in all regions except for a region located about 1.5 to 3.3 kb on the oligonucleotide maps corresponding to the viral envelope gene. Within this region no oligonucleotides were shared. Such a relationship is typical of many dualtropic viruses with respect to their ecotropic parents (Faller and Hopkins 1978; Rommelaere et al. 1978; Shih et al. 1978) and is shown schematically in Fig. 1.

II. Structural Relationship of SFFV to F-MCF

Initial hybridization results of SFFV RNA with F-MCF cDNA indicated that virtually all SFFV RNA is closely related to the F-MCF genome; however, only 14 of 24 SFFV oligo-

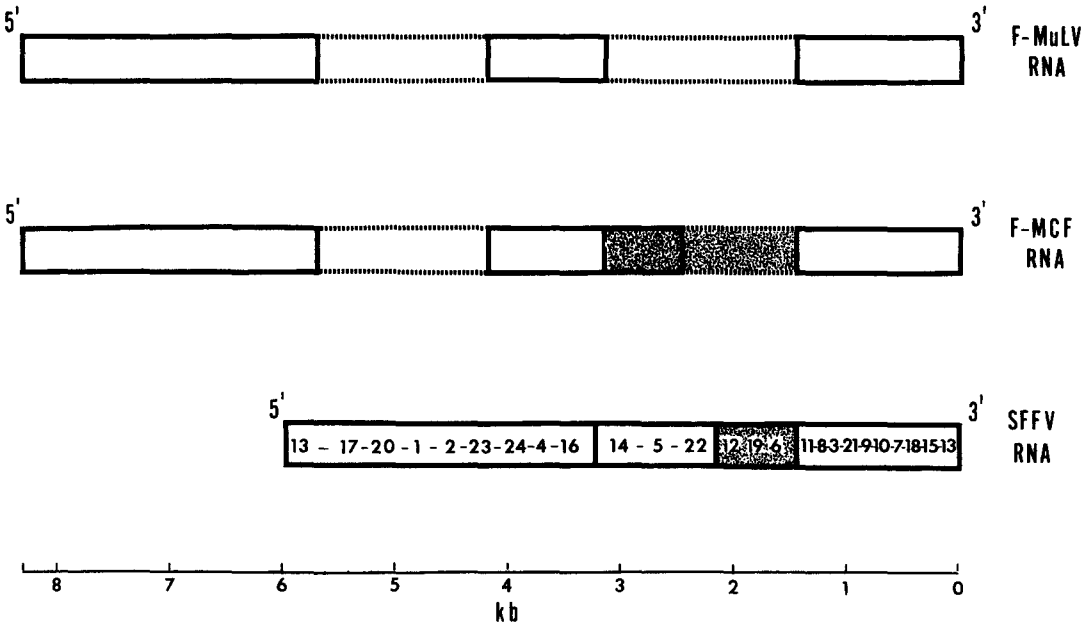

Fig. 1. Structural relationships of F-MuLV, F-MCF, and SFFV RNAs. RNA sequences of each viral genome which are shared with ecotropic F-MuLV RNA are *unshaded*. RNA sequences shared with F-MCF RNA but not with F-MuLV RNA are *shaded*. Sequences shared with SFFV RNA are bordered by *solid lines* and those not shared with SFFV RNA are bordered by *dashed lines*. The positions of RNase T_1-resistant oligonucleotides (designated by Nos. 1 through 24) are shown for SFFV RNA

nucleotides have identical counterparts in F-MCF. To determine the F-MCF-related oligonucleotides in SFFV, the oligonucleotides present in an SFFV RNA-F-MCF cDNA hybrid were identified. It was found that all SFFV oligonucleotides were hybridized; thus, our prediction that all SFFV sequences may be contained in a single replication-competent retrovirus was confirmed.

Since F-MCF does not cause an acute disease, but contains all sequences of SFFV, including those responsible for Friend disease, it would appear that the pathogenicity of SFFV is the result of a particular configuration of F-MCF-related sequences. It is, therefore, important to determine what structural features distinguish F-MCF sequences from F-MCF-related sequences in SFFV. To determine which T_1-oligonucleotides correspond to SFFV-related sequences in F-MCF, the oligonucleotides in a hybrid of F-MCF RNA with molecularly cloned SFFV DNA (Linemeyer et al. 1980; also see below) were determined. This analysis indicated that the structure of $SFFV_{LS}$ corresponds to an F-MCF which has deleted two large contiguous sequences (Fig. 1): one region which extends from 1.5 to 2.5 kb from the 3′ end in F-MCF and corresponds to about one-half of the dualtropic sequences and a second region extending from approximately 4.2 to 5.8 kb which would include most of the polymerase gene and probably a portion of the *gag* gene which encodes the major virion core proteins.

D. Molecular Cloning of SFFV-Specific DNA

The structural analyses described above imply that the SFFV genome may encode defective *gag* gene and *env* gene products. Both defective *gag* proteins (Ruscetti et al. 1980; Bernstein et al. 1977; Barbacid et al. 1978) and defective *env* proteins (Dresler et al. 1979; Ruscetti et al. 1979) have been described in SFFV-infected cells. *Gag* gene related proteins have been detected in cells infected with only certain strains of SFFV, and these proteins vary considerably in size and in their antigenic properties. In contrast, cells infected with any strain of SFFV always express an *env* gene related protein of 52,000–55,000 daltons which contains antigenic determinants of dualtropic viral envelope glycoproteins. No evidence exists to determine directly whether either protein is involved in the SFFV erythroid disease. Thus, we have taken a molecular approach to study the genetics of the erythroproliferation induced by this virus.

I. Molecular Cloning of Complete SFFV Proviral DNA

The SFFV proviral DNA has been recently molecularly cloned in the plasmid vector pBR322 (Linemeyer et al. 1980). Briefly, a culture of normal rat kidney fibroblasts was infected with a helper viral pseudotype of $SFFV_{LS}$. The unintegrated viral DNA was extracted by the Hirt procedure (Hirt 1967), electrophoresed through a 1% agarose gel, and the fractions enriched for the linear form III and closed circular form I SFFV-specific DNA molecules were collected and pooled. An analysis of the 6.3 kilobase pair (kbp) unintegrated linear SFFV DNA was performed using single and double restriction endonuclease digestions to develop the physical map of restriction enzyme recognition sites shown in Fig. 2A. The map was oriented 5′ to 3′ according to the viral genomic RNA by hybridizing enzyme digestion fragments to a ^{32}P-labeled cDNA prepared from the 3′ end of F-MuLV RNA, which shares homology to the 3′ end of SFFV RNA (Evans et al. 1979).

A single *Hind* III digestion site was found to be located 2.6 kbp from the 3′ end of the linear molecule (Fig. 2A). The closed circular form I SFFV DNA was then linearized by cleavage at this single Hind III site, and SFFV-specific DNA molecules were cloned in *Escherichia coli* with the plasmid vector pBR322 using the unique *Hind* III site as described (Linemeyer et al. 1980). The clones of SFFV proviral DNA generated by this protocol were, thus, circularly permuted about the *Hind* III site with respect to the in vivo linear DNA. After growth of the bacterial clones and extraction of the recombinant plasmid SFFV DNA, physical restriction enzyme maps of the cloned SFFV DNAs were determined. The map of one clone (clone 4–1a3) is shown in Fig. 2B. This molecule is only 5.7 kbp in size and lacks 0.6 kbp of DNA and one *Kpn* I restriction enzyme site found in the in vivo linear DNA. This variation can be accounted for by the absence of one copy of the terminally redundant sequences which contain the *Kpn* I recognition site and which have been previously reported for murine and avian retroviruses (Gilboa et al. 1979; Hager et al. 1979; Hsu et al. 1978). To demonstrate the completeness of this 5.7 kbp clone of DNA, we showed that it was able to hybridize to all the T_1-oligonucleotides of SFFV genomic RNA (Evans et al. 1980), thus indicating that it contains at least one copy of all the sequences present in the SFFV genome.

II. Molecular Cloning of a Subgenomic Fragment of SFFV Proviral DNA

Using the 5.7-kbp clone of SFFV DNA we have now molecularly cloned a subgenomic fragment of SFFV DNA, again using the plasmid vector pBR322. We accomplished this by cleaving the recombinant circular pBR322-SFFV-cloned DNA with *Pst* I and inserting the resulting DNA fragments into fresh pBR322 molecules which were linearized by digestion at the unique *Pst* I site. The resulting SFFV DNA fragment pBR322 molecules were cloned in *E. coli* and identified as containing SFFV sequences by hybridization to a ^{32}P-labeled cDNA which has homology to SFFV. The plasmid DNA of various clones was then isolated and restriction endonuclease digestion products were analyzed. One clone was found to contain, along with an extra *Pst* I to *Hind* III

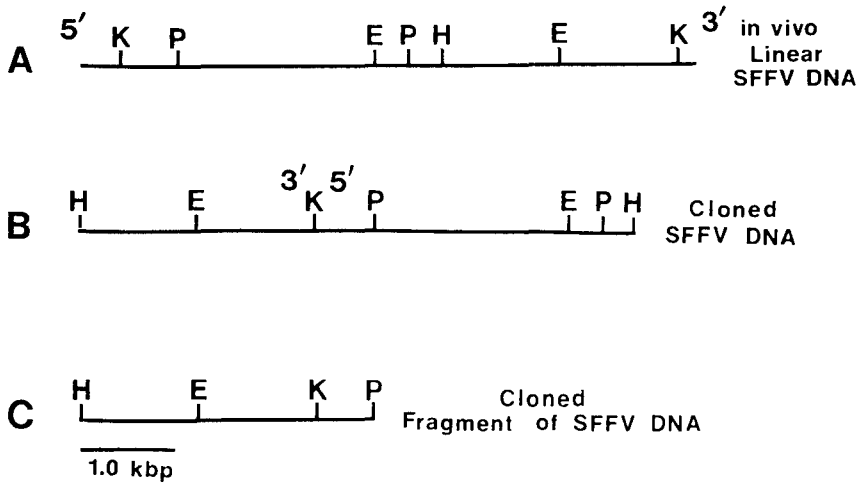

Fig. 2. Schematic map of restriction endonuclease recognition sites located on SFFV DNA. The relative positions of the recognition sites for the enzymes *Eco*RI *(E), Hind* III *(H), Kpn* I *(K),* and *Pst* I *(P)* are shown on **(A)** unintegrated linear SFFV proviral DNA, **(B)** molecularly cloned complete SFFV DNA, and **(C)** molecularly cloned subgenomic fragment of SFFV DNA. The areas homologous to the 3′ and 5′ ends of the SFFV genomic RNA are also shown

fragment of pBR322 DNA, a 3.0-kbp SFFV-specific DNA fragment (Fig. 2C) with restriction enzyme sites analogous to the lefthand *Hind* III to *Pst* I portion of the cloned SFFV DNA shown in Fig. 2B. This fragment of SFFV DNA should, therefore, contain 2.0 kbp of information analogous to the 3′ end of the SFFV viral genome, one copy of the 0.6 kbp terminally redundant sequences, and 0.4 kbp of information analogous to the 5′ end of the viral genome.

To further substantiate the portion of the SFFV proviral genome cloned, we hybridized the cloned fragment DNA to ^{32}P-labeled SFFV genomic RNA and fingerprinted the T_1-oligonucleotides which formed hybrids. Fifteen out of 24 large SFFV T_1-oligonucleotides were found to hybridize, and these oligonucleotides correspond to a contiguous segment of the SFFV T_1-oligonucleotide map which extends from the 3′ end to approximately 3 kb (oligonucleotides No. 5 to No. 15 including the terminally redundant oligonucleotide No. 13, see Fig. 1). It is especially important to note that these oligonucleotides include Nos. 6, 19, and 12, which are unrelated to F-MuLV, but have identical counterparts in dualtropic MuLVs (Evans et al. 1979, 1980; Rommelaerre et al. 1978; Shih et al. 1978). Thus, these results indicate that the cloned subgenomic fragment of SFFV DNA represents the 3′ half of the SFFV RNA genome and the terminally redundant sequences of the proviral DNA and contains very little of the 5′ *gag* gene sequences.

E. Biologic Activity of Cloned DNA

To determine whether the cloned SFFV proviral DNA had biologic activity, NIH 3T3 fibroblasts were transfected with recombinant pBR322-SFFV DNA which had been digested with *Hind* III to linearize the plasmid molecule and to release the SFFV DNA from the vector. Since SFFV does not transform fibroblasts, it was necessary to assay for the biologic activity of the cloned DNA in the mouse. Since SFFV is also replication defective, a replication-competent helper virus must be used to rescue the SFFV sequences and allow infection of the adult mice used for the assay. Therefore, it is essential that the helper virus used does not cause a erythroid disease in adult mice.

It is possible to rescue the transfected SFFV sequences from the transfected fibroblasts by superinfecting these cells with competent helper viruses, but we have found that this rescue is accomplished more efficiently by a cotransfection protocol. In this procedure the fibroblasts are transfected with both molecularly cloned infectious helper viral DNA and cloned SFFV DNA after the two DNAs are precipitated together using $CaCl_2$ as described (Linemeyer et al. 1980). After the cotransfected cells begin to produce virus, measured by release of reverse transcriptase (1 to 3 weeks), the cell-free supernatant of these cultures is injected intravenously into adult NIH Swiss mice. The mice are then observed for the characteristics of the SFFV-induced disease from 2 to 4 weeks after injection. The results obtained from such cotransfection studies are shown in Table 1. Cotransfections using the complete 5.7-kbp cloned SFFV DNA and either cloned F-MuLV helper virus DNA (Oliff et al. 1980) or cloned Moloney MuLV (Mo-MuLV) helper virus DNA (Wei et al., unpublished work) yield virus preparations which induce splenomegaly, polycythemia, and splenic foci in the adult mice. These disease characteristics are identical to those induced by actual virus preparations of $SFFV_{LS}$. Importantly, transfections of either helper virus DNA alone produce viruses which do not induce the characteristics of SFFV-induced disease (see Table 1).

Nearly identical results are produced when the 3.0 kbp *Hind* III to *Pst* I fragment of SFFV DNA is used in the cotransfections instead of the 5.7 kbp DNA (Table 1). These results indicate that this 3′ subgenomic fragment of SFFV proviral DNA contains the information responsible for the induction of the erythroproliferative disease.

F. Protein Expression of Cells Transfected with Cloned SFFV DNA

We were interested in whether the subgenomic fragment of SFFV DNA, which contained the 3′ *env* gene related sequences, encoded the information necessary to express the *env* gene related gp52 SFFV protein. Cells from a culture of NIH 3T3 fibroblasts cotransfected with Mo-MuLV DNA and the SFFV DNA fragment were cloned. Twenty-three single cell

Table 1. Biologic properties of virus produced from cells transfected with cloned SFFV DNA released from the plasmid by *Hind* III digestion[a]

Exp. No.	Transfection of *Hind* III-digested cloned SFFV DNA	Rescue of SFFV activity by cotransfection with DNA	Days after mouse injection	Spleen wt[b] (g)	Hemato-crit[b]	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	−

[a] The characteristics of disease were monitored in intravenously injected 6- to 8-week-old NIH Swiss mice. NT = not tested

[b] The values indicated are from individual mice

clones were obtained. One clone was found to produce virus which induced the SFFV disease after injection into adult mice. These cells were labeled with ^{35}S-methionine and the extract of these cells was analyzed for gp52 by immune precipitation. Two other cell clones, derived from the same experiment, which were not producing SFFV were labeled as controls. Gp52 was detected in the SFFV-positive cell clone only. This gp52 could be precipitated by an antiserum specific for MCF viral envelope determinants which are present on the gp52, but not by normal nonimmune serum. It has the same antigenic properties and sodium dodecyl sulfate-polyacrylamide gel migration pattern as the gp52 expressed in normal rat kidney cells nonproductively infected with actual $SFFV_{LS}$ (Ruscetti et al. 1979) and is clearly not expressed by cells transfected with the helper Mo-MuLV DNA alone. The gp52 was also expressed in cells from diseased spleens of mice infected with the virus progeny of the fibroblasts cotransfected with the fragment of SFFV DNA and F-MuLV DNA.

G. Conclusions

In summary, we have shown that the genome of SFFV contains sequences homologous to the helper virus F-MuLV and sequences nonhomologous to F-MuLV. These latter sequences which are unrelated to F-MuLV are related to envelope gene sequences of dualtropic MuLVs. These structural analyses indicate that $SFFV_{LS}$ is a deletion mutant of a dualtropic Friend MCF virus. Using a molecularly cloned subgenomic fragment of $SFFV_{LS}$ proviral DNA, we have shown that sequences present in the 3′ half of the SFFV genome are responsible for the SFFV-induced erythroproliferative disease. These 3′ sequences contain the dualtropic MuLV *env* gene related sequences and encode the SFFV-specified gp52 *env* gene related protein. Although it is possible that other sequences closely linked to those encoding gp52 are required for the SFFV biologic activity, such as the terminally redundant sequences, the results are consistent with the hypothesis that the gp52 plays a role in initiation of the erythroproliferation.

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