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Three Laboratory Strains of Spleen Focus-Forming Virus: Comparison of Their Genomes and Translational Products

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The molecular properties of three laboratory strains of the spleen focus-forming virus were compared. All strains contain genetic sequences related to the *env* gene of mink cell focus-inducing murine type C leukemia viruses, and each strain codes for a glycoprotein of 50,000 to 52,000 daltons which shares specific immunological properties with the gp70's of mink cell focus-inducing viruses. In contrast to this constancy, *gag* gene products coded for by these strains vary significantly. The *gag* and *env* gene products are synthesized from separate mRNA's, and the mRNA for the *env* gene product is approximately 18S. Unlike other acute leukemia viruses, which can transform various undifferentiated cells, have large unique sequence cellular gene inserts fused to helper virus *gag* genes, and have one known genome-length intracellular mRNA, the spleen focus-forming virus transforms only specific hematopoietic stem cells, is an *env* gene rather than a *gag* gene recombinant virus, and has a second distinct and smaller class of intracellular mRNA. Our data therefore indicate that the Friend strain of the spleen focus-forming virus is a unique replication-defective acute leukemia virus.

The spleen focus-forming virus (SFFV) is a replication-defective murine type C retrovirus. When inoculated as appropriate pseudotypes into adult mice of susceptible strains, SFFV causes a rapid leukemic transformation of erythroid precursor cells (30). Although SFFV is present in both Friend (9) and Rauscher (21) viral complexes, a detailed history of the generation of SFFV is somewhat difficult to obtain from the literature. In 1957, Friend isolated from Swiss mice a virus preparation which had the capacity to induce rapid erythroleukemia characterized by splenomegaly and anemia after inoculation into adult mice (9). In the years after the inoculation of highly leukemogenic virus, samples of Friend virus were distributed to many investigators in different laboratories, where independent stocks of Friend virus were maintained by serial mouse to mouse passage of leukemic spleen homogenates in mice of susceptible strains, such as Swiss and DBA/2 mice. Many of these different passage lines of Friend virus were subsequently referred to as strains (19), primarily because variation in the host ranges and pathogenic properties of the different virus stocks were detected. For example, Mirand noted that some preparations of Friend virus passaged in HaICR Swiss mice consistently induced polycythemia and not anemia (18). This preparation of Friend virus was thus designated the Mirand strain of polycythemia-inducing Friend virus, or FV-P (Mirand), in order to distinguish it from the anemia-inducing strain of Friend virus (FV-A) originally obtained from Friend. Similarly, a stock of Friend virus which had been obtained by A. Axelrad from the American Type Culture Collection and passaged in C3H/Bi mice became known as the Axelrad strain of FV-P, or FV-P (Axelrad), since it also was found to induce polycythemia in inoculated mice (19). The Axelrad strain of FV-P was the virus preparation which was used by Axelrad and Steeves to develop a 9-day in vivo focus formation assay which was based upon the observation that this virus stock induced discrete macroscopic foci of transformed cells in the spleens of inoculated mice (2). Lilly and Steeves (15), using a preparation of Friend virus obtained from Friend and passaged in HaICR Swiss mice and DBA/2 mice, derived a B-tropic pseudotype of SFFV by passage of N-tropic Friend virus along with B-tropic murine leukemia virus (MuLV) in BALB/c mice. This strain of Friend virus, which was designated B-tropic strain of Friend virus, also induced polycythemia and discrete foci in BALB/c mice. Thus, the above investigators used stocks of Friend virus which had been obtained independently to derive different preparations of the Friend virus complex, each of which had a different passage history but was found to induce polycythemia, splenic foci, and rapid erythroleukemia in adult mice of susceptible strains.

To investigate the molecular biology of SFFV, we devised an experimental procedure by which it was possible to clone SFFV free of replicating

murine type C helper virus. Using the B-tropic strain of Friend virus derived by Lilly and Steeves, FV-P (Lilly-Steeves), we isolated a series of nonproducer mouse and rat cells containing SFFV (32, 34). Molecular hybridization studies on this cloned SFFV demonstrated that SFFV was a recombinant between Friend type C helper virus and a portion of the *env* gene of a murine xenotropic virus (32, 33). In further studies, we found that this isolate of SFFV codes for a protein which shares immunological determinants with the gp70 product of mink cell focus-inducing (MCF) strains of MuLV (25, 26). From these studies we concluded that SFFV shared several molecular features with the helper-independent MCF strains of MuLV derived from ecotropic AKR MuLV (10), Moloney MuLV (M-MuLV) (8, 33), and Friend MuLV (F-MuLV) (36), and we speculated that SFFV arose by a recombinational event which was similar to that which generated the MCF viruses. More recently, the genomic RNA of the Lilly-Steeves strain of SFFV has been examined by Evans et al. (7; personal communication), using oligonucleotide fingerprinting. These investigators confirmed the presence of MCF-specific oligonucleotides in the Lilly-Steeves strain of SFFV and mapped these oligonucleotides at approximately 2.0 to 2.5 kilobases from the 3′ end of the viral genome.

Since all of the above molecular studies had been carried out with the Lilly-Steeves strain of SFFV, we were interested in determining whether the MCF envelope gene-related sequences and the product of this gene were associated with strains of SFFV with other passage histories. In addition, we were also interested in further characterizing a p12-containing protein that is coded for by the Lilly-Steeves strain of SFFV (3) and determining whether it too was coded for by all strains of SFFV.

In this paper, we report our studies of three separate pathogenic strains of SFFV that were compared by molecular hybridization techniques as well as by characterization of both the *env* and *gag* gene-related proteins coded for by each strain. Our results demonstrate that all strains of SFFV that induce rapid erythroleukemia in mice contain sequences that are homologous to F-MuLV, as well as xenotropic and MCF envelope gene-related sequences, and that they all code for a glycoprotein that contains MCF gp70 determinants. In contrast, there is considerable variability in the expression of *gag* gene-related proteins in cells infected with these viruses. Our data also demonstrate that the *gag* and *env* gene-related proteins coded for by SFFV can be distinguished from one another both immunologically and by their in vitro translation by two distinct size classes of SFFV RNA.

MATERIALS AND METHODS

Cells. The sources of normal rat kidney (NRK) cells, Fisher rat embryo (FRE) cells, NIH 3T3 mouse embryo fibroblasts, and mink lung cells (CCL-64) have been described previously (32, 33). Cells were maintained in Dulbecco modified Eagle medium containing either 10% calf serum or 10% fetal calf serum. All cells were negative for mycoplasma (Flow Laboratories, Rockville, Md.).

Viruses and virus assays. F-MuLV clone 201, an ecotropic NB-tropic Friend type C helper virus, has been described previously (35). This virus was originally cloned from a preparation of the anemia-inducing strain of Friend virus (FV-A) obtained from Charlotte Friend, Mt. Sinai School of Medicine, New York, N.Y. F-MuLV clone 201 was propagated on NIH 3T3 or NRK cells. Fr-MCF-1 and Mol-MCF_{83}, which are helper-independent *env* gene recombinant MCF viruses derived from F-MuLV clone 201 and ecotropic M-MuLV, respectively, have been described previously (33, 36). These viruses were grown on NIH 3T3 or mink lung cells. Replicating helper viruses were assayed by XC plaque assays (24), by the assay of supernatant medium for reverse transcriptase activity (34), and by MCF-type cytopathic effects on mink cells (10). SFFV was assayed biologically by the spleen focus formation assay (2).

Isolation of nonproducer cells containing different strains of SFFV. (i) Nonproducer cells containing SFFV derived from FV-P (Mirand). The Mirand strain of polycythemia-inducing virus, FV-P (Mirand), was kindly provided by E. A. Mirand, Roswell Park Memorial Cancer Institute, Buffalo, N.Y. This virus had been routinely passaged in DBA/2 mice since 1968 and was received as a spleen homogenate. This virus was cloned first in NIH 3T3 cells and then in FRE clone 2 cells by using previously described procedures (32, 34). Nonproducer cells of this strain of SFFV are designated SFFV-P-NIH clone 1 and SFFV-P-FRE clone 1, respectively, and the SFFV from these cells is referred to as $SFFV_M$.

(ii) Nonproducer cells containing SFFV derived from FV-P (Axelrad). The cell line designated SFFV-NRK NP 501 was derived by Bernstein and Mak by using the Axelrad strain of FV-P and has been described previously (4). These cells were generously provided by these investigators. The SFFV from these cells is referred to as $SFFV_{AX}$.

(iii) Nonproducer cells containing SFFV derived from FV-P (Lilly-Steeves). Isolation of the SFFV contained in the B-tropic strain of Friend virus obtained from Lilly and Steeves, FV-P (Lilly-Steeves), has been described previously (32, 34). These nonproducer cells are designated SFFV-BALB 3T3 1902 B clone 4, SFFV-NRK clone 1, and SFFV-FRE clone 1, and the SFFV derived from these cells is designated $SFFV_{LS}$. The cell line SFFV-FRE clone 1 was the source of virus used to prepare complementary DNA_{SFFV} ($cDNA_{SFFV}$) as described below.

When rescued with F-MuLV clone 201, the SFFVs

from SFFV-P-FRE clone 1, SFFV-NRK NP 501, and SFFV-NRK clone 1 all induced splenomegaly, polycythemia, and rapid erythroleukemia in adult NIH Swiss mice.

Preparation of cDNA probes. ^{3}H-labeled cDNA probes were prepared in endogenous reverse transcriptase reactions primed with calf thymus DNA fragments as previously described (34). To prepare $cDNA_{SFFV}$, an endogenous cDNA product was made from RNA extracted from virions released from SFFV-FRE clone 1 which had been superinfected with ecotropic F-MuLV clone 201. The cDNA was then hybridized to saturating amounts of 70S RNA derived from F-MuLV clone 201 grown on NRK cells, after which the unhybridized portion of the $cDNA_{SFFV}$ was obtained by hydroxylapatite chromatography. The hybridization profile of $cDNA_{SFFV}$ obtained in this way was similar to that of the $cDNA_{SFFV}$ described in earlier reports (32, 33). $cDNA_{SFFV}$ prepared from SFFV-FRE clone 1 cells hybridized 100% to SFFV-NRK clone 1 cellular RNA, but failed to hybridize appreciably (8.8%) to cellular RNA from control NRK cells or NRK cells infected with F-MuLV clone 201 (16%). Furthermore, $cDNA_{SFFV}$ hybridized extensively to viral RNA from two strains of MCF viruses, Fr-MCF-1 and Mol-MCF_{83} (100 and 88%, respectively), whereas it hybridized poorly to viral RNA from the ecotropic type C viruses from which these MCF viruses were derived (3 to 6%).

A nucleic acid probe was also prepared from F-MuLV clone 201 grown on Sc-1 cells. This cDNA probe was hybridized to saturating amounts of SFFV-NRK clone 1 cellular RNA, and the hybridized portion was obtained by hydroxylapatite chromatography. This cDNA probe, designated $cDNA_{F\text{-}MuLV+,SFFV+}$, measures the portion(s) of F-MuLV which is contained in the replication-defective SFFV genome. This cDNA probe hybridized 100% to F-MuLV/NRK cellular RNA and also hybridized extensively to SFFV-NRK clone 1 cellular RNA (80%), but failed to hybridize appreciably to control NRK cells (7.5%). $cDNA_{F\text{-}MuLV+,SFFV+}$ served as an independent measurement for the levels of SFFV-specific RNA in the cellular RNA of SFFV-containing nonproducer cells since it detects a portion(s) of SFFV which is not detected by $cDNA_{SFFV}$.

Viral and cellular RNAs. The extractions of 70S viral RNA and total cellular RNA were performed as previously described (32).

Molecular hybridizations. RNA-[^{3}H]cDNA hybridizations were performed at 66°C in 0.75 M NaCl by methods which have been described previously (32), and hybridization was assayed with S1 nuclease and analyzed as a function of RNA concentration and time (C_rt) (5) or volume of virus-containing supernatant fluid and time (V_0t) (22).

Sera. Antiserum to Mol-MCF_{83} gp70 was prepared by immunization of goats with purified gp70 from Mol-MCF_{83}. Characterization of this serum both before and after absorption with cloned ecotropic M-MuLV has been reported previously (26). Antiserum to Fr-MCF virus was obtained by immunizing rabbits with viable rabbit corneal cells (SIRC) infected with Fr-MCF-1 virus and has been described previously (25). Goat antiserum to Rauscher MuLV p12 was provided by Roger Wilsnack, Huntingdon Research Labs, Brooklandville, Md.

For some of the experiments described in this paper, antisera made against SFFV-NRK clone 1 cells were used. Rat anti-SFFV NRK clone 1 antiserum was prepared by injecting 0.5×10^6 to 1.0×10^6 viable SFFV-NRK clone 1 cells subcutaneously into 10-day-old Osborne-Mendel rats. Most of the rats so inoculated developed tumors at the site of injection within 1 to 3 months; these animals were bled, and the sera were assayed for the presence of antibodies reactive with iodinated MCF gp70. Sera with high titers of MCF gp70 precipitating antibodies were used at limiting antibody concentrations for radioimmunoassays. Additional results using these anti-SFFV NRK clone 1 antisera for antibody immunoprecipitation studies in antibody excess have been described previously (26).

Radioimmunoassays. Competition radioimmunoassays and serum titrations for Fr-MCF gp70 and F-MuLV gp70 were performed in double antibody precipitation experiments as previously described (25).

Metabolic labeling of cells, immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Metabolic labeling of cells and analysis of immune precipitates were carried out as previously described (26). Briefly, subconfluent monolayers of cells grown in 100-mm petri dishes were labeled with 300 μCi of [^{35}S]methionine (400 Ci/mmol; New England Nuclear Corp.) per plate, 400 μCi of [^{3}H]mannose or [^{3}H]galactose (10 Ci/mmol; New England Nuclear Corp.) per plate, or 500 μCi of ^{32}P (500 Ci/mmol; New England Nuclear Corp.) per plate. Extracts of cells were then preabsorbed with normal serum and *Staphylococcus aureus* to reduce nonspecific background precipitation and immune precipitated with specific antisera and *S. aureus*, and the washed precipitates were electrophoresed on 7% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Gels were then fixed, fluorographed, and exposed to X-ray film at −70°C.

In vitro translation. In vitro translation of SFFV-specific cytoplasmic RNA was performed by using a nuclease-digested rabbit reticulocyte system as previously described (29). To prepare virus-specific RNA from cells infected with SFFV, the polyadenylic acid-containing RNA was prepared by following the procedure of Hayward (12). This RNA was heated in 0.01 M Tris-hydrochloride, pH 7.8, for 2 min at 80°C and centrifuged through a 10 to 30% sucrose gradient. Virus-specific RNA was localized on the gradient by hybridization kinetic analysis with $cDNA_{F\text{-}MuLV}$ and then translated in a rabbit reticulocyte system as previously described. Proteins translated were then immune precipitated, and the precipitates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as previously described (26).

RESULTS

Hybridization of $cDNA_{SFFV}$ and $cDNA_{F\text{-}MuLV+,SFFV+}$ to cellular RNA from SFFV-containing nonproducer cells. With each of the two cDNA's prepared as described above, molecular hybridizations were performed with total cellular RNA from nonproducer rat cells con-

taining SFFV isolated from FV-P (Mirand), FV-P (Axelrad), and FV-P (Lilly-Steeves) (Fig. 1). Figure 1A shows that $cDNA_{SFFV}$ failed to hybridize appreciably to cellular RNA from F-MuLV clone 201/NRK cells, whereas $cDNA_{F\text{-}MuLV+,SFFV+}$ hybridized extensively (100%) to F-MuLV/NRK cellular RNA. Figure 1B demonstrates that both cDNA probes hybridized well to total cellular RNA from SFFV-NRK clone 1 cells, which contained SFFV isolated from FV-P (Lilly-Steeves). The $C_rt_{1/2}$ value for RNA detectable with either cDNA probe was approximately 100 mol·s/liter, and complete saturation (100%) of both cDNA probes was achieved at a C_rt value of 2,000 to 3,000 mol·s/liter. Figure 1C shows that both cDNA probes hybridized well to total cellular RNA from SFFV-P-FRE clone 1 cells, which contained SFFV isolated from FV-P (Mirand). The $C_rt_{1/2}$ of SFFV-specific RNA in cellular RNA from SFFV-P-FRE clone 1 was approximately 400 to 500 mol·s/liter, suggesting that these cells contain a somewhat lower concentration of SFFV RNA than do SFFV-NRK clone 1 cells. Finally, Figure 1D demonstrates that cellular RNA from SFFV-NRK NP 501 cells, which contained SFFV isolated from FV-P (Axelrad), hybridized to both cDNA probes, although only 50 to 60% saturation of each cDNA was obtained even at a C_rt value of greater than 3,000 mol·s/liter. These results suggest that the levels of SFFV RNA are significantly lower in SFFV-NRK NP 501 than they are in either SFFV-NRK clone 1 or SFFV-FRE clone 1. It should be pointed out that, although the concentrations of SFFV RNA varied considerably in each of the three different SFFV-containing nonproducer cells, both cDNA's detected essentially identical concentrations of SFFV RNA within cellular RNA from each nonproducer cell line.

To determine whether the incomplete hybridization of both $cDNA_{SFFV}$ and $cDNA_{F\text{-}MuLV+,SFFV+}$ to cellular RNA from SFFV-NRK NP 501 was due to a lower concentration of SFFV RNA in this cell line, we superinfected SFFV-NRK NP 501 cells with woolly monkey leukemia virus (WoLV) and assayed the viral RNA released from the superinfected cells with each of the cDNA's used above (Fig. 2). Each cDNA hybridized to a value of 100% to viral RNA released from SFFV-NRK NP 501/WoLV at a V_0t value of approximately 10^2 ml·h. These results indicate that the partial hybridization of $cDNA_{SFFV}$ to cellular RNA from SFFV-NRK NP 501 was

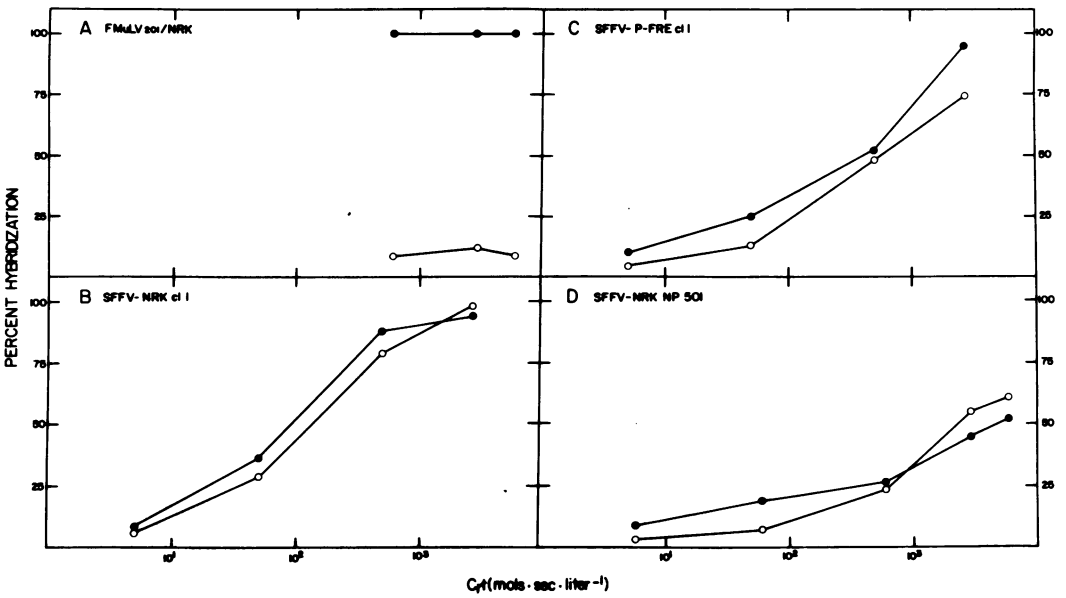

FIG. 1. *Hybridization of $cDNA_{SFFV}$ and $cDNA_{F\text{-}MuLV+,SFFV+}$ to cellular RNA from cells infected with various strains of SFFV. Various concentrations of total cellular RNA from each nonproducer cell line and F-MuLV/NRK cells were hybridized to $cDNA_{SFFV}$ (○) or $cDNA_{F\text{-}MuLV+,SFFV+}$ (●), and percent hybridization was determined with S1 nuclease. In (A) the percent hybridization values shown reflect the final extent of hybridization of each cDNA to respective positive controls; specifically, for $cDNA_{SFFV}$ 962 counts hybridized to SFFV-NRK clone 1 cellular RNA equaled 100% hybridization, and for $cDNA_{F\text{-}MuLV+,SFFV+}$ 2,380 counts hybridized to F-MuLV/NRK RNA equaled 100% hybridization. In (B) through (D) 100% hybridization reflects values which have been corrected for hybridization of each cDNA to SFFV-NRK clone 1 cellular RNA (962 counts for $cDNA_{SFFV}$, 1,920 counts for $cDNA_{F\text{-}MuLV+,SFFV+}$).*

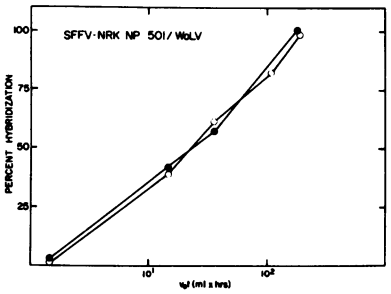

FIG. 2. *Hybridization of $cDNA_{SFFV}$ and $cDNA_{F\text{-}MuLV+,\ SFFV+}$ to viral RNA from SFFV-NRK NP 501/WoLV. To obtain viral RNA, 250 ml of 24-h supernatant medium was harvested from each of six 150-cm^2 culture flasks of SFFV-NRK NP 501 which had been superinfected with WoLV. Virus-containing supernatant from these cells was centrifuged at 3000 rpm for 10 min, after which virus was pelleted by centrifugation at 25,000 rpm for 90 min at 4°C. Viral pellets were resuspended in TNE buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride, 0.001 M EDTA) containing 0.1% SDS and extracted three times with phenol and chloroform-isoamyl alcohol (20:1). The RNA was then dialyzed against distilled water, lyophilized, and resuspended in 0.5 ml of distilled water, resulting in a 500-fold concentration of viral RNA from the virus-containing supernatant medium. Samples of viral RNA were then hybridized to $cDNA_{SFFV}$ (○) or $cDNA_{F\text{-}MuLV+,\ SFFV+}$ (●), and the results of each hybridization were expressed as a function of V_0t (ml·h). Viral RNA contained in NRK cells was determined in the same way.*

due to a lower concentration of SFFV RNA in this cell line and not to a lack of genetic information homologous to $cDNA_{SFFV}$ in this isolate. In a control for this study, neither cDNA probe hybridized appreciably to RNA from WoLV grown in NRK or FRE cells, achieving a final extent of hybridization of less that 5% at V_0t values of greater than 10^3 ml·h (data not shown).

The results of these molecular hybridization studies demonstrate that SFFV isolated from three different strains of Friend virus complex, namely FV-P (Mirand), FV-P (Axelrad), and FV-P (Lilly-Steeves), contain a set of genetic sequences which we have previously designated as SFFV specific and which have been shown to hybridize specifically to genomic RNA from murine xenotropic and MCF strains of MuLV's.

Immunological analysis of SFFV gene products by competition radioimmunoassay. We recently developed a radioimmunoassay specific for the gp70 of MCF MuLV's and demonstrated an antigen in nonproducer cells infected with the Lilly-Steeves strain of SFFV that cross-reacts specifically in that immunoassay (25). This was the first evidence that the SFFV isolated from FV-P (Lilly-Steeves) encodes a protein which shares antigenic determinants with the gp70's of MCF viruses. To determine whether SFFV isolated from other strains of the Friend virus complex also encode for an MCF-related *env* gene product, we analyzed extracts of nonproducer cells containing SFFV derived from FV-P (Mirand) and FV-P (Axelrad) at limiting dilutions of antibody in a competition radioimmunoassay which detects only MCF gp70 antigenic determinants (Fig. 3). In this radioimmunoassay, triton-disrupted extracts of Fr-MCF virus competed extensively (80% competition with 1.0 μg of protein), whereas extracts of F-MuLV clone 201 failed to compete appreciably, confirming that the radioimmunoassay is MCF gp70 type specific. Triton-disrupted extracts of SFFV-NRK clone 1 cells, SFFV-P-FRE clone 1 cells, and SFFV-NRK NP 501 cells all competed extensively in this radioimmunoassay, with each extract achieving a final level of 60 to 65% at approximately 10 to 30 μg of competing antigen. In contrast, uninfected NRK cells showed no competition at a comparable level of antigen (20 to 50 μg). Furthermore, relative levels of competing antigen in each nonproducer cell correlated with the amounts of SFFV RNA in the different nonproducer cell lines (Fig. 1).

These results demonstrate that SFFVs derived from three distinct strains of Friend virus encode for gene products which share antigenic determinants with the gp70 *env* gene product of MCF viruses, providing further evidence that

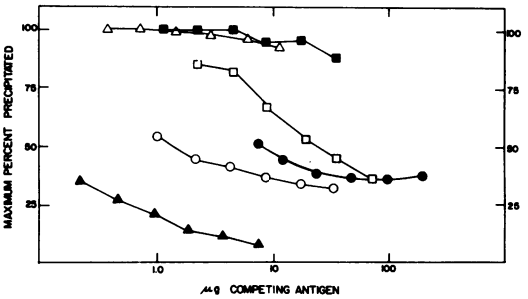

FIG. 3. *Detection of MCF-related antigen in SFFV nonproducer cells. This competition assay was carried out as described in the text by using ^{125}I-labeled Mol-MCF gp70 and a 1:1,600 dilution of goat anti-Mol-MCF gp70 antiserum absorbed with ecotropic M-MuLV. Absorbed antiserum precipitated 10 to 15% of the ^{125}I-labeled counts per minute of Mol-MCF gp70 at a dilution of 1:1,600, but failed to precipitate labeled ecotropic M-MuLV or F-MuLV gp70 at any concentration. Cell extracts used as competing antigens in this assay included NRK (■), SFFV-NRK clone 1 (○), SFFV-P-FRE clone 1 (●), and SFFV-NRK 501 (□). Virus extracts used in this assay included FR-MCF-1 (▲) and F-MuLV clone 201 (△).*

each isolate of SFFV contains MCF-related *env* gene sequences.

Antisera made against SFFV-NRK clone 1 cells precipitated MCF-related gp70's. To provide further evidence that SFFV encodes for a gene product which is antigenically related to MCF gp70, we made antisera to SFFV-encoded gene product(s) by immunization of Osborne-Mendel rats with SFFV-NRK clone 1 cells. The immunization procedure consisted of one or more subcutaneous inoculations of viable SFFV-NRK clone 1 cells into 10-day-old rats. Although these NRK cells are not morphologically transformed by SFFV, a large percentage of the inoculated rats formed tumors at the site of injection. Sera were obtained from these tumored animals and were tested for the presence of antibodies to the gp70's of ecotropic F-MuLV and Fr-MCF virus. A serum titration using a pool of rat anti-SFFV-NRK clone 1 serum is shown in Fig. 4. Antisera made against SFFV-containing nonproducer cells contained antibodies which immunoprecipitated 20% of the ^{125}I counts per minute of labeled Fr-MCF gp70 at a dilution of 10^{-1}. The same antisera failed to immunoprecipitate the gp70 of the ecotropic F-MuLV at any dilution, whereas anti-F-MuLV antiserum or other antisera with broader specificity precipitated over 80% of the gp70 of ecotropic F-MuLV at much higher dilutions of antibody (data not shown). Similarly, anti-SFFV-NRK clone 1 antisera were able to immunoprecipitate up to 30% of the input counts per minute of ^{125}I-labeled gp70 from a Moloney MCF virus (Mol-MCF_{83}) but precipitated only 2% of input counts per minute of ecotropic M-MuLV gp70 (data not shown). Sera from normal rats did not contain antibodies which could immunoprecipitate labeled gp70 from either ecotropic or MCF MuLV. These results demonstrate that SFFV-containing nonproducer cells express a gene product(s) which contains determinants which are predominantly MCF gp70 type specific and not ecotropic gp70 type specific and that antisera to this SFFV-encoded gene product can be generated by inoculation of such SFFV-containing nonproducer cells into rats.

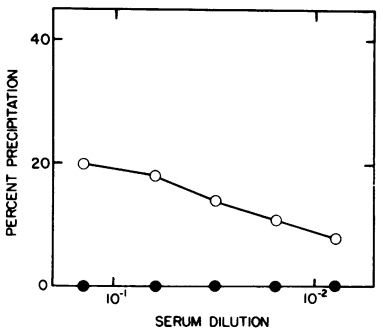

FIG. 4. *Immune precipitation of purified gp70's by rat anti-SFFV-NRK antisera. ^{125}I-labeled gp70 from either Fr-MCF or F-MuLV was reacted with various dilutions of a pool of rat anti-SFFV-NRK clone 1 antisera and was analyzed as described in the text. Symbols: ●, ^{125}I-labeled F-MuLV gp70; ○, ^{125}I-labeled Fr-MCF-1 gp70.*

Use of anti-SFFV antisera in competition radioimmunoassays. To further demonstrate the specificity of the antisera made against SFFV-containing nonproducer cells, sera which contained anti-MCF gp70 antibodies were pooled from several rats and were used in a competition radioimmunoassay. In these experiments, purified gp70 from Fr-MCF virus was used as the labeled antigen. As Fig. 5 shows, Fr-MCF virus competed extensively in this competition assay, whereas ecotropic F-MuLV showed no competition. These results indicate that this competition radioimmunoassay is MCF type specific when antisera to SFFV-encoded gene product(s) are used. Furthermore, Fig. 5 also shows that extracts of rat nonproducer cells containing each of the three isolates of SFFV were able to compete extensively in this radioimmunoassay when anti-SFFV-NRK clone 1 antisera were used, whereas control NRK cells failed to compete to the same degree. These results demonstrate that each isolate of SFFV derived from three separate strains of Friend virus synthesizes cross-reacting gene products which share immunological determinants with the *env* gene products of MCF strains of MuLV.

Precipitation of different metabolically labeled SFFV nonproducer cells by MCF-specific antisera. In addition to showing the expression of an MCF gp70-related protein in cells nonproductively infected with SFFV by

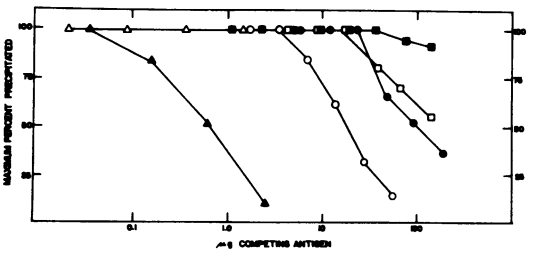

FIG. 5. *Analysis of SFFV nonproducer cells with rat anti-SFFV-NRK clone 1 antisera: competition radioimmunoassay using ^{125}I-labeled Fr-MCF gp70 and a 1:64 dilution of pooled rat antisera against SFFV-NRK clone 1 cells. Virus extracts used included Fr-MCF-1 (▲) and F-MuLV clone 201 (△). Cell extracts used included NRK (■), SFFV-NRK clone 1 (○), SFFV-P-FRE clone 1 (●), and SFFV-NRK NP 501 (□).*

using competition radioimmunoassays, we were able to identify a 52,000-dalton glycoprotein in $SFFV_{LS}$ nonproducer cells metabolically labeled with [^{35}S]methionine and immune precipitated with MCF gp70 type-specific antisera (26). We were interested in whether this same protein could be detected in cells nonproductively infected with the other two strains of SFFV and therefore pulsed cells nonproductively infected with either the Lilly-Steeves strain of SFFV, the Mirand strain of SFFV, or the Axelrad strain of SFFV and precipitated the labeled extracts with anti-Moloney-MCF gp70 serum that had been absorbed with ecotropic M-MuLV (Fig. 6A). A 50,000- to 52,000-dalton protein precipitable with MCF gp70-specific antiserum was present in all cells infected with SFFV (Fig. 6A, lanes 1 through 3), with the Mirand and Axelrad strains encoding for slightly smaller proteins than the Lilly-Steeves strain. No such protein was present in uninfected cells (lane 4), nor was it precipitated by normal goat serum from any of these cells (lanes 5 through 8). We have demonstrated previously that this protein is absent from cells infected with ecotropic or MCF MuLV's (26). Since we had shown previously that the 52,000-dalton protein coded for by the Lilly-Steeves strain of SFFV was glycosylated, we labeled the three different SFFV nonproducer cell lines with [^{3}H]mannose or [^{3}H]galactose and precipitated the extracts with the MCF-specific gp70 antiserum. As Fig. 6B and C show, mannose and galactose were incorporated into the anti-MCF gp70 precipitable proteins of all three SFFV strains. Galactose labeling (Fig. 6C, lanes 1 through 3) showed for the first time that a small percentage of the gp52 of all three strains is further processed by the addition of terminal sugars to a 62,000- to 68,000-dalton form. In addition to precipitating a 50,000- to 52,000-dalton protein from the SFFV nonproducer cells with goat antiserum to MCF gp70, we could also specifically precipitate the same size proteins from those cells with anti-SFFV-NRK clone 1 sera (data not shown).

Precipitation of different SFFV nonproducer cells with anti-p12 serum. It has been reported previously that the Lilly-Steeves strain of SFFV codes for p15 and p12 proteins of F-MuLV and that the p12 is attached to another protein that cannot be accounted for by known type C virus helper structural proteins (3). The molecular weights of the p12-containing proteins, as determined by guanidine hydrochloride agarose column chromatography, were >100,000, 50,000, and 30,000, whereas the p15-containing protein migrated in the molecular weight range of 15,000. We were interested in further characterizing the p12-linked protein of the Lilly-Steeves strain of SFFV as well as determining whether a similar protein was coded for by the other two strains of SFFV. Thus, cells nonproductively infected with the three strains of SFFV were pulsed with [^{35}S]methionine and then immune precipitated with anti-p12 antiserum (Fig. 7A). Only the Lilly-Steeves strain of

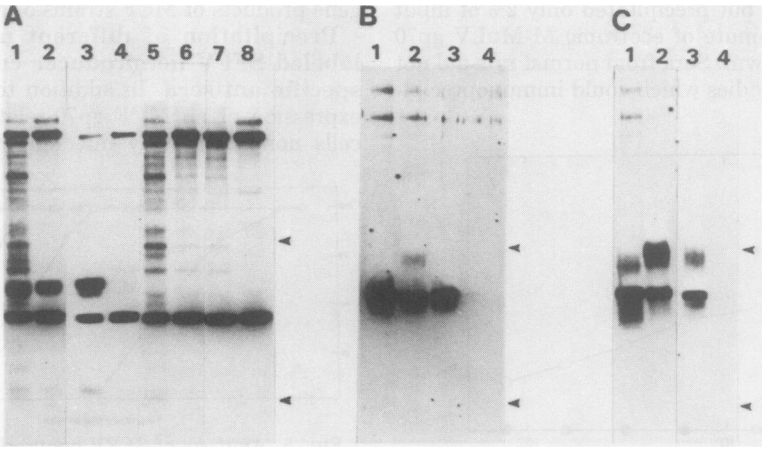

FIG. 6. *Autoradiographs of various metabolically labeled SFFV nonproducer cell extracts immunoprecipitated with anti-MCF gp70 serum. SFFV-P-FRE clone 1 (lane 1), SFFV-NRK NP 501 (lane 2), SFFV-NRK clone 1 (lane 3), or NRK (lane 4) cells were labeled with [^{35}S]methionine (A), [^{3}H]mannose (B), or [^{3}H]-galactose (C) and then immune precipitated with goat anti-Moloney MCF gp70 serum that had been absorbed with ecotropic M-MuLV. Lanes 5 through 8 represent [^{35}S]methionine-labeled extracts precipitated with normal goat serum in the same order. Precipitates were then run on 7% SDS-polyacrylamide gels, and the gels were autoradiographed as described in the text. Molecular weight markers used were iodinated bovine serum albumin (68,000) (top arrows) and Rauscher MuLV p30 (30,000) (bottom arrows).*

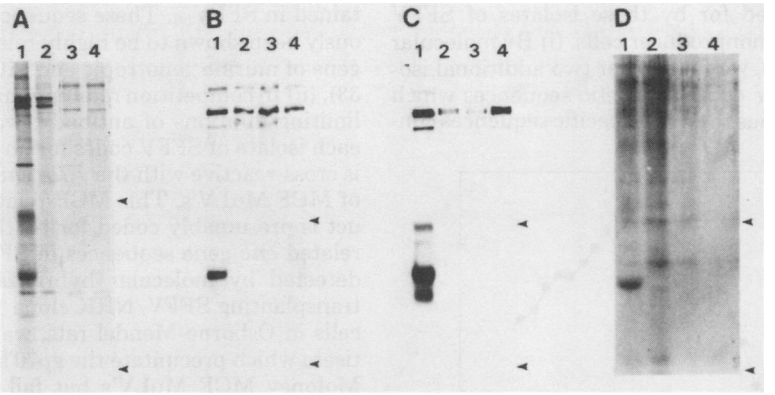

FIG. 7. *Autoradiographs of various metabolically labeled SFFV nonproducer cell extracts immunoprecipitated with anti-Rauscher MuLV p12 serum. SFFV-NRK clone 1 (lane 1), SFFV-P-FRE clone 1 (lane 2), SFFV-NRK NP 501 (lane 3), and NRK (lane 4) cells were labeled with [^{35}S]methionine (A), [^{3}H]mannose (B), [^{3}H]galactose (C), or ^{32}P (D) and then immune precipitated with goat anti-Rauscher MuLV p12 serum. Precipitates were then run on 7% SDS-polyacrylamide gels, and the gels were autoradiographed as described in the text. Molecular weight markers used were iodinated bovine serum albumin (68,000) (top arrows) and Rauscher MuLV p30 (30,000) (bottom arrows).*

SFFV coded for a protein that was precipitable with anti-p12 antiserum (Fig. 7A, lane 1). Cells nonproductively infected with the Axelrad strain (lane 2) or the Mirand strain (lane 3) of SFFV did not code for a p12-linked protein.

In an attempt to further characterize the p12-containing protein of the Lilly-Steeves strain of SFFV, we pulsed cells nonproductively infected with this virus with [^{3}H]mannose, [^{3}H]galactose, and ^{32}P. As Fig. 7B through D show, this virus coded for a 45,000-dalton protein that incorporated ^{32}P (Fig. 7D, lane 1), incorporated [^{3}H]-mannose (Fig. 7B, lane 1), and was further glycosylated to a 65,000-dalton protein by the addition of terminal galactose sugars (Fig. 7C, lane 1).

In vitro translation of virus-specific RNA from cells nonproductively infected with the Lilly-Steeves strain of SFFV. Since the Lilly-Steeves strain of SFFV codes for two distinct proteins, an *env*-related protein and a *gag*-related protein, we were interested in determining whether these two proteins were translated from two separate mRNA's in the SFFV-NRK clone 1 cells. We first separated the polyadenylic acid-containing cytoplasmic RNA from SFFV-NRK clone 1 cells by sucrose density centrifugation and isolated the virus-specific RNA by molecular hybridization (Fig. 8). Two distinct size classes of SFFV RNA were detected: 26S and 18S species. When the two RNA species were translated across the gradient in a nuclease-digested rabbit reticulocyte in vitro translating system and the proteins were immune precipitated with goat anti-MCF gp70 antiserum or goat anti-p12 antiserum, the results shown in Fig. 9 were obtained. Fractions around the 26S peak of SFFV RNA coded for 45,000- and 40,000-dalton proteins which were precipitable with anti-p12 serum but not anti-MCF gp70 serum. In contrast, fractions around the 18S peak coded for a 46,000-dalton protein which was precipitable with anti-MCF gp70 serum but not anti-p12 serum. Similar results were also obtained with the Mirand strain of SFFV (data not shown). Although no *gag* gene product was detected with anti-p12 serum, an 18S mRNA was detected in SFFV$_M$-infected cells, and this RNA coded for an MCF gp70-related protein when translated in vitro.

DISCUSSION

The current studies are an extension of our previous work in which we analyzed the genetic composition and gene products coded for by the Friend strain of SFFV. In our previous experiments, SFFV was isolated from the B-tropic strain of FV-P derived by Lilly and Steeves (15). In the current studies we analyzed SFFVs which were isolated from two other distinct strains of the Friend virus complex, each of which had been maintained as an independent passage line of Friend virus. One isolate of SFFV was derived from a preparation of the Mirand strain of FV-P (18) and was initially cloned in NIH 3T3 cells and later recloned in FRE clone 2 cells. The second isolate of SFFV was derived from the Axelrad strain of FV-P (2) and was cloned in NRK cells by Alan Bernstein, Ontario Cancer Institute, Toronto, Canada (4).

We applied four different approaches to the analyses of the genomic compositions of and the

proteins coded for by these isolates of SFFV contained in nonproducer cells. (i) By molecular hybridization, we found that two additional isolates of SFFV contain genetic sequences which are homologous to SFFV-specific sequences contained in $SFFV_{LS}$. These sequences have previously been shown to be highly related to the *env* gene of murine xenotropic and MCF viruses (32, 33). (ii) In competition radioimmunoassays using limiting dilutions of antibody, we showed that each isolate of SFFV codes for an antigen which is cross-reactive with the gp70 *env* gene product of MCF MuLV's. This MCF-related gene product is presumably coded for by the xenotropic-related *env* gene sequences in SFFV which are detected by molecular hybridization. (iii) By transplanting SFFV/NRK clone 1 nonproducer cells in Osborne-Mendel rats, we produced antisera which precipitate the gp70's of Friend and Moloney MCF MuLV's but fail to precipitate the gp70's of ecotropic F-MuLV and M-MuLV. These results provide further evidence that SFFV codes for a gene product which shares antigenic determinants with the gp70's of MCF MuLV's. (iv) Finally, by pulse-labeling each SFFV nonproducer cell line with [^{35}S]methionine and following this with immune precipitation with excess antibody, we can precipitate with MCF-specific antisera, as well as with rat anti-SFFV-NRK clone 1 sera, a glycoprotein which has a molecular weight of 50,000 to 52,000,

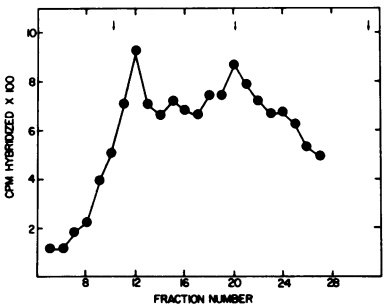

FIG. 8. *Size distribution of SFFV-specific RNA in SFFV nonproducer cells. Polyadenylic acid-containing RNA from SFFV-NRK clone 1 cells was prepared and fractionated by sucrose density gradient centrifugation, and samples from each fraction were hybridized to ^{3}H-labeled $cDNA_{F\text{-}MuLV}$ as described in the text. Arrows indicate sedimentation values calculated from rRNA run on a parallel gradient and are (left to right) 28S, 18S, and 4S.*

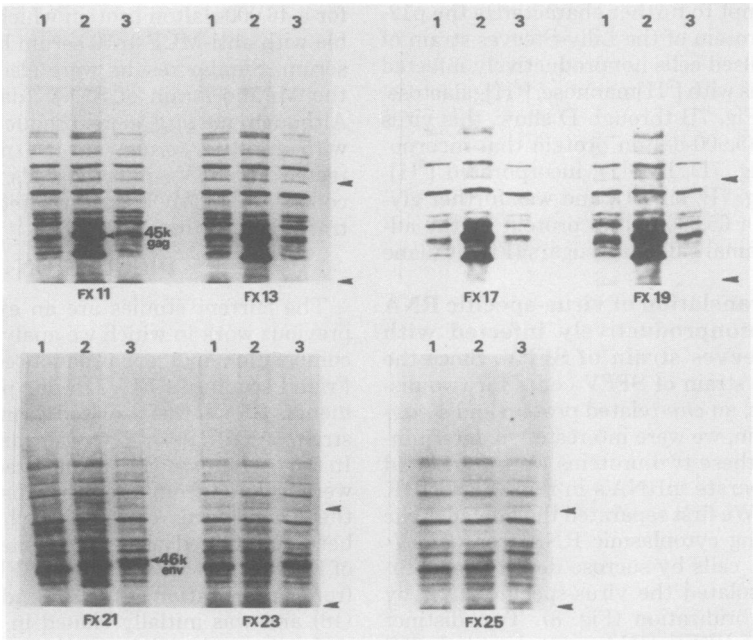

FIG. 9. *Autoradiograph of immune precipitates of in vitro translated SFFV-NRK RNA products. Various fractions from the SFFV-NRK clone 1 RNA gradient in Fig. 8 were translated in a nuclease-digested rabbit reticulocyte in vitro translating system, and the products were immune precipitated with normal goat serum (lane 1), goat anti-Rauscher MuLV p12 serum (lane 2), goat anti-Moloney MCF gp70 serum that had been absorbed with ecotropic M-MuLV (lane 3). Precipitates were then run on 7% SDS-polyacrylamide gels, and the gels were autoradiographed as described in the text. Molecular weight markers used were iodinated bovine serum albumin (68,000) (top arrows) and Rauscher MuLV p30 (30,000) (bottom arrows).*

as determined by SDS-polyacrylamide gel electrophoresis. This MCF gp70-related glycoprotein is found in nonproducer cells containing SFFV isolated from FV-P (Lilly-Steeves), FV-P (Mirand), and FV-P (Axelrad).

Thus, on the basis of the combined results of our analysis of three different strains of SFFV, we again conclude that SFFV arose by recombination between F-MuLV and the *env* gene of a murine xenotropic or MCF virus and that such MCF-related *env* gene sequences acquired in the formation of SFFV code for a 50,000- to 52,000-dalton glycoprotein. It is important to emphasize that these conclusions are not based solely on the preparation and fractionation of cDNA probes by hydroxylapatite chromatography, since the product of the MCF-related *env* gene sequences in SFFV can be demonstrated without recourse to molecular cycling procedures. These results cannot be reconciled readily with those of Mak et al. (16), who were unable to demonstrate xenotropic sequences with their $cDNA_{SFFV}$ synthesized from murine cells, nor can they be reconciled with the results of Pragnell et al. (20), who were unable to detect xenotropic-related *env* gene sequences in SFFV released from murine erythroleukemia cells.

In contrast to the presence of an MCF *env*-related gene product in all strains of SFFV tested, there is considerable variability in the expression of the *gag* gene-related proteins. Whereas both the Lilly-Steeves and Axelrad strains of SFFV have been shown to code for F-MuLV p15 (3, 4), only the Lilly-Steeves strain codes for F-MuLV p12 (3), and the p12 reactivity is attached to another protein moiety that gives it an electrophoretic mobility of 45,000 daltons. The studies presented in this paper indicate that the p12-containing 45,000-dalton protein coded for by the Lilly-Steeves strain of SFFV is either phosphorylated or glycosylated. The glycosylated form appears to be further processed to a 65,000-dalton terminally glycosylated protein. These data are consistent with the guanidine hydrochloride chromatography data presented by Barbacid et al. (3), which indicated that NRK cells nonproductively infected with the Lilly-Steeves strain of SFFV expressed p12-containing proteins of 50,000 and 30,000 daltons. The p12 reactivity migrating near the void of their column may have been the 65,000-dalton glycosylated protein. The pulse-labeling data are also consistent with our in vitro translation of a 45,000-dalton p12-containing protein from SFFV-specific cytoplasmic RNA. In addition to p12 determinants, the 45,000-dalton SFFV-encoded protein appears to contain some determinants of p30 since a large percentage of rats injected with SFFV nonproducer rat cells contain high titers of anti-p30 antibodies (unpublished data). However, SFFV must code for only a portion of the p30 molecule, for antisera prepared against purified p30 fail to precipitate the 45,000-dalton SFFV-encoded protein (unpublished data). The 45,000-dalton protein does not contain determinants of F-MuLV p10 and also appears to lack p15 determinants (unpublished data), although the Lilly-Steeves strain of SFFV has been shown to code for F-MuLV p15 (3). It is possible (depending upon how much of the p30 gene SFFV contains) that the 45,000-dalton p12-containing protein of $SFFV_{LS}$ contains as much as 23,000 daltons of protein that cannot be accounted for by any known MuLV structural proteins. We therefore cannot rule out the possibility that, although two of the three strains of SFFV studied do not code for a p12-containing polyprotein, they may all code for the currently unknown portion of this molecule. To determine this, we are attempting to develop specific antisera directed to the non-*gag* portion of the $SFFV_{LS}$ 45,000-dalton protein.

Although $SFFV_{LS}$ codes for a *gag*-related protein, this protein does not appear to be the typical high-molecular-weight polyprotein that has been shown to be coded for by a number of defective viruses, such as the Abelson leukemia virus (38), the feline sarcoma virus (27, 31), and the acute avian leukemia viruses (11, 37). Whereas these other defective viruses have been shown both by metabolic labeling and in vitro translation to code for 75,000- to 130,000-dalton *gag*-related polyproteins, SFFV appears to code for a much smaller *gag*-related protein with a molecular weight of only 45,000. The protein data are consistent with the molecular data, however. Most of the defective leukemia and sarcoma viruses contain large inserts of newly acquired sequences after recombination with helper viruses (14, 17, 29; A. Shields et al., manuscript in preparation), and these inserts appear to code for the high-molecular-weight *gag* gene-related polyprotein. In contrast, SFFV contains a small portion of its genome (in the *env* region) which is newly acquired, and the remainder is closely related or identical to sequences present in the parental F-MuLV (7). Accordingly, SFFV does not code for a high-molecular-weight *gag* gene-related polyprotein. The organization of the SFFV genome and its gene products are more reminiscent of that of the Moloney sarcoma virus. Like SFFV, there are strains of Moloney sarcoma virus which code for a 60,000- to 70,000-dalton *gag*-related protein, as well as other strains which do not code for any *gag* gene products (23). In the Moloney sarcoma virus,

the newly acquired (*src*) sequences have been shown to be located toward the 3′ end of the genome (1, 13). Although a product of this *src* gene has been rigorously sought but not identified, a candidate mRNA, which is smaller than genomic RNA and might code for the product of this gene, has been detected recently in Moloney sarcoma virus-transformed cells (6).

Although our data indicate that $SFFV_{LS}$ codes for two gene products, it is not known whether either of these proteins plays a role in the rapid erythroleukemia induced by SFFV. Although no direct evidence is currently available to implicate the gp52 as the "leuk" gene product of SFFV, the relative constancy of this protein for all of the pathogenic strains of SFFV, the locations of the newly acquired sequences in SFFV, and the molecular analogy to Moloney sarcoma virus seem to strengthen the hypothesis that the gp52 is a key protein in the leukemogenicity of this virus.

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