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Characterization of a Protein Found in Cells Infected with the Spleen Focus-Forming Virus That Shares Immunological Cross-Reactivity with the gp70 Found in Mink Cell Focus-Inducing Virus Particles

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Previously we detected an antigen in cells infected with the spleen focus-forming virus (SFFV) with a radioimmunoassay specific for the gp70's of murine leukemia mink cell focus-inducing (MCF) viruses. This antigen has now been characterized in competition radioimmunoassays with limiting dilutions of antibody and in pulse-labeling studies under conditions of antibody excess. Both methods of analysis indicate that the SFFV-encoded antigen is a glycoprotein with a molecular weight of approximately 52,000. The gp52 shared immunological reactivity and methionine-containing tryptic peptides with the gp70 of a Friend MCF virus and was expressed on the surface of SFFV-infected cells as well as in the cytoplasm. The gp52 could be detected (i) in fibroblastic cell lines from several species when these cells were infected with SFFV; (ii) in several established erythroleukemic cell lines; and (iii) in the spleens of mice recently infected with SFFV. Although it shared immunochemical properties with the gp70 of Friend MCF virus, the gp52 could be distinguished from the MCF gp70 (i) by its apparent lack of group and interspecies immunological determinants compared with MCF virus-derived gp70's; (ii) by its failure to be released from cells infected with SFFV or SFFV plus helper virus; (iii) by its molecular weight; and (iv) by tryptic peptide analysis. The results indicate that SFFV codes for an MCF gp70-related gp52 which is apparently no longer a virion structural protein like the MCF gp70 from which it was originally derived.

The Friend strain of the spleen focus-forming virus (SFFV) has been shown to be a replication-defective leukemia virus associated with the development of rapid splenomegaly, splenic foci, and erythroleukemia when injected as a pseudotype into susceptible mice (1, 7, 26). After the SFFV was cloned free of helper-independent type-C virus in rat cells (28), the genome of SFFV was extensively analyzed by molecular hybridization. The SFFV genome has been shown to consist of genetic sequences homologous to Friend helper viruses with which it was originally associated, as well as SFFV-specific sequences that are partially homologous to three separate strains of mouse xenotropic viruses, and highly related to the genomes of mink cell focus-inducing viruses (MCF), which are intragenic envelope gene recombinants between ecotropic and xenotropic viruses (28, 29). Thus, the Friend strain of the SFFV, due to is homology with MCF viruses, appears to be a recombinant virus containing sequences derived from a portion of the Friend murine leukemia virus (F-MuLV) genome and RNA sequences that are highly related to the *env* gene of xenotropic and MCF viruses.

We have been interested in the proteins coded for by SFFV. Earlier studies from several laboratories demonstrated that SFFV codes for F-MuLV p15 and p12 (2, 3), and a recent paper from our laboratory reported the detection of an antigen in cells nonproductively infected with SFFV that cross-reacts with the gp70 of MCF MuLV's (21). In the present paper, we report a more sensitive immunoassay for the detection of the SFFV MCF gp70-related protein as well as results of our efforts to further characterize this protein and show its clear association with the SFFV genome.

MATERIALS AND METHODS

Viruses. The sources of the ecotropic, xenotropic, and MCF viruses used in these studies have been previously described in detail (21). Briefly, ecotropic

viruses used include F-MuLV clone 201 and an ecotropic clone of Moloney MuLV (M-MuLV). MCF viruses include Friend MCF-1 (F-MCF) and Moloney MCF clone 83 (M-MCF). Xenotropic viruses include BALB:virus-2 and a xenotropic virus isolated from induced NZB cells. All viruses were isopycnically banded in sucrose gradients before use and concentrated approximately 1,000-fold over starting supernatant cultures. The cells in which the viruses were grown are listed below.

Cells. Uninfected cells used in these studies include NIH 3T3 (10), NRK (6), FRE clone 2 (23), mink lung fibroblasts (CCL 64) (23), and a cell line derived from a dog thymus, Cf_2Th (23). Nonproducer cells used include SFFV-infected NRK cells (SFFV-NRK clone 1) (28) and SFFV-infected FRE cells (SFFV-FRE clone 2) (28). The source of the SFFV in these nonproducer cells is the Lilly-Steeves preparation of Friend virus complex (14). Also used were NIH 3T3 cells nonproductively infected with SFFV derived from the Mirand strain of polycythemia-inducing Friend virus (SFFV-P-NIH) (15; D. H. Troxler et al., submitted for publication) as well as NIH 3T3 or mink cells nonproductively infected with the Kirsten sarcoma virus (Ki-NIH and Ki-mink, respectively) (22).

F-MuLV clone 201 was grown either in NRK cells (F-MuLV/NRK) or NIH 3T3 cells (F-MuLV/NIH). LLV-S is another ecotropic strain (N-tropic) of F-MuLV obtained from Richard Steeves (27) and was twice terminally diluted on NIH/3T3 cells and then used to infect SFFV-P-NIH cells. This producer cell line is designated LLV/SFFV-P-NIH. Friend MCF-1 virus was grown in mink lung cells (F-MCF/mink). Woolly monkey virus (M55) was grown on dog thymus cells, and the virus released from these cells was used to infect SFFV-NRK clone 1. The virus coming out of the M55/SFFV-NRK cells was then used to infect mink lung cells, resulting in the M55/SFFV-mink cell line. M55 was also grown in Ki-mink cells. A culture of F-MuLV/KiSV was prepared by superinfecting Ki-NIH cells with F-MuLV clone 201 that had previously been grown in NIH 3T3 cells.

Three erythroleukemic cell lines established from the spleens of F-MuLV/SFFV-infected mice were also used in these studies. They included T_3Cl_2, which was obtained from and previously described by Y. Ikawa, Tokyo, Japan (9), as well as DS19 (25) and DR10 (16) cell lines obtained from R. Reuben, Columbia University. For induction studies, hexamethylenebisacetamide was obtained from R. Reuben and has previously been shown to cause differentiation of DS19 cells (19).

All fibroblastic cell lines were grown in Dulbecco's modification of Eagle medium containing either 10% calf serum or 10% fetal calf serum. Erythroleukemic cells were grown in Eagle basal medium with Earle balanced salt solution and 15% fetal calf serum.

Sera. Rat antiserum to SFFV-NRK clone 1 cells was prepared by injecting 0.5×10^6 to 1×10^6 SFFV-NRK clone 1 cells subcutaneously into 10-day-old Osborne-Mendel rats. Those animals developing tumors were bled, and the sera were titrated against the gp70's purified from F-MCF-1 and F-MuLV clone 201. Those sera having high-titered antibodies to F-MCF gp70 were pooled and used as rat anti-SFFV-NRK serum in the studies reported in this manuscript. More details about these sera will be given below as well as in subsequent publications.

Rabbit antiserum which precipitated F-MCF gp70 was obtained by immunizing rabbits with viable normal rabbit corneal cells (SIRC) infected with F-MCF-1 virus. Characterization of this serum has been reported previously (21).

Goat antiserum to M-MCF gp70 was prepared by immunizing goats with gp70 purified from Triton X-100-disrupted M-MCF clone 83 virus. This serum has a high titer of antibodies against M- and F-MCF gp70's (1:6,400) as well as to the gp70 of F-MuLV clone 201 (1:1,600). To obtain a more MCF-specific reagent, this serum was absorbed by previously described procedures (21) with cloned Moloney ecotropic MuLV that had been propagated on NIH 3T3 cells. The titer of the absorbed serum is 1:3,200 against the gp70's of M- or F-MCF gp70's and less than 1:10 against F-MuLV gp70.

Goat antiserum to F-MuLV and to Rauscher MuLV (R-MuLV) gp70 was kindly provided by Roger Wilsnack, Huntingdon Research Labs, Brooklandville, Md. The exact pedigree of the viruses used by Wilsnack is not available.

Competition radioimmunoassays. Competition radioimmunoassays for F-MCF and F-MuLV clone 201 gp70's were performed in double antibody precipitation experiments as previously described (21).

Metabolic labeling of cells and immunoprecipitation. Subconfluent monolayers of cells grown in 100-mm petri dishes were labeled with [^{35}S]methionine (New England Nuclear Corp., 400 Ci/mmol) in 2 ml of methionine-free Dulbecco-modified Eagle medium containing 1% dialyzed fetal calf serum (frozen immediately after dialysis and thawed just before use to avoid proteolysis) and 300 μCi of isotope per ml. Pulsing was carried out for 30 min at 37°C. For pulse-chase experiments, isotope was removed after the pulse, and the cells were maintained in isotope-free complete medium containing 10% undialyzed calf serum for the indicated period of time. For studies of proteins chased into extracellular fluid, cells were chased in complete medium containing only 1% calf serum.

Cells were labeled with [^{3}H]mannose (New England Nuclear Corp., 10 Ci/mmol) by incubating subconfluent monolayers of cells grown in 100-mm petri dishes in 2 ml of glucose-free medium containing 1% dialyzed fetal calf serum and 200 μCi of isotope per ml. Cells were labeled for 2 h at 37°C.

Plates of pulse-labeled cells were rinsed twice with 5 ml of 0.15 M phosphate-buffered saline, pH 7.6, and then lysed in 2 ml of extraction buffer (10 mM sodium phosphate buffer, pH 7.6, containing 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) and dounced 10 times in a glass homogenizer. Extracts were then spun at $2,000 \times g$ for 20 min to remove nuclei and cell debris. Approximately 50×10^6 to 100×10^6 trichloroacetic acid-precipitable cpm were obtained from each [^{35}S]methionine-labeled plate, and 20×10^6 to 40×10^6 precipitable cpm were obtained from each [^{3}H]mannose-labeled plate. To reduce the background due to nonspecific precipitation, extracts were incubated for 16 h at 4°C with 5 μl of normal serum and 50 μl of *Staphylococcus aureus* (a 10% Formalin-fixed

solution of Cowan strain I [12]) per ml of extract and then clarified by centrifugation at 2,000 × *g* for 20 min at 4°C. Immune precipitation was then carried out by incubating 10×10^6 to 20×10^6 cpm of each extract for 16 h at 4°C with 5 μl of undiluted serum. Antigen-antibody complexes were isolated by the addition of 50 μl of a 10% solution of Formalin-fixed *S. aureus* and incubation for 2 h at 4°C. Reaction tubes were then spun at 1,000 × *g* for 15 min at 4°C, and the pellets were washed three times with extraction buffer. The pellets were then resuspended and boiled for 1 min in electrophoresis sample buffer (50 mM Tris-hydrochloride [pH 6.8], 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue) in preparation for SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis. Polyacrylamide gels (7%) with 3.5% stacks were prepared, and electrophoresis was carried out by the Laemmli procedure (13) as previously described (24). Molecular weight markers run with each gel included iodinated bovine serum albumin (68,000), bovine immunoglobulin G (50,000 and 23,500), and R-MuLV p30 (30,000). Gels were then fixed and fluorographed by the method of Bonner and Laskey (5) and exposed to Kodak Royal Blue X-Omat film at −70°C.

Radioiodination of cell surfaces. Surface labeling of cells was carried out by a previously described (20) modification of the lactoperoxidase method as described by Kennel et al. (11). Briefly, 10^7 erythroleukemic cells or a confluent monolayer of fibroblastic cells in a 100-mm petri dish was washed twice with 5 ml of phosphate-buffered saline, and then labeled in 1 ml of phosphate-buffered saline containing 20 μg of lactoperoxidase and 1 to 2 mCi of ^{125}I (Amersham/Searle). The reaction was catalyzed by the addition of two portions of hydrogen peroxide 1 min apart so that the final concentration of hydrogen peroxide was 45 mM. After incubation for 10 min at room temperature, the reaction was stopped, and the cells were washed five times with phosphate-buffered saline and then lysed by the addition of phosphate-buffered saline containing 0.5% Triton X-100. After incubation for 30 min at room temperature, the extracts were centrifuged at 2,000 × *g* for 15 min at 4°C to remove cell debris and nuclei. The supernatants were then used as labeled cell membrane protein preparations and precleared and immunoprecipitated as described above for metabolically labeled cells.

Tryptic peptide fingerprints. Methionine-containing tryptic peptides from the gp70's of F-MuLV clone 201 and F-MCF-1 viruses as well as from the gp52 encoded for by SFFV were obtained by labeling virus-infected mink or NRK cells with [^{35}S]methionine and identifying particular bands by immune precipitation, electrophoresis, and autoradiography. Bands were cut from the gels, eluted with diphenyl carbamyl chloride-trypsin (Sigma Chemical Co.), and subjected to two-dimentional peptide analysis on cellulose thin-layer plates as previously described (30).

RESULTS

Competition radioimmunoassays with MCF-specific antisera: limiting antibody studies. In our previous manuscript, we developed an MCF gp70-specific immunoassay with iodinated gp70 from an F- or M-MCF virus and an antiserum made in rabbits against rabbit cells infected with F-MCF MuLV (21). This antiserum was useful at the time in allowing us to detect for the first time an MCF-related antigen encoded for by SFFV. We have subsequently attempted to produce sera which are also MCF specific but react better with the SFFV MCF-related protein. This was done by transplanting into Osborne-Mendel rats NRK cells nonproductively infected with SFFV-NRK clone 1. Sera from the majority of these rats contain precipitating antibodies to the iodinated gp70's of F- or M-MCF viruses but not to the gp70's from ecotropic F-MuLV or M-MuLV (data not shown). When such sera were utilized with MCF gp70's in a competition radioimmunoassay, they were found to be even more discriminating than the previously described rabbit anti-MCF sera. As shown in Fig. 1A, F- and M-MCF viruses compete completely in an assay with iodinated F-MCF gp70 and a limiting dilution of rat anti-SFFV-NRK serum; ecotropic and xenotropic viruses react little, if at all.

To determine whether the anti-SFFV-NRK rat sera would react with proteins coded for by SFFV, NRK cells nonproductively infected with SFFV were compared with uninfected NRK cells as competing antigens in the same competition immunoassay as described above. As shown in Fig. 1B, extracts of the SFFV nonproducer cells are clearly competitive, competing to virtually the same final extent as banded F-MCF virus. Uninfected NRK cells did not compete at all. In contrast, when these same cellular extracts are analyzed in our previously reported MCF immunoassay with antiserum against F-MCF-infected rabbit cells (Fig. 1C), they are only partially competitive. Since MCF viruses compete equally as well in both immunoassays (Fig. 1B and C), it is possible to distinguish cells infected with F- and M-MCF viruses from those infected with SFFV by a combination of the two MCF-specific immunoassays.

Detection of an MCF gp70 cross-reacting protein in spleens of SFFV-infected mice and in erythroleukemic cell lines. In addition to analyzing the rat kidney cells infected with SFFV, the spleens from adult mice infected with the F-MuLV/SFFV complex were also analyzed for the presence of MCF gp70 cross-reacting proteins. The results appear in Fig. 2A. Adult BALB/c mice infected with the F-MuLV/SFFV complex showed markedly enlarged spleens at 14 days post-inoculation, and these spleens competed completely in the MCF gp70 immunoassay with rat anti-SFFV-NRK serum.

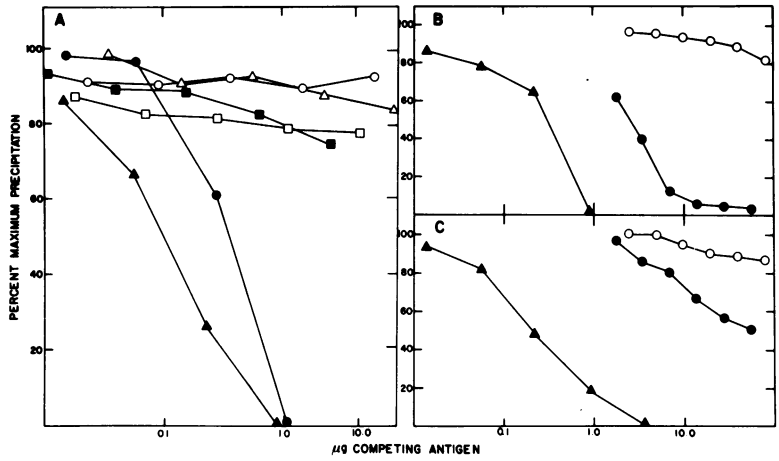

FIG. 1. *Competition radioimmunoassays with F-MCF gp70. (A) Competition radioimmunoassays were carried out as recently described (21) with 50,000 cpm of ^{125}I-labeled F-MCF gp70, a 1:128 dilution of rat anti-SFFV-NRK serum and the following viruses as competing antigens: F-MuLV$_{eco}$ (○); M-MuLV$_{eco}$ (△); NZB xenotropic MuLV (■); BALB:virus-2 xenotropic MuLV(□); Friend MCF MuLV (●); and Moloney MCF MuLV (▲). (B) Competition radioimmunoassays were carried out with ^{125}I-labeled Friend MCF gp70, a 1:128 dilution of rat anti-SFFV-NRK serum, and extracts of SFFV nonproducer NRK cells (●), uninfected NRK cells (○), and F-MCF virus (▲) as competing antigens. (C) Competition radioimmunoassays were carried out with ^{125}I-labeled F-MCF gp70, a 1:400 dilution of rabbit anti-F-MCF serum and SFFV nonproducer cells (●), uninfected NRK cells (○), and F-MCF virus (▲) as competing antigens.*

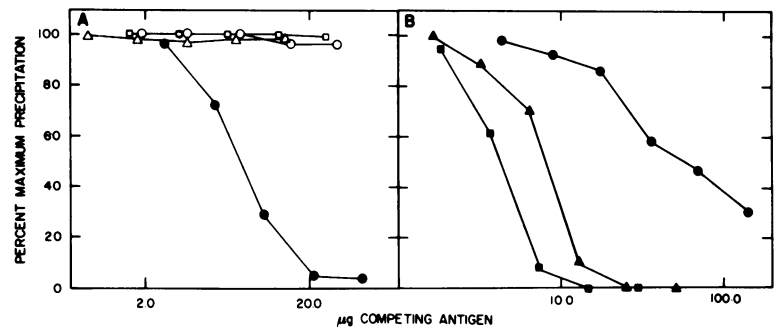

FIG. 2. *Detection of MCF gp70-related antigen in spleens of infected mice and erythroleukemic cell lines. (A) Competition radioimmunoassays were carried out with 50,000 cpm of ^{125}I-labeled MCF gp70, a 1:128 dilution of rat anti-SFFV-NRK serum, and as competing antigens extracts of spleens from 7-week-old BALB/c mice infected 14 days earlier with F-MuLV (○), with an F-MuLV pseudotype of SFFV (●), with an F-MuLV pseudotype of the Kirsten sarcoma virus (△), or uninfected (□). (B) Competition radioimmunoassay with ^{125}I-labeled F-MCF gp70, a 1:128 dilution of rat anti-SFFV-NRK serum, and erythroleukemic cell lines T_3Cl_2 (●), DS19 (■), and DR10 (▲) as competing antigens.*

In contrast, the spleens from uninfected or F-MuLV-infected mice or the enlarged and transformed spleens of mice infected with the F-MuLV pseudotype of the Kirsten sarcoma virus did not compete in the MCF gp70 immunoassay, although the latter spleens had high titers of F-MuLV ecotropic gp70 detectable in parallel immunoassays (data not shown). Like the SFFV-NRK cells shown in Fig. 1C, the spleens from the F-MuLV/SFFV-infected mice competed only partially in the MCF gp70 immunoassay with rabbit anti-MCF serum (data not shown), further suggesting that the MCF gp70 reactivity in these spleens is due to the presence of an SFFV gene product and not solely to the presence of MCF viruses that may have been generated in vivo during the course of the infection.

In addition, several erythroleukemic cells developed from the spleens of F-MuLV/SFFV-infected mice were analyzed for the presence of MCF gp70 cross-reacting proteins. As shown in Fig. 2B, T_3Cl_2, DR10, and DS19 cells all compete effectively in the MCF gp70 immunoassay with rat anti-SFFV/NRK sera. This reactivity appears to be due to the presence of SFFV and not solely to MCF viruses which could potentially

be present in these cells (data not shown).

Sizing of the MCF gp70 immunoreactivity in SFFV-NRK cells and binding to concanavalin A-Sepharose. To estimate the size of the MCF gp70-related reactivity in the NRK cells nonproductively infected with SFFV, extracts of these cells were subjected to guanidine-hydrochloride-agarose chromatography, and the resulting fractions were analyzed in the MCF gp70 immunoassay. The results appear in Fig. 3. The MCF gp70 reactivity in SFFV-NRK clone 1 migrates as a peak in the molecular weight range of 50,000 to 60,000. Recovery of immunoreactivity from the column was in the range of 30 to 50% of that contained in the crude extract.

In addition to analyzing the SFFV nonproducer cells by sizing chromatography, extracts of these cells were also subjected to chromatography on concanavalin A-Sepharose. In data not shown, the MCF gp70-related reactivity in the SFFV nonproducer cells specifically bound to concanavalin A-Sepharose and was eluted with alpha-methyl mannoside, suggesting that the MCF gp70-related 50,000- to 60,000-dalton (50 to 60K) protein in the SFFV nonproducer cells is glycosylated.

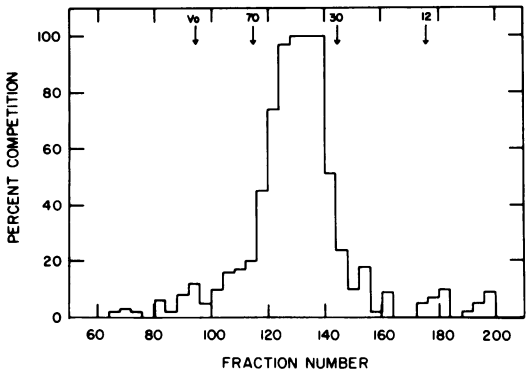

FIG. 3. *Molecular size analysis of MCF gp70 reactivity in SFFV nonproducer NRK cells. A 200-μg amount of an SFFV-NRK cell extract prepared as described for radioimmunoassays (21) was lyophilized and resuspended in 50 mM Tris-hydrochloride, pH 8.0, containing 8 M guanidine-hydrochloride, 1 mM EDTA, and 20 mM dithiothreitol. The sample was heated for 30 min at 37°C and then spun at 15,000 × g for 20 min at 4°C to remove any insoluble material. The sample was then applied to an A-5 agarose column (100 to 200 mesh, 2.5 by 90 cm) equilibrated with 6 M guanidine-hydrochloride, 10 mM dithiothreitol, and 20 mM sodium phosphate buffer, pH 6.5. Fractions (2 ml) were collected and dialyzed against 10 mM potassium phosphate buffer, and 100-μl portions were analyzed in a competition radioimmunoassay with ^{125}I-labeled F-MCF gp70 and a 1:128 dilution of rat anti-SFFV-NRK serum. Molecular weight markers included tracer amounts of ^{125}I-labeled F-MuLV gp70 (70,000), R-MuLV p30 (30,000), and F-MuLV p12 (12,000).*

Effect of pronase on the MCF-specific gp70 reactivity. Since both the MCF gp70 and the SFFV-encoded MCF gp70-related protein are glycosylated, we were interested in determining whether our MCF-specific gp70 immunoassay was detecting antigens present on the protein or the sugar moiety of the glycoproteins. We therefore digested purified F-MCF virus with pronase and tested it in comparison with untreated virus in the MCF gp70 immunoassay with rat anti-SFFV-NRK serum. As a control, F-MuLV was digested with pronase and used as a competing antigen along with the untreated virus in an F-MuLV gp70 type-specific immunoassay. The determinants being detected on the F-MuLV gp70 in this assay have previously been shown to be on the protein part of the molecule and not the carbohydrate (4). The results appear in Fig. 4. Pronase completely destroyed the gp70 immunoreactivities from both F-MCF and Friend ecotropic viruses. Control assays with pronase in the absence of viruses indicate that neither the iodinated gp70's nor the antibodies are being destroyed by residual pronase. The results indicate that glycoproteins competing in the MCF-specific gp70 immunoassay are doing so by virtue of determinants present on the protein moiety of the molecule.

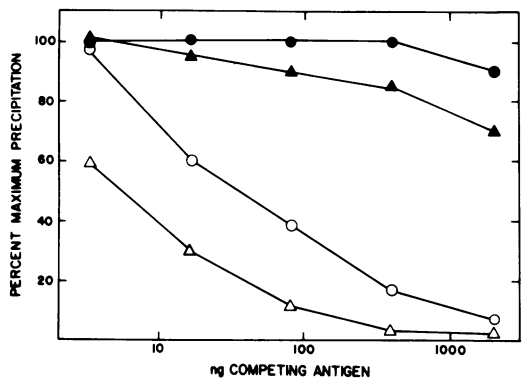

FIG. 4. *Effect of pronase on MCF gp70 reactivity. F-MCF and F-MuLV were digested with freshly prepared self-digested pronase (Sigma Chemical Co.) for 2 h at 37°C with a 1:40 (wt/wt) enzyme to substrate ratio. After incubation, the viruses were diluted in radioimmunoassay buffer for analysis in the appropriate competition radioimmunoassays. A 50,000-cpm quantity of ^{125}I-labeled F-MuLV gp70 and a 1:51,200 dilution of goat anti-F-MuLV serum were used to test untreated F-MuLV (△) or pronase-treated F-MuLV (▲) as competing antigens. A 50,000-cpm quantity of ^{125}I-F-MCF gp70 and a 1:128 dilution of rat anti-SFFV-NRK serum were used to test untreated F-MCF (○) or pronase-treated F-MCF (●) as competing antigens. Pronase incubated without virus and then tested at concentrations comparable to pronase-treated viruses had no effect on the maximum immune precipitation in either assay.*

Precipitation of pulse-labeled SFFV nonproducer cells by various antisera: antibody excess studies. To further characterize the MCF gp70-related reactivity in the SFFV-NRK nonproducer cells, these cells were pulse-labeled with [^{35}S]methionine, and the labeled proteins were then immune precipitated with various anti-gp70 sera. Controls included uninfected NRK cells as well as NRK cells infected with ecotropic F-MuLV. The resulting precipitates were subjected to electrophoresis on SDS-polyacrylamide gels, and autoradiographs of these gels appear in Fig. 5. As shown in Fig. 5A, a number of goat sera prepared against MuLV gp70's were employed to precipitate extracts of pulse-labeled cells. Goat antiserum prepared against M-MCF gp70, which has been shown by radioimmune precipitation to contain both anti-ecotropic and anti-MCF gp70 reactivities, immunoprecipitates the F-MuLV Pr85^{env} (lane 1) as well as a 52K protein present only in the SFFV-NRK cell (lane 2). When this serum is absorbed with ecotropic M-MuLV, it is MCF specific and precipitates only the 52K protein in the SFFV-NRK cell (lane 5) and not the F-MuLV Pr85^{env} (lane 4). Antiserum obtained from Roger Wilsnack and prepared in goats against the gp70 of an R-MuLV with an unknown pedigree precipitates both the F-MuLV Pr85^{env} (lane 7) and the SFFV p52 (lane 8). In studies not presented, antisera prepared in goats against AKR-MuLV, wild mouse ecotropic MuLV, or feline leukemia virus failed to precipitate the 52K protein from SFFV-NRK cells, whereas sera prepared against NZB or BALB:virus-2 xenotropic viruses were able to do so.

To further characterize the antibodies in the anti-R-MuLV gp70 serum that are precipitating the SFFV p52, competition assays were carried out with [^{35}S]methionine-labeled SFFV-NRK cell extracts, a limiting dilution of anti-R-MuLV gp70 serum, and various concentrations of F-MCF, F-MuLV, and NZB xenotropic viruses. As shown in Fig. 5B, both the MCF (lanes 2 to 4) and NZB (lanes 8 to 10) viruses are able to compete out the reactivity of the anti-R-MuLV gp70 serum for the 52K SFFV-specific protein to a much greater extent than F-MuLV (lanes 5 to 7), suggesting that the serum is reacting with the SFFV protein primarily by virtue of MCF-specific or xenotropic-specific antibodies present in the serum.

In addition to goat anti-gp70 sera, we also tested the earlier described rat anti-SFFV-NRK sera against [^{35}S]methionine-labeled SFFV-NRK cells. As shown in Fig. 5C, two such sera specifically precipitate a 52K protein as well as a 45K protein from SFFV-NRK cell extracts (lanes 1 and 3). The 45K protein is also precipitated by goat anti-R-MuLV p12 serum (data not shown) and appears to be related to the previously described SFFV-encoded, p12-containing polyprotein (2).

Precipitation of different pulse-labeled

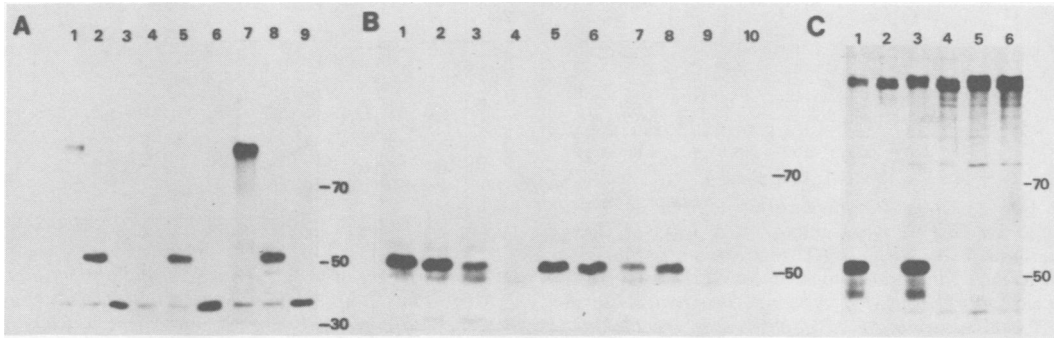

FIG. 5. *Autoradiographs of [^{35}S]methionine-labeled SFFV-nonproducer cell extracts precipitated with various sera. Cells were pulsed with [^{35}S]methionine and then immune precipitated as follows. (A) Goat anti-M-MCF gp70 serum to precipitate F-MuLV/NRK cells (lane 1), SFFV-NRK cells (lane 2), and NRK cells (lane 3); goat anti-M-MCF gp70 serum absorbed with ecotropic M-MuLV to precipitate F-MuLV/NRK (lane 4), SFFV-NRK (lane 5), and NRK (lane 6); and goat anti-R-MuLV gp70 serum to precipitate F-MuLV/NRK (lane 7), SFFV-NRK (lane 8), and NRK (lane 9). (B) Competition assays were set up by reacting 1% Trition X-100-disrupted virus preparations with a limiting dilution (1:3,200) of goat anti-R-MuLV gp70 serum for 2 h at 4°C and then performing immune precipitation with [^{35}S]methionine-labeled SFFV-NRK cells. Competing antigens were F-MCF virus at 1 μg (lane 2), 10 μg (lane 3), and 100 μg (lane 4); F-MuLV at 1 μg (lane 5), 10 μg (lane 6) and 100 μg (lane 7); and NZB xenotropic virus at 1 μg (lane 8), 10 μg (lane 9), and 100 μg (lane 10). Lane 1 represents precipitation in the absence of any added competing antigen. (C) Rat anti-SFFV-NRK sera precipitating SFFV-NRK cells (lanes 1 and 3) and NRK cells (lanes 2 and 4); and normal rat serum precipitating SFFV-NRK (lane 5) and NRK cells (lane 6).*

SFFV-infected cells by MCF-specific antisera. To demonstrate that the presence of the MCF gp70-related p52 segregates with the presence of SFFV in a given cell, a variety of cells infected with SFFV were pulse-labeled with [^{35}S]methionine and then immune precipitated with MCF-specific serum as well as with serum having reactivity with both ecotropic F-MuLV and MCF gp70's. The same cells infected either with F-MuLV or F-MCF viruses were also labeled for comparative purposes. The results appear in Fig. 6A. A 52K protein precipitated with MCF-specific antiserum is present in NRK, FRE, and mink cells infected with SFFV (lanes 2b, 4b, and 7b). NRK cells infected with F-MuLV contain a Pr85^{env} (lane 1a), whereas mink cells infected with F-MCF virus contain a presumptive Pr80^{env} (lanes 6a and b) which is precipitable with anti-MCF gp70 serum. Neither cell contains a p52 unless infected with SFFV. Mink cells transformed by the Kirsten sarcoma virus contain no specific proteins precipitable by anti-MCF gp70 serum (lanes 8a and b).

In addition to fibroblasts infected with SFFV, erythroleukemic DS19 cells were pulsed with [^{35}S]methionine and then immune precipitated with MCF-specific sera. As shown in Fig. 6B, unabsorbed anti-M-MCF gp70 serum precipitates both the F-MuLV Pr85^{env} and the SFFV p52 (lane 1), whereas the MCF-specific gp70 serum precipitates only the SFFV p52 (lane 7). Since these erythroleukemic cells can be induced to differentiate in the presence of various inducers, including hexamethylenebisacetamide, we sought to determine whether there was any change in the synthesis of the SFFV p52 at various times after induction. As shown in lanes 2 to 6 and 8 to 12, both the F-MuLV Pr85^{env} and the SFFV p52 continue to be synthesized throughout the induction period, even as late as 96 h when the majority of the cells have been induced to synthesize hemoglobin.

To demonstrate that the 52K SFFV-encoded protein was glycosylated, as suggested by the binding of the SFFV MCF gp70-related protein to concanavalin A-Sepharose, NRK cells infected with F-MuLV or nonproductively infected with SFFV were pulse-labeled for 2 h with [^{3}H]mannose and then immune precipitated with anti-M-MCF gp70 serum. The results appear in Fig. 6C. This serum precipitates a 70K glycosylated protein from F-MuLV/NRK cells (lane 1) as well as a 52K protein from mannose-labeled SFFV-NRK cells (lane 2), confirming the glycoprotein nature of this SFFV-encoded protein.

Analysis of SFFV gp52 by pulse-chase and extracellular protein studies. Since the SFFV gp52 is related to the MCF gp70, we were interested in determining whether, like the envelope gene product of helper-independent viruses, the gp52 chases into a lower-molecular-weight protein in nonproducer cells and whether this protein eventually leaves the cell to enter into extracellular fluids and/or virus when the nonproducer cells are superinfected with helper

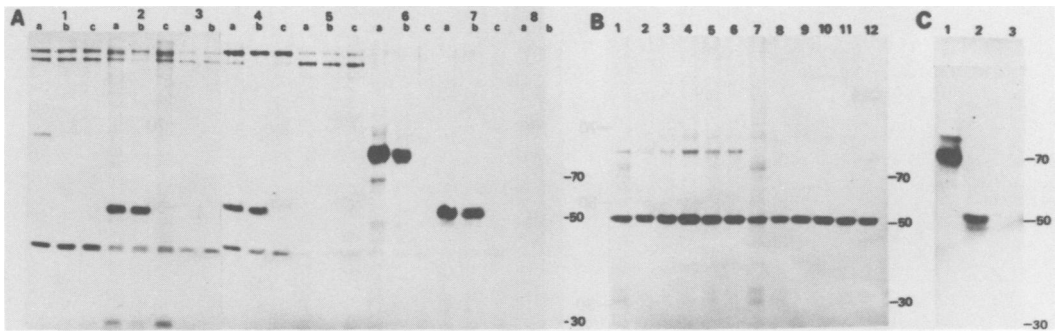

FIG. 6. *Autoradiographs of immune precipitates of [^{35}S]methionine- and [^{3}H]mannose-labeled cell extracts. (A) The following cell lines were pulsed with [^{35}S]methionine and then immune precipitated with (a) goat anti-M-MCF gp70 serum, (b) goat anti-M-MCF serum absorbed with ecotropic M-MuLV, and (c) normal goat serum: F-MuLV/NRK (lane 1), SFFV-NRK (lane 2), NRK (lane 3), SFFV-FRE (lane 4), FRE (lane 5), F-MCF/mink (lane 6), M55/SFFV-mink (lane 7), and M55/Ki-mink (lane 8). (B) DS19 erythroleukemic cells were pulsed with [^{35}S]methionine at various times after induction with 5 mM of the differentiation inducer hexamethylenebisacetamide, and the extracts were immune precipitated as follows: goat anti-M-MCF gp70 serum to precipitate DS19 cells at 0 h (lane 1), 16 h (lane 2), 24 h (lane 3), 48 h (lane 4), 72 h (lane 5), and 96 h (lane 6) after induction; and goat anti-M-MCF gp70 serum absorbed with ecotropic M-MuLV to precipitate DS19 cells at 0 h (lane 7), 16 h (lane 8), 24 h (lane 9), 48 h (lane 10), 72 h (lane 11), and 96 h (lane 12) after induction. Normal goat serum did not precipitate any major proteins. (C) The following cells were pulsed for 2 h with [^{3}H]mannose and then immune precipitated with goat anti-M-MCF gp70 serum: F-MuLV/NRK (lane 1), SFFV-NRK (lane 2), and NRK (lane 3).*

virus. To do this, we first examined SFFV nonproducer NRK cells compared with F-MuLV-infected NRK cells that had been pulsed for 30 min and then chased for various periods of time. As shown in Fig. 7A, although the $Pr85^{env}$ from F-MuLV chases into a 70K protein (lanes 1 to 4), the gp52 present in SFFV nonproducer cells changes molecular weight only slightly during the chase (lanes 5 to 8), resulting in a protein with a molecular weight of approximately 50,000. To determine whether the gp52 eventually enters the extracellular fluid, F-MuLV-superinfected SFFV nonproducer cells were pulsed for 30 min, and the supernatants were examined after chasing for various periods of time. As shown in Fig. 7B, F-MuLV gp70 is readily detectable in the supernatant after an 18-h chase (lane 1), whereas no MCF gp70-specific protein is detected at any molecular weight region of the gel (lane 3) when compared with uninfected NIH 3T3 cells (lane 4), whether the cells were chased for 30 min, 1 h, or 18 h (only 18-h data shown). Importantly, both the gp52 (lanes 6 and 8) and the F-MuLV gp70 (lane 6) are present intracellularly in these same cells after a 30-min pulse. In other studies not shown, extracts of 1,000-fold-concentrated supernatants from F-MuLV-superinfected SFFV-FRE cells were examined by competition radioimmunoassay for both MCF gp70 and F-MuLV gp70 reactivity. Consistent with the pulse-chase data, only the F-MuLV gp70 could be detected in the supernatants from these cells.

Tryptic peptide comparison of SFFV gp52 with F-MCF and F-MuLV gp70's. Since both the SFFV gp52 and the F-MCF gp70 are precipitated with the MCF-specific antisera, the proteins should be expected to show some chemical similarities. Thus, the [^{35}S]methionine-labeled bands precipitated from the SFFV nonproducer cells and the F-MCF-infected cells by MCF-specific sera were eluted from the gels and compared by tryptic fingerprinting analysis. The results appear in Fig. 8. As expected, the SFFV gp52 (Fig. 8B) and the F-MCF gp70 (Fig. 8A) share a number of tryptic peptides (spots B1 and A1, B4 and A2, B7 and A7, B8 and A6, B9 and A9, and B10 and A15). Both glycoproteins, however, have peptides unique to themselves (for example, spots 3, 5, and 6 in the SFFV gp52), consistent with the size difference in the two proteins as well as antigenic differences detected in immunoassays. In contrast to its relatedness with the F-MCF gp70, the SFFV gp52 shares little similarity in tryptic peptides with the gp70 of ecotropic F-MuLV (Fig. 8C). Although a few of the F-MuLV gp70 spots are present in the gp52 (spots 1 and 2), the majority of the F-MuLV gp70 spots are absent.

Detection of an MCF-gp70-related protein on the surface of SFFV nonproducer NRK cells and erythroleukemic cells. To

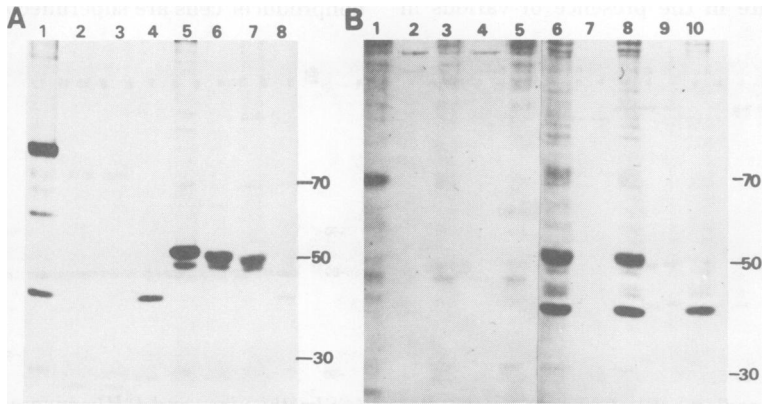

FIG. 7. *Autoradiographs of immune precipitates of pulsed and chased SFFV-infected cell lysates and extracellular fluids. (A) SFFV-NRK and control cells were pulse-labeled for 30 min with [^{35}S]methionine and then chased in isotope-free medium for the indicated periods of time. Extracts were then immune precipitated with goat anti-M-MCF gp70 serum. F-MuLV/NRK cells chased for 0 h (lane 1), 2 h (lane 2), 4 h (lane 3) and 18 h (lane 4); SFFV-NRK cells chased for 0 h (lane 5), 2 h (lane 6), 4 h (lane 7), and 18 h (lane 8). (B) LLV/SFFV-P-NIH and NIH 3T3 cells were pulsed for 30 min with [^{35}S]methionine and then chased for 30 min or for 18 h. Both the cells (after a 30-min chase) and the extracellular fluids (after an 18-h chase) were analyzed by immune precipitation and autoradiography as follows: goat anti-M-MCF gp70 serum to precipitate LLV/SFFV-P-NIH extracellular fluids (lane 1) or cells (lane 6) and NIH 3T3 extracellular fluids (lane 2) or cells (lane 7); goat anti-M-MCF gp70 serum absorbed with ecotropic M-MuLV to precipitate LLV/SFFV-P-NIH extracellular fluids (lane 3) or cells (lane 8) and NIH 3T3 extracellular fluids (lane 4) or cells (lane 9); and normal goat serum to precipitate LLV/SFFV-P-NIH extracellular fluids (lane 5) or cells (lane 10).*

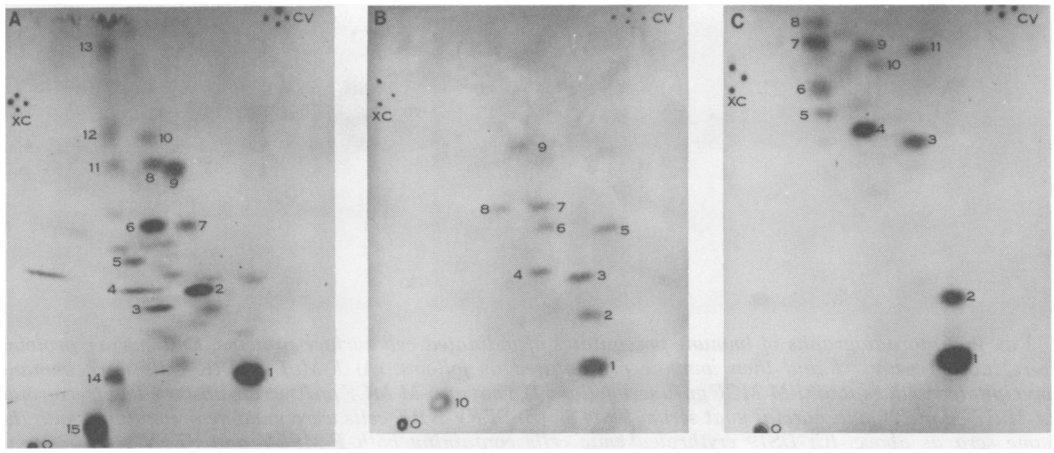

FIG. 8. *Fingerprint analysis of the peptides of F-MCF and F-MuLV gp70's compared with SFFV gp52. NRK cells infected with F-MuLV or nonproductively with SFFV as well as mink cells infected with F-MCF-1 were pulse-labeled with [^{35}S]methionine. The gp70's of F-MuLV and F-MCF and the gp52 of SFFV were identified by immune precipitation with goat anti-M-MCF gp70 serum and autoradiography and then cut from the gels, digested with trypsin, and subjected to two-dimensional peptide analysis as described in the text. A 0 indicates the origin, and the dotted upper circles indicate the dye markers xylene cyanol (XC) and crystal violet (CV) included in each sample run. (A) F-MCF gp70; (B) SFFV gp52; (C) F-MuLV gp70.*

determine whether the MCF-related SFFV protein is present on the surface of cells nonproductively infected with SFFV, the surface of SFFV-NRK cells was iodinated with the lactoperoxidase method, and the labeled extract was immune precipitated with anti-MCF gp70 serum. The results appear in Fig. 9A and B. Unabsorbed goat anti-M-MCF gp70 serum precipitates a 60K protein from the surface of SFFV-NRK nonproducer cells (Fig. 9B, lane 1). This surface protein is somewhat smaller than the 70K protein precipitated from the surface of NRK cells infected with F-MuLV (Fig. 9A, lane 1). Neither of these proteins is present on the surface of uninfected NRK cells (data not shown). Ecotropic M-MuLV-absorbed goat anti-M-MCF gp70 serum precipitates only the 60K SFFV-encoded (Fig. 9B, lane 2), demonstrating that this cell surface antigen, like the gp52 in the cytoplasm of the SFFV-NRK cell, contains MCF gp70-related determinants.

In addition to fibroblasts nonproductively infected with SFFV, we also analyzed the surface of the F-MuLV- and SFFV-producing DS19 erythroleukemic cell line. As shown in Fig. 9C, unabsorbed goat anti-M-MCF gp70 serum precipitates two bands, a 70K protein as well as a 60K protein, from the surface of these erythroleukemic cells. Both proteins are present on the surface of uninduced cells (lane 1) as well as cells that have been induced to differentiate in the presence of hexamethylenebisacetamide (lane 2), with the expression of the 60K protein being somewhat greater in the induced cells. Ecotropic M-MuLV-absorbed anti-M-MCF gp70 serum still precipitates the 70K protein in this cell as well as the 60K protein (lanes 3 and 4). Since this same absorbed serum fails to precipitate the F-MuLV gp70 on the surface of F-MuLV/NRK cells (Fig. 9A, lane 2), it seems likely that the 70K proteins in lanes 3 and 4 of Fig. 9C are either derived from the gp70 of an endogenous xenotropic virus or an MCF virus also present in this mouse erythroleukemic cell line. Based on the results in Fig. 9B as well as those in Fig. 9C, the 60K protein is most likely the MCF gp70-related, SFFV-encoded protein.

DISCUSSION

Earlier work from this laboratory has demonstrated that SFFV is a recombinant virus whose genome is derived from portions of ecotropic F-MuLV and portions of the envelope gene of a murine xenotropic virus (28, 29). Recently, we developed a radioimmunoassay specific for the gp70 of MCF MuLV and demonstrated an antigen in nonproducer cells infected with SFFV that cross-reacts specifically in the MCF gp70 radioimmunoassay (21). In this manuscript, we describe results characterizing this MCF cross-reactive antigen with a variation of our original MCF gp70 radioimmunoassay as well as metabolic labeling of SFFV-containing nonproducer cells. The radioimmunoassays measure steady-state levels of antigen with limiting dilutions of antibody, whereas the pulse-labeling studies measure newly synthesized molecules with conditions of antibody excess. In the

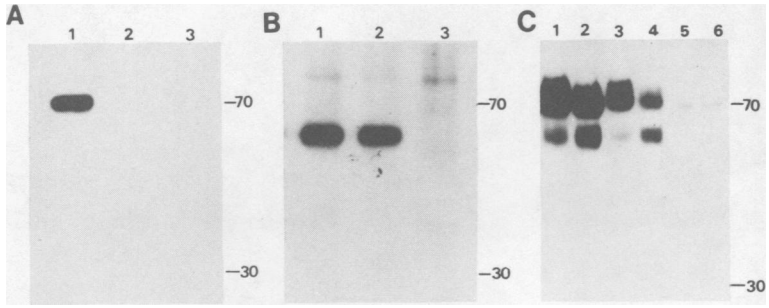

FIG. 9. *Autoradiographs of immune precipitates of iodinated cell surface proteins. Cell surface proteins were labeled with ^{125}I and then immune precipitated as follows. (A) F-MuLV/NRK cells were immune precipitated with goat anti-M-MCF gp70 serum (lane 1), goat anti-M-MCF gp70 serum absorbed with ecotropic M-MuLV (lane 2), and normal goat serum (lane 3). (B) SFFV-NRK cells were immune precipitated with the same sera as above. (C) DS19 erythroleukemic cells containing both F-MuLV and SFFV were immune precipitated as follows: goat anti-M-MCF gp70 serum to precipitate uninduced cells (lane 1) and hexamethylenebisacetamide-induced cells (lane 2); goat anti-M-MCF gp70 serum absorbed with ecotropic M-MuLV to precipitate uninduced cells (lane 3) and hexamethylenebisacetamide-induced cells (lane 4); and normal goat serum to precipitate uninduced cells (lane 5) and hexamethylenebisacetamide-induced cells (lane 6).*

SFFV-containing nonproducer rat cell, we have characterized the antigen extensively by both assays and the techniques that can be employed with each of them. First, a new MCF gp70 immunoassay has been developed with rat sera from rats transplanted with SFFV-containing rat nonproducer cells. This assay detects protein determinants on the MCF gp70 molecules and detects more readily the SFFV cross-reacting protein. The SFFV antigen elutes on a guanidine-hydrochloride–agarose column at a molecular weight of 50,000 to 60,000 and binds specifically to concanavalin A–Sepharose. In the pulse-labeling studies, the SFFV antigen labels with [^{35}S]methionine and [^{3}H]mannose and migrates after immunoprecipitation at a molecular weight of 52,000 on SDS gels. This gp52 shares several methionine-containing tryptic peptides with the gp70 of an F-MCF virus and is accessible to iodination with lactoperoxidase on the surface of SFFV-NRK nonproducer cells. In addition to detecting this SFFV-specific protein in infected fibroblasts, we have also detected the same protein in the cytoplasm of three different erythroleukemic cell lines as well as on the cell surface of one of them and in the spleens of mice newly infected with SFFV. We therefore conclude that SFFV codes for a 52K glycoprotein which shares immunochemical homology to the protein portion of MCF gp70 molecules.

Our results with metabolic labeling and immune precipitation in antibody excess are very similar to those obtained by Racevskis and Koch (17, 18) with a number of erythroleukemic cell lines. Using antiserum against R-MuLV gp70, they were able to precipitate a gp55 in erythroleukemic cell lines that contain both F-MuLV and SFFV but not in cells containing only R-MuLV. They therefore speculated that the gp55 might be a translational product of the RNA of SFFV. Our data would confirm their speculation. They also reported that the gp55 in the erythroleukemic cell lines was stable for up to 24 h and did not chase either into a lower-molecular-weight protein or into the cell-free culture medium or virus. Our observations with SFFV nonproducer cells are completely consistent with their earlier work on erythroleukemic cell lines carrying the SFFV genome.

Our observation that pronase virtually destroyed the antigenic reactivity of the MCF gp70 is strong evidence that the MCF gp70 determinant(s) being measured in the radioimmunoassays described is present on the protein part of the molecule. By extrapolation, we can also conclude that the MCF gp70-related determinants present in SFFV-infected cells are also proteinatious. Preliminary data from our laboratory with endoglycosidase-treated F-MCF gp70 as well as endoglycosidase and 2-deoxy-D-glucose-treated, [^{35}S]methionine-labeled SFFV-NRK cell extracts are consistent with the conclusion that the carbohydrate moieties of neither the MCF gp70's nor the SFFV gp52 are involved in the reactivity with the MCF-specific gp70 sera described. These data are consistent with previous conclusions reached on the antigenicity of F-MuLV gp70 (4).

The presence of an MCF cross-reacting protein on the surface of both SFFV nonproducer cells and erythroleukemic cell lines is consistent with data obtained by Gillis and his co-workers. Using immune lymphocytes from mice injected with syngeneic SFFV nonproducer cells, they

were able to demonstrate specific cytotoxicity against a variety of SFFV-containing cells (8), suggesting that the targets of the immune lymphocytes are SFFV-specific antigens on the cell surface. In more recent studies in cooperation with our laboratory, they were able to show that this specific lymphocyte cytotoxicity could be blocked by MCF gp70-specific antisera but not by normal serum or serum to "*gag*"-related proteins of F-MuLV (Gillis et al., Virology, in press). It therefore appears likely that the target of these immune lymphocytes is the MCF gp70-related antigen that we can detect on the surface of SFFV-infected cells.

Although both the SFFV gp52 and the MCF gp70 contain cross-reacting determinants, there are a number of biological and molecular differences that are now apparent between these viruses. Biologically, after injecting comparable pseudotypes of SFFV and F-MCF viruses into mice, SFFV is much more leukemogenic (D. Troxler, unpublished data). On a more molecular level, the F-MCF virus is a typical helper-independent virus coding for the *gag*-related proteins, polymerase, and envelope proteins, whereas SFFV is replication defective and codes for a still poorly characterized polyprotein linked to p15 and/or p12 (2) as well as the gp52 which shares cross-reactivity with the MCF envelope gene product. The envelope gene products have a number of differences in addition to their different molecular weights. First of all, the two proteins can be distinguished by their differential reactivity in immunoassays employing F-MCF gp70 and either rabbit anti-MCF serum or rat anti-SFFV-NRK serum. Second, the MCF gp70 possesses all of the antigenic determinants usually associated with gp70's (type, group, and interspecies), allowing for the detection of this protein in a wide range of immunoassays. In contrast, the SFFV gp52, while containing MCF type-specific determinants, appears to lack both the predominant group and interspecies determinants that are present on MCF gp70's (21). Finally, tryptic peptide analysis of the SFFV gp52 and the F-MCF gp70 demonstrates that these proteins contain common as well as unique methionine-containing tryptic peptides. Although the origin of the unique peptides in the SFFV gp52 is presently unknown, several possibilities exist. First of all, these unique peptides could simply be a consequence of diversity resulting from different recombinational events in the *env* gene, which would yield proteins with similar as well as unique peptides. In this model, the gp52 unique peptides would still be derived from a product of an envelope gene. Alternatively, the gp52 could represent a fusion product coded for by a gene in SFFV which originated by recombination between MCF *env* gene sequences and some other unique genetic sequence unrelated to the *env* gene of any MCF virus. In this case, SFFV would potentially represent a double recombinant, with an F-MCF virus being its parent. The gp52 would then be an *env*-linked polyprotein analogous to the *gag*-linked polyprotein of such other defective leukemia viruses as Abelson (31) and MC-29 (P. K. Vogt, K. Bister, S. S. F. Hu, and M. J. Hayman, *In* Y. Ikawa, ed., *Oncogenic Viruses and Host Cell Genes,* in press). Which of the differences between MCF viruses and SFFV accounts for the increased leukemogenicity of SFFV is unknown. However, it is striking that the MCF gp70-related gp52 accumulates intracellularly and is not released from cells as is the gp70 of helper-independent MCF viruses. It is tempting to speculate that this property of the gp52, acquired by recombination within the envelope gene, may play an important role in the pathological potential of SFFV. Further studies on the genetics of SFFV and the characterization and metabolism of its proteins are in progress in an attempt to test this speculation.

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