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Glycoprotein Encoded by the Friend Spleen Focus-Forming Virus

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The Friend spleen focus-forming virus (F-SFFV) released from cultured erythroleukemia cells (cell line F4-6/K) was cloned free of its helper lymphatic leukemia virus (F-MuLV). After allowing adsorption to Sc-1 fibroblasts at a low multiplicity of infection, the cells were seeded individually into wells of a microtiter test plate and the resulting colonies were grown into large cultures. Among 14 of these cell cultures that have been analyzed thoroughly, 6 contained F-SFFV alone, 1 contained F-MuLV plus F-SFFV, and 7 were uninfected. Each of the Sc-1 cell lines which had been infected with cloned F-SFFV contained a glycoprotein with an apparent molecular weight of 55,000 (gp55) that was absent from the cell lines that lacked F-SFFV. gp55 was also present in Friend erythroleukemia cells and in fibroblasts infected with an F-SFFV that had been doubly cloned in another laboratory. These results indicate that gp55 is encoded by the F-SFFV genome. gp55 has the following additional properties. It can be immunoprecipitated with antiserum made to the F-MuLV virion envelope glycoprotein (gp75). Its unglycosylated polypeptide, formed in cells treated with 2-deoxy-D-glucose, has a molecular weight of approximately 45,000. Its tryptic peptide map contains peptides in common with F-MuLV gp75 but it also contains unique peptides. It appears to be absent or present in only low concentrations in erythroleukemia cell plasma membranes as determined by lactoperoxidase-catalyzed iodination, and it accumulates intracellularly in large amounts. In addition, it is absent from released virions. The majority of the cellular gp55 has an isoelectric point of 8.5 to 9.0. These results are consistent with the idea that an *env* gene recombination event was involved in the origin of F-SFFV.

The Friend strain of murine acute erythroleukemia virus consists of at least two components. One is a replication-defective virus which is responsible for the rapid transformation of erythropoietic stem cells in vivo (1, 18, 44) or in cell culture (6, 8). This component is called the spleen focus-forming virus (F-SFFV) because of its ability to induce discrete foci of transformed cells in the spleens of mice which have been innoculated with the Friend virus complex. The other virus component, called the lymphatic leukemia virus (F-MuLV), is a replication-competent helper virus which by itself causes lymphatic leukemia of T-cells after a relatively long, several-month, latent period (7). These two Friend virus components have been clonally separated (4, 44, 48).

Little is known about the mechanism of malignant transformation by the retroviruses which cause leukemia. However, recent studies have indicated that several highly leukemogenic MuLVs may have arisen by recombination between less leukemogenic lymphatic leukemia viruses and unidentified endogenous xenotropic MuLVs (10, 16, 17, 20, 24, 36, 47, 49). For example, the onset of leukemia in mice infected with F-MuLV has been correlated with expression of endogenous xenotropic MuLV and with appearance of recombinants between F-MuLV and an endogenous xenotropic virus (15–17, 48, 49). A similar phenomenon occurs during the development of spontaneous leukemia in AKR mice (20, 24). In all of these cases the evidence suggests that the recombination event has occurred in the genetic region of the two parental virions which encodes their membrane envelope glycoproteins (i.e., their *env* genes) (9, 10, 13, 17, 20, 36, 47, 49). Consequently, it has been suggested that recombinant *env* genes or their encoded glycoproteins might play a causal role in leukemogenesis (10, 16, 20).

In view of the above evidence, it is interesting that Troxler et al. (27, 46, 47) have recently

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obtained evidence based on nucleic acid hybridization analyses that F-SFFV may be an *env* gene recombinant between F-MuLV and an endogenous xenotropic virus. In contrast with early reports (2, 4, 46), a recent radioimmunoassay study has detected envelope glycoprotein-related antigens in nonproducer fibroblasts infected with cloned F-SFFV (38). However, the antigens were not characterized at the metabolic or molecular levels. In addition, there have been reports that the F-SFFV genome encodes a 15,000-dalton protein that may be identical to the p15 protein encoded by F-MuLV (2, 4). In addition, there is evidence that some but apparently not all leukemogenic F-SFFV genomes encode a large polypeptide chain that is immunologically cross-reactive with antiserum made to F-MuLV p12 (2). Furthermore, there is a new membrane antigen on the surface of cells infected with F-SFFV (19).

In this paper we present evidence that F-SFFV encodes a glycoprotein (gp55, a glycoprotein with an apparent molecular weight of 55,000). Although it is structurally related to the F-MuLV membrane envelope glycoprotein gp75, the gp55 molecule has distinctive structural features. Furthermore, gp55 accumulates intracellularly and it is not incorporated extensively into either the cellular membranes or into released virions.

MATERIALS AND METHODS

Cells and viruses. The F4-6 erythroleukemia cell line was generously supplied by W. Ostertag (Max-Planck-Institut fur experimentelle Medizin, 34 Gottingen, Germany). The F4-6/K subline has been grown in suspension culture in this laboratory for several years (43). Sc-1 cells derived from a feral mouse embryo and showing no Fv-1 restriction were provided by contract E-73-2001-NO1 within the Special Virus-Cancer Program, National Institutes of Health, Public Health Service, through the countesy of J. Weaver (Cell Culture Laboratory, University of California School of Public Health, Oakland, Calif.). The Sc-1 cells were maintained in monolayer culture with McCoy modified 5A medium (Grand Island Biological Co.) supplemented with 10% complement-inactivated fetal calf serum and antibiotics (Grand Island Biological Co.).

NRK cells, BALB/c 3T3 cells, and the SFFV nonproducers BALB clone 4 and NRK clone 1 were obtained through the courtesy of D. Troxler (Laboratory of Tumor Virus Genetics, National Cancer Institute, Bethesda, Md.). The derivation of these SFFV nonproducer clones has been described (46, 48). These cells were maintained as monolayers in Dulbecco modified Eagle medium supplemented with 10% complement-inactivated fetal calf serum and antibiotics.

Friend virus (original strain) was obtained from the American Type Culture Collection (Rockville, Md.). The F-MuLV (clone F12) used for cell infections was obtained by cloning the virus from F4-6/K cells. This cloned F-MuLV induces lymphatic leukemia in Swiss mice by 6 months after innoculation (unpublished observation).

Virus assays. The MuLV titer was measured using a variation (43) of the S^+L^- method of Bassin et al. (3).

SFFV was assayed by methods described previously (1, 37, 43). As described elsewhere (43) and below, different clones of F-SFFV differ in their ability to cause macroscopic spleen foci by 9 days after innoculation. Sc-1 cell lines that had been used for F-SFFV cloning were superinfected with cloned F-MuLV (clone F12). A 0.5-ml volume of culture fluid containing rescued virus from exponentially growing cells was subsequently assayed for F-SFFV by inoculation into the tail vein of three different 4- to 6-week-old NIH/Swiss female mice. Spleens were examined 14 to 21 days after injection for macroscopic foci (1) and for enlargement (37). All spleens not showing extensive signs of Friend disease at 21 days were examined histologically (28). The latter diagnosis was based on the presence of foci of large cells with large nuclei having a distinct nuclear membrane with one to two nucleoli and a moderate amount of pale eosinophilic cytoplasm. These foci involved the red pulp as either large nodules or more diffusely.

Virus cloning. Virus was cloned by a modification of previously published methods (4, 48). A monolayer of Sc-1 cells (3×10^5 cells) was inoculated with 0.5 ml of culture medium from F4-6/K cells containing 1.7×10^5 infectious F-MuLV per ml and 25 μg of DEAE-dextran per ml. After 30 min, the culture medium was removed and the cells were incubated for 2 h in fresh medium. The cells were then trypsinized, counted, and diluted to obtain a cell concentration of 5 cells per ml. Two Falcon Microtiter II dishes containing 96 wells per dish were seeded with 0.1 ml of the cell suspension, per well. Wells containing a single cell colony were grown up to form clonal cell lines. Fifty-seven cloned cell lines were thereby obtained, and 16 were selected for more thorough analysis.

Labeling and extraction of cells. F4-6/K cells were preincubated for 10 min in methionine-free minimal essential medium (MEM, Grand Island Biological Co.) and then labeled in methionine-free MEM supplemented with 15 to 100 μCi of L-[^{35}S]methionine per ml. When pulse-chase studies were performed, the labeling medium was replaced with complete medium and incubation was continued for the appropriate time. F4-6/K cells were labeled with D-[^{3}H]glucosamine by adding the isotope directly to a growing culture (17 μCi/ml) for 7 h. Glycosylation inhibition studies were performed by preincubating (2 h) and then labeling (30 min) the cells with L-[^{35}S]methionine in medium containing 15 mM 2-deoxy-D-glucose (Calbiochem). The labeled F4-6/K cells were then lysed in a volume of immune buffer A (0.01 M NaH_2PO_4 [pH 7.6]-0.001 M disodium EDTA-1% Triton X-100-0.5% sodium deoxycholate-0.1% sodium dodecyl sulfate [SDS]) equal to 20% of the volume of the cell culture sample. The cell extracts were centrifuged at 200,000 $\times$ *g* in a Beckman 65 rotor for 20 min.

Monolayer cell cultures were washed twice with phosphate-buffered saline (Grand Island Biological

Co.) and incubated in methionine-free MEM for 10 min prior to labeling. Pulse labeling was performed by adding methionine-free MEM supplemented with L-[^{35}S]methionine (15 to 50 μCi/ml) for the appropriate time. The cells were lysed in immune buffer A, and the cell extracts were centrifuged as described above.

Cell surface radioiodination. Cell surface labeling with [^{125}I]iodine was performed at 0°C by a modification (26) of the method of Vitetta et al. (51). Growing cells were used for the labeling.

Immune precipitation procedure. All samples were precipitated by secondary immune precipitations. Both the antisera and methods used have been thoroughly described (12). The goat antiserum to Rauscher MuLV gp70 was generously provided by J. Gruber (National Cancer Institute, Bethesda, Md.). The goat antiserum to F-MuLV (Eveline strain) envelope glycoprotein was generously given by J. Collins (Duke University, Durham, N.C.).

Electrophoresis. Polyacrylamide gel electrophoresis was performed by a modification of the procedure of Laemmli (25). Immune precipitates were dissolved in sample buffer containing 0.05 M Tris-hydrochloride (pH 6.8), 1% SDS, 1% beta-mercaptoethanol, 20% glycerol, and 6 M urea and heated at 100°C for 90 s before electrophoresis. Ten percent polyacrylamide separating gels were made in 0.1875 M Tris-hydrochloride (pH 8.8) containing 0.1% SDS and 8 M urea. Five percent polyacrylamide stacking gels were made in 0.0625 M Tris-hydrochloride (pH 6.8) containing 0.1% SDS and 6 M urea. Ten to 20% polyacrylamide gradient slab gels containing 0.1% SDS but lacking urea were made using the Laemmli buffer system. It was found that these gradient gels were preferable for separation of gp55 away from nonspecific components. Two-dimensional polyacrylamide gel electrophoresis was performed essentially as described by O'Farrell (30) with minor modification in the first dimension: 9 M urea (ultrapure, Schwarz/Mann), 1 M electrode solutions, and 1% ampholytes (LKB): pH 3.5 to 10/pH 9 to 11/pH 4 to 6/pH 5 to 7 in ratio 14:2:1:1. After electrophoresis, all gels were fixed overnight in 12.5% trichloroacetic acid and processed for fluorography (5). Molecular weights of proteins were estimated by comparing their electrophoretic mobilities in polyacrylamide gels containing SDS with the mobilities of standard proteins of known molecular weights (12).

Peptide maps. Peptide maps of L-[^{35}S]methionine-labeled proteins were performed by a modification of the procedure of Elder et al. (11). Radioactive proteins that had been resolved in a polyacrylamide gradient slab gel (see above) were located by autoradiography, and the gel fragments containing these proteins were subsequently washed and trypsinized in situ as described by Elder et al. (11). The tryptic peptides were analyzed on CEL 300 thin-layer plates (20 by 20 cm, 0.25 mm thick) (Brinkman). The chromatography dimension employed the developing solvent isoamyl alcohol-pyridine-water-ethanol-acetic acid (70:70:60:40:10). Electrophoresis in the second dimension was performed in 0.5% pyridine-5% acetic acid at 220 V for the time required for the fast major dye component of acid fuchsin to migrate 15.5 cm (approximately 7 h). The dried thin-layer plates were processed for fluorography as described by Randerath (34).

RESULTS

gp55 in Friend erythroleukemia cells. As shown in Fig. 1, extracts of L-[^{35}S]methionine-labeled F4-6/K Friend erythroleukemia cells contain three prominent radioactive components that precipitate specifically with monospecific antiserum made to the F-MuLV virion envelope glycoprotein. All of these proteins can also be precipitated by antibody to Rauscher MuLV envelope glycoprotein. Two of these components, gp75 and gPr90^{env} (the biosynthetic precursor of gp75 and p15E) have been well characterized and are known to be encoded by the F-MuLV genome (14, 23, 31, 50, 52; M. J. Murray and D. Kabat, J. Biol. Chem., in press).

The third prominent component in Fig. 1, which has an apparent molecular weight of 55,000 and is also a glycoprotein (see data be-

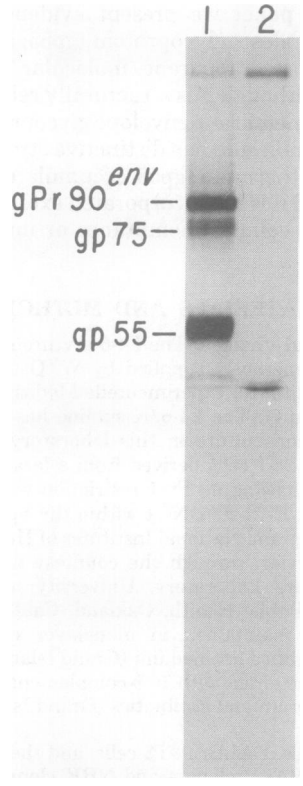

FIG. 1. *Labeling analysis of gp75-specific proteins in F4-6/K cells. F4-6/K cells (10^6 cells/ml) were pulse labeled with [^{35}S]methionine for 1 h. A cellular lysate was immunoprecipitated with antiserum to the F-MuLV envelope glycoprotein (lane 1) and was analyzed by electrophoresis on 10 to 20% polyacrylamide gradient gels containing SDS as described in the text. This gel as well as the other polyacrylamide gels were processed for fluorography (5). Lane 2 is a normal goat serum control of F4-6/K cells.*

low), has only been observed (32, 33, 42) in different erythroleukemia cell lines obtained from the spleens of mice infected with the Friend virus complex. As shown in Fig. 2, gp55 is rapidly labeled during brief exposure of cells to L-[^{35}S]methionine and it only slowly loses its radioactivity during a subsequent cold chase with nonradioactive methionine. This suggests that gp55 may be a primary translation product rather than a derivative of either gPr90^{env} or gp75. Genetic evidence described below supports this interpretation. Furthermore, the results in Fig. 2 are consistent with the idea that gp55 is relatively stable and that it is not rapidly exported from the cells.

Cloning of F-SFFV. To identify the virus component that encodes gp55, we cloned the virus released from the F4-6/K erythroleukemia cells and examined the resulting cell lines for F-SFFV, F-MuLV, and for gp55 synthesis. The F4-6/K virus was adsorbed for 2 h onto Sc-1 fibroblasts at a multiplicity of F-MuLV per cell of approximately 0.3, and the cells were then seeded individually into the wells of a microtiter test plate and grown up to form clonally derived cell lines (see Materials and Methods). Sixteen of these cell lines were then studied more thoroughly, and the results are summarized in Table 1. Fourteen cell lines gave unambiguous results. One of these contained F-MuLV plus F-SFFV, six contained SFFV alone, and the other seven were uninfected. Two other cell lines that contained F-MuLV (clones 3E1 and 3F1) released virus that caused neither macroscopic spleen foci nor enlargement; however, the spleens were histologically ambiguous and may have contained small erythroleukemic foci (see Table 1).

Most of the F-SFFV-containing virus samples that were rescued from the cell lines by F-MuLV superinfection caused a typical rapid Friend disease with marked spleen enlargement by 14 days, as illustrated in Fig. 3C. However, as shown in Fig. 3B, the virus rescued from the 2B1 cell line caused only a few small foci (an average of eight foci per spleen) on the spleen surface after 21 days and no increase in spleen weight as compared with controls (Fig. 3A). Histological examination confirmed the diagnosis of mild Friend erythroleukemia.

gp55 Synthesis in the cell lines containing cloned MuLV and SFFV. Each of the 16 Sc-1 cell lines that had been derived from the F-SFFV cloning experiment was also analyzed for gp55 synthesis. This was more difficult than with erythroleukemia cells because the gp55 was produced in relatively small amounts in the fibroblasts and because there are several Sc-1 cell proteins that co-precipitate nonspecifically with

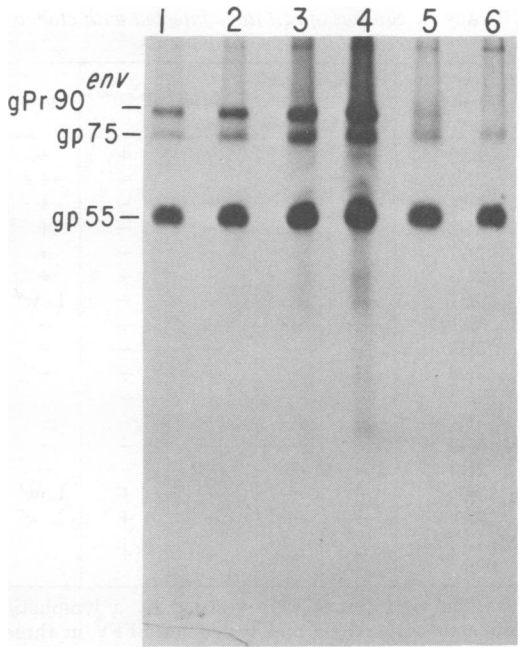

FIG. 2. *Pulse-chase analysis of gp75-specific proteins in F4-6/K cells. F4-6/K cells (10^6) were pulse labeled with [^{35}S]methionine for various time intervals and chased by addition of unlabeled methionine. Samples taken during the experiment were precipitated with antiserum to the F-MuLV envelope glycoprotein and subjected to electrophoresis in 10% polyacrylamide gels containing 8 M urea and SDS. The samples correspond to immunoprecipitates obtained from cells after a (1) 5-min pulse, (2) 10-min pulse, (3) 20-min pulse, (4) 30-min pulse, (5) 30-min pulse and 30-min chase, or (6) 30-min pulse and 120-min chase.*

antigen-antibody complexes. Figure 4 shows a typical electrophoretic analysis of the immunoprecipitates from 10 cloned Sc-1 cell lines. The gp55 band is well separated from contaminating proteins, and its synthesis clearly segregates from the synthesis of gPr90^{env}.

As summarized in Table 1, all of the cell lines that contained F-SFFV also contained gp55, whereas the cell lines that lacked F-SFFV lacked gp55. As expected, the cell lines with F-MuLV all contained gPr90^{env} and gp75. These results, and concordant evidence described below, strongly support the idea that gp55 synthesis segregates with the F-SFFV genome.

We have observed that the amount of gp55 differed in different cell lines that contain F-SFFV. For example, the 2B1 cell line (Fig. 4, lane 2) contained a small amount of gp55 that was clearly seen only after relatively prolonged exposure of the X-ray film. Interestingly, the mild erythroleukemia illustrated in Fig. 3B was

TABLE 1. *Studies of cell lines infected with cloned Friend virus components*

Clone of infected Sc-1 cells	Friend disease[a]		F-MuLV[b]	gPr90^{env}[c]	gp55[c]
	Day 14	Day 21			
2G3	+++		+	+	+
2F2	+++		−	−	+
2E4	+++		−	−	+
3G2	−	++	−	−	+
2A1	−	++	−	−	+
2C5	+++		−	−	+
2B1	−	−+[d]	−	−	Low[d]
3A12	−	−−	−	−	−
3H9	−	−−	−	−	−
3D8	−	−[e]	−	−	−
3G3	−	−−	−	−	−
3F8	−	−−	−	−	−
3G9	−	−−	−	−	−
2B2	−	−−	−	−	−
3E1	−	−−[f]	+	+	Low[f]
3F1	−	−−[f]	+	+	Low[f]
MuLV control	−	−−	+	+	−

[a] The viral clones were rescued by a lymphatic leukemia virus clone and tested for SFFV in three mice (see text). Friend Disease was determined by screening one mouse for splenic foci on day 14. If foci were observed, all three mice were killed and analyzed. If no foci were observed, the remaining mice were kept until day 21. All spleens which showed no foci at 21 days were examined histologically (see text). Each notation (+ or −) indicates the diagnosis of one mouse.

[b] F-MuLV was assayed by the S^+L^- method as described in the text.

[c] gPr90^{env} and gp55 were detected as described in Fig. 4.

[d] gp55 was detected in clone 2B1 only after a longer exposure of the autoradiogram shown in Fig. 4. Only one spleen from the mice injected with SFFV from clone 2B1 showed foci (see Fig. 3). Histological examination confirmed the presence of Friend disease in that spleen.

[e] Data from only two mice are available for clone 3D8.

[f] Clones 3E1 and 3F1 contain gp55 which was detected only after prolonged exposure of the autoradiogram in Fig. 4. Although the 3E1 and 3F1 spleens showed no macroscopic signs of Friend disease, histological examination gave an equivocal diagnosis. In particular, small foci of cells were observed in the red pulp which appeared similar to those of Friend disease (see text). These were, however, difficult to unambiguously distinguish from the hematopoietic foci that occur normally in mouse spleens. We therefore cannot make a positive diagnosis for Friend Disease for these samples.

also caused by virus rescued from this cell line. These results suggest that there may be a correlation between gp55 synthesis and leukemogenic potential of different F-SFFV-infected nonproducer cell lines.

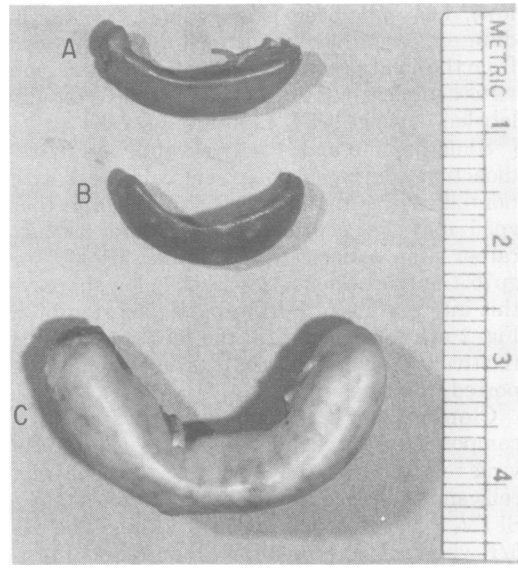

FIG. 3. *Spleens of mice injected with rescued virus from Friend virus clones. Shown above are the spleens of mice injected with clone 2G3 rescued with F-MuLV (C), clone 2B1 rescued with F-MuLV (B), and clone 3G3 rescued with F-MuLV (A). Spleen A was removed at 14 days whereas spleens B and C were removed at 21 days after injection. Histological examination of spleen B confirmed the diagnosis of mild Friend disease.*

Other cell lines infected with Friend virus SFFV. We have examined other cell lines infected with Friend virus. For example, Friend virus (original strain) was used to infect Sc-1 cells. As shown in Fig. 5 (lane 10), this cell line also synthesizes gp55. In addition, we examined two F-SFFV nonproducer clones, BALB clone 4 and NRK clone 1, derived by D. Troxler. The BALB clone 4 cell line was obtained by cloning a Friend virus complex having a B-tropic helper in BALB/3T3 cells (48); and the virus rescued from BALB clone 4 was later cloned a second time to derive NRK clone 1 (46). As shown in Fig. 5 (lanes 6 and 8), the F-SFFV in NRK clone 1 encodes a protein antigenically related to gp75 that is very similar to gp55. BALB clone 4 also contains a similar glycoprotein (data not shown). These results strongly support the idea that gp55 is encoded by the F-SFFV genome.

Careful examination of several gels indicates that the gp55-like protein in NRK clone 1 cells (Fig. 5, lanes 6 and 8) is slightly smaller than the gp55 that is produced in F4-6/K cells (lane 2) or in Friend virus-infected Sc-1 cells (lanes 3 and 10). Similarly, the mature viral envelope glycoprotein gp75 formed in F4-6/K cells is larger than the envelope glycoprotein that is formed in

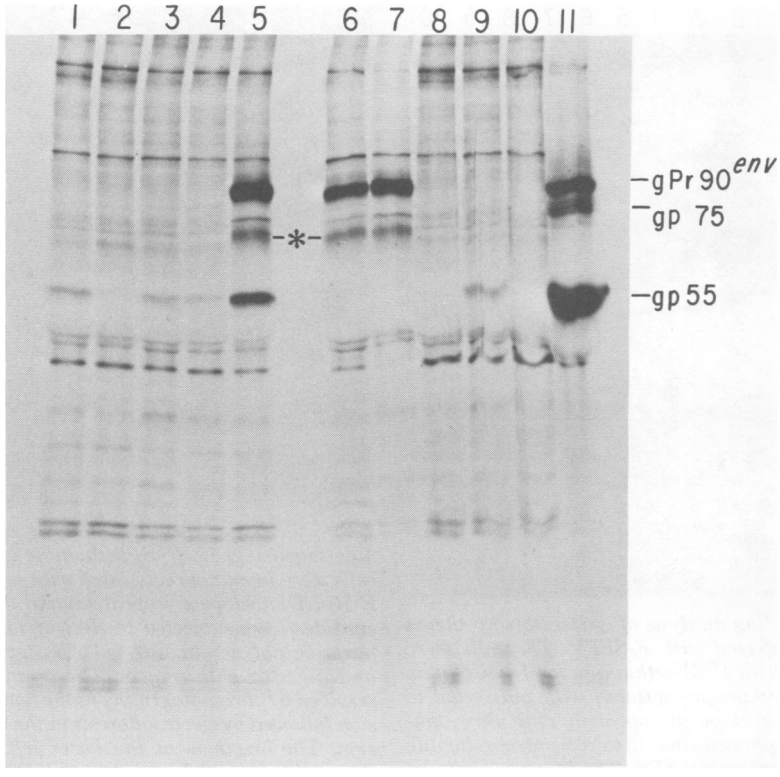

FIG. 4. *Labeling analysis of gp75-related proteins in Sc-1 fibroblasts containing F4-6/K viral clones. The clones were pulse labeled with [^{35}S]methionine for 1 h. Cellular lysates were immunoprecipitated with antisera to the F-MuLV envelope glycoprotein and analyzed on a 10 to 20% polyacrylamide gradient gel containing SDS. The lanes correspond to immunoprecipitates from (1) clone 2A1, (2) clone 2B1, (3) clone 2E4, (4) clone 2F2, (5) clone 2G3, (6) clone 2E1, (7) clone 3F1, (8) clone 3F8, (9) clone 3G2, (10) clone 3G3, and (11) uncloned F4-6/K cells. It is interesting to note that the viral envelope-related protein gp75 from F4-6/K cells migrates at a lower apparent molecular weight when cloned in Sc-1 fibroblasts (compare the gp75 band in lane 11 with the bands in lanes 5, 6, and 7 denoted by the asterisk). This molecular weight difference may be due to different glycosylation processing in the two cell lines. Lanes 2 and 7 contained only a small amount of gp55 that could be seen by relatively prolonged exposure of the X-ray film.*

Sc-1 cells (e.g., compare Fig. 4, lanes 5, 6, and 7, where the glycoprotein is labeled with an asterisk, with the gp75 that occurs in lane 11). These size differences are probably caused by differences in processing (presumably glycosylation) because they depend upon the cell line in which the virus is grown.

Peptide maps of gp55 and gp75. As mentioned above, F-SFFV may be an *env* gene recombinant between F-MuLV and an unidentified xenotropic MuLV (27, 46, 47). Therefore, it is possible that gp55 might contain amino acid sequences corresponding to either or both of these parental type *env* genes. Two-dimensional peptide maps of the L-[^{35}S]methionine-labeled tryptic peptides of gp55 and of gp75 are shown in Fig. 6 and 7, respectively. Both of these glycoproteins contain the peptides A, B, C, and D. However, two peptides in gp55 (E and F) which migrate only in the horizontal chromatographic dimension are absent from gp75. Similarly, gp75 contains several peptides that are absent from gp55 (e.g., G, H, I, J). These results support the idea that gp55 contains amino acid sequences that occur in F-MuLV gp75 and are also consistent with the possibility that it may contain additional amino acid sequences. However, some of the peptide differences between these glycoproteins could be caused by differences in their oligosaccharide moieties (see below).

Processing of the gp55 glycoprotein. The carbohydrate labeling and processing of gp55 were analyzed by several techniques. Figure 8 shows a two-dimensional isoelectric focusing/SDS-polyacrylamide gradient gel electrophoresis analysis of a D-[^{3}H]glucosamine-labeled erythroleukemia cell lysate that had been immunoprecipitated with antibody to gp75. The

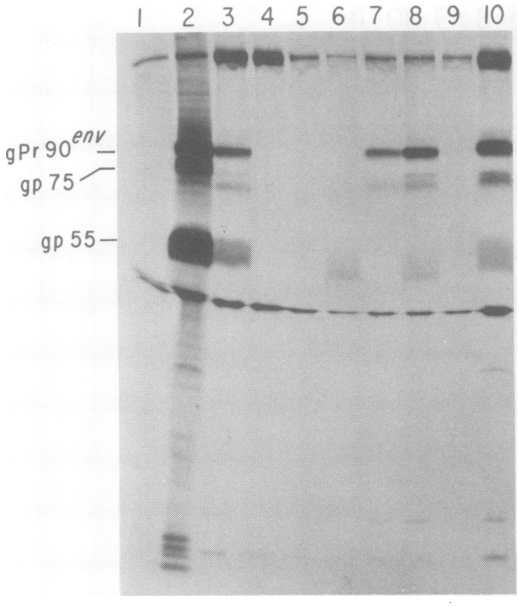

FIG. 5. *Labeling analysis of gp75-related proteins in cell lines infected with F-SFFV. The cells were pulse labeled with [^{35}S]methionine for 1 h. Cellular lysates were immunoprecipitated with antiserum to the F-MuLV envelope glycoprotein and were analyzed by electrophoresis in a 10 to 20% polyacrylamide gradient gel containing SDS. The lanes correspond to (1) normal goat serum control of F4-6/K cells, (2) F4-6/K cells, (3) F4-6/K virus-infected Sc-1 fibroblasts, (4) normal goat serum control of Sc-1 cells, (5) uninfected NRK cells, (6) NRK clone 1 provided by D. Troxler, (7) F-MuLV-infected NRK cells, (8) F-MuLV superinfected in NRK clone 1, (9) normal goat serum control of NRK cells, (10) Friend virus (original strain)-infected Sc-1 fibroblasts.*

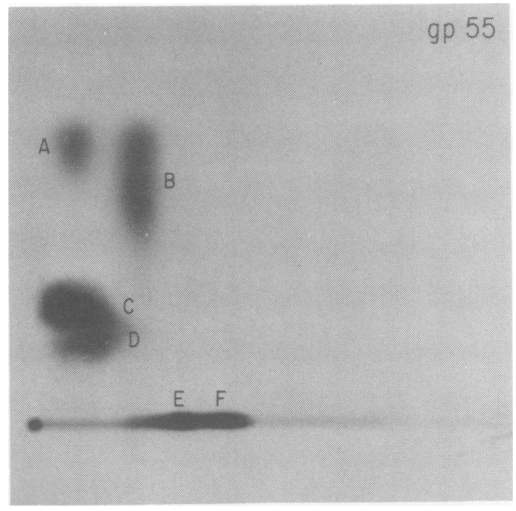

FIG. 6. *Tryptic peptide analysis of [^{35}S]methionine-labeled gp55. [^{35}S]methionine-labeled F4-6/K cells were immunoprecipitated with antiserum to the F-MuLV envelope glycoprotein, and the immunoprecipitates were subjected to electrophoresis on a preparative polyacrylamide gel. The gp55 was eluted and digested with trypsin. The tryptic digests were resolved by chromatography in the horizontal dimension followed by electrophoresis in the vertical dimension. The origin is at the lower left. The peptides detected by autoradiography are labeled for comparison with the gp75 peptides (Fig. 7). Peptides A, B, C, and D are also detected in digests of gp75. Peptides E and F are unique to gp55.*

radioactive gPr90^{env} (component 1) and the row of discrete gp75 components (component 3) that differ in sialic acid content and correspondingly in size have been thoroughly described elsewhere (Murray and Kabat, J. Biol. Chem., in press).

Approximately 90% of the radioactive gp55 usually focuses in the basic region (pH 8.5 to 9.5) of the isoelectric focusing dimension (component 2). However, there is also a row of gp55-related components (components 4) that have a slightly larger size and appear beneath the gp75 components in the more acidic region of the gel. In addition, this row of components 4 seems to extrapolate back to a minor component that has the same isoelectric point as component 2 but a slightly larger size. Preliminary studies using very brief labeling with L-[^{35}S]methionine suggest that component 2 is the primary translational product (unpublished result). Our tenta-

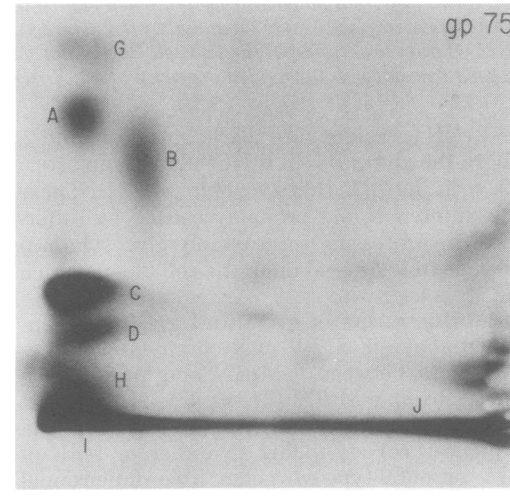

FIG. 7. *Tryptic peptide analysis of [^{35}S]methionine-labeled gp75. The peptides were prepared and resolved by the method used in Fig. 6. Peptides G, H, I, and J are unique to gp75.*

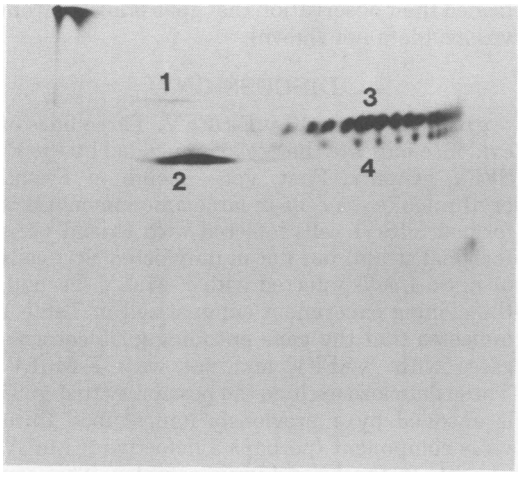

FIG. 8. *Two-dimensional gel electrophoresis of [^{35}H]glucosamine-labeled F4-6/K cells. F4-6/K cells were pulse labeled for 7 h with [^{3}H]glucosamine. The cellular lysates were immunoprecipitated with antiserum to the F-MuLV envelope glycoprotein, and the immunoprecipitates were analyzed by two-dimensional gel electrophoresis as described in the text. Component 1 corresponds to gPr90^{env}, the viral-envelope glycoprotein precursor. Components 3 are the multiply sialylated forms of gp75. Component 2 corresponds to the majority of gp55 which migrates in the basic region of the gel. Component 4 contains the more extensively processed forms of gp55.*

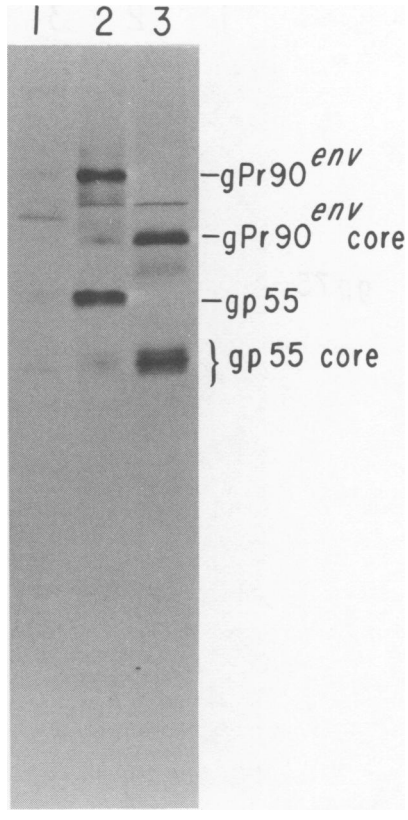

FIG. 9. *Labeling analysis of gp75-specific proteins in F4-6/K cells grown in the presence of the glycosylation inhibitor 2-deoxy-D-glucose. F4-6/K cells were pulse labeled with [^{35}S]methionine for 30 min in the presence of 15 mM 2-deoxy-D-glucose, as described in the text. The cellular lysates were immunoprecipitated with antiserum to the F-MuLV envelope glycoprotein, and the immunoprecipitates were analyzed on a 10 to 20% polyacrylamide gradient gel containing SDS. The lanes correspond to immunoprecipitates of (1) normal goat serum control, (2) F4-6/K cells, and (3) F4-6/K cells grown in the presence of 2-deoxy-D-glucose.*

tive interpretation of these results is that most of the gp55 (component 2) is only partially glycosylated and that it may remain at its site of synthesis in the rough endoplasmic reticulum and contain only the core carbohydrate units which are added in that organelle (21, 35, 45). On the other hand, a minor fraction of gp55 may reach the smooth endoplasmic reticulum and Golgi apparatus which are the sites for addition of galactose, fucose, and sialic acids onto the periphery of the complex type asparagine-linked oligosaccharides (21, 41). Additional evidence that gp55 processing is inhibited is presented below.

Previous studies have indicated that preincubation of cells with 15 mM 2-deoxyglucose blocks glycosylation of the MuLV envelope glycoproteins (40; Murray and Kabat, J. Biol. Chem., in press). As shown in Fig. 9, erythroleukemia cells treated with 2-deoxyglucose did not synthesize gPr90^{env} or gp55. Rather, these cells incorporated L-[^{35}S]methionine predominantly into their unglycosylated polypeptide chains. Consistent with previous results (40), the core polypeptide portion of gPr90^{env} (labeled "gPr90^{env} core") has a molecular weight of approximately 70,000. On the other hand, the unglycosylated gp55 polypeptide has an approximate molecular weight of 45,000.

Analyses of gp55 on the cell surface and in virions. The surface membrane of F4-6/K erythroleukemia cells was labeled at 0°C by lactoperoxidase-catalyzed iodination with [^{125}I]iodine, and the radioactive cell extracts were then analyzed by immunoprecipitation and electrophoresis. As shown in Fig. 10, a [^{125}I]-labeled immunoreactive protein that coelectrophoreses with gp75 is present on the cell surface whereas radioactive material the size of gp55 is not detected (lane 2). Furthermore, proteins that react with antibody to p30 are also not detected

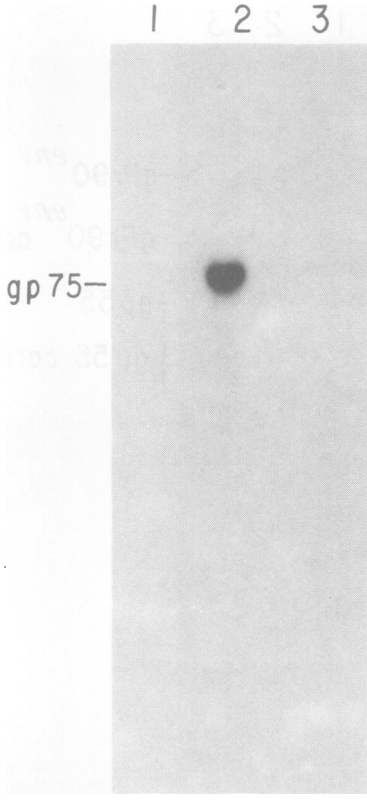

FIG. 10. *Analysis of lactoperoxidase-catalyzed ^{125}I-iodinated cell surface proteins related to the viral proteins p30 and gp75. The surface of F4-6/K was labeled with ^{125}I using the lactoperoxidase-catalyzed iodination method of Vitteta et al. (51). The cellular lysates were immunoprecipitated with monospecific antisera to the viral proteins, and the immunoprecipitates were analyzed on a 10% polyacrylamide gel containing 8 M urea and SDS. The lanes correspond to lysates immunoprecipitated with (1) antiserum to p30, (2) antiserum to the F-MuLV envelope glycoprotein, and (3) normal goat serum control.*

on the surface membrane (lane 1). When the radioactive gel used in this experiment was allowed to overexpose X-ray film for a prolonged period, a small amount of heterogeneously sized radioactive material of 55,000 to 63,000 daltons was detected in lane 2. Conceivably, this material might consist of some of the larger-sized gp55 components detected in Fig. 8. However, additional experiments will be required to test this possibility. These results suggest either that gp55-related molecules occur on the erythroleukemia cell surface to a much lesser extent than gp75 or that the cell surface gp55 is relatively inaccessible to the [^{125}I]iodine labeling procedure. Using the same techniques described by Racevskis and Koch (33), we have also confirmed their observation that gp55 is absent from virions (data not shown).

DISCUSSION

gp55 is encoded by F-SFFV. Three lines of evidence indicate that gp55 is encoded by the F-SFFV genome. First, gp55 occurs in Friend erythroleukemia cells in large amounts and it is formed in Sc-1 cells infected with Friend virus (original strain) but not in uninfected Sc-1 cells or in Sc-1 cells infected with F-MuLV. Second, the cloning experiment summarized in Table 1 indicates that the gene encoding gp55 cosegregates with F-SFFV and not with F-MuLV. These data also exclude the possibility that gp55 is encoded by a previously undescribed third virus component (perhaps a defective F-MuLV variant) in the Friend virus complex. This can be readily seen if we consider only the 14 clonally infected cell lines that gave unequivocal results in the F-SFFV assays. For example, if such a hypothetical gp55-encoding virus were as prevalent as F-SFFV in the virus preparation used for cloning (so that half of the resulting cell lines would be infected by it), the probability that it would have cosegregated with F-SFFV in all cases would be less than 10^{-4} [i.e., probability = $(0.5)^{14}$]. Third, a nonproducer cell line infected with a doubly cloned F-SFFV that had been isolated in another laboratory (46) also synthesizes gp55 (Fig. 5). We therefore conclude that the F-SFFV genome codes for gp55.

Other proteins encoded by F-SFFV. The recent availability of cell lines infected with cloned F-SFFV, with F-MuLV, or with both virus components has provided a system for identifying F-SFFV-encoded proteins. Using such cell lines and radioimmunoassay techniques for detecting viral antigens, it has been reported that F-SFFV encodes a protein that is structurally and immunologically indistinguishable from the p15 polypeptide encoded by F-MuLV (2, 4). In addition, evidence has recently been presented for a large F-SFFV-encoded polypeptide chain that cross-reacts with antibody to the F-MuLV-encoded protein p12 but not with antiserum to other MuLV-encoded proteins (2, 39). Other evidence suggests that there is a new surface antigen that can be recognized by killer T-cells on nonproducer cells infected with F-SFFV (19). However, it is not known whether this antigen is encoded by the virus.

In contrast with our results, it was reported that F-SFFV-infected nonproducer cell lines do not contain any protein that is detectable using a radioimmunoassay procedure that employs [^{125}I]-labeled Rauscher MuLV gp70 and antibody made to Rauscher MuLV gp70 (2, 4, 46).

Subsequently, a cross-reacting SFFV gene product has been detected in SFFV nonproducer cell lines using a radioimmunoassay specific for a mink cell focus-inducing virus gp70 (38). However, the molecular properties of the antigen have not yet been presented. An advantage of the immunoprecipitation procedure used here is that the antibody is present in excess, and even weakly cross-reactive antigens can be quantitatively precipitated. In addition, this method is preferable for metabolic studies. Using this procedure, we detected gp55 in the same F-SFFV-infected cell lines that had been studied by Troxler et al. (2, 46) (Fig. 5). Control immunoprecipitations clearly indicated that the gp55 in these cells is encoded by F-SFFV and not by cellular or by F-MuLV genes (Fig. 5).

Properties of gp55 in leukemia cells. The gp55 glycoprotein is produced in F4-6/K erythroleukemia cells in large amounts (estimated by immunoprecipitation as approximately 0.5% of total cellular protein synthesis), and it is relatively stable in the cells compared with $gPr90^{env}$ or gp75 (Fig. 2). Additionally, our results suggest that only small amounts of gp55 may reach the cell surface and that gp55 is absent from virions. Nevertheless, gp55 must embark on the secretory pathway and enter the rough endoplasmic reticulum because it is related to gp75 and because its newly synthesized polypeptide chain is rapidly glycosylated. These results imply that the processing of gp55 for export may be inhibited in the erythroleukemia cells.

We do not yet have definitive evidence concerning the intracellular sites of accumulation or the processing defects in gp55. However, the two-dimensional electrophoresis maps of gp55 (e.g., Fig. 8) are consistent with the idea that the processing of its oligosaccharides may be inhibited compared with gp75. In particular, the component 2 of gp55 which is rapidly formed in brief L-[^{35}S]methionine pulse-labeling studies is the major intracellular form of this glycoprotein. On the contrary, gp75 is first formed as a larger precursor $gPr90^{env}$ (component 1) that contains core high-mannose type oligosaccharides, and it is subsequently processed by proteolysis and glycosylation in the smooth endoplasmic reticulum and Golgi apparatus to form the fucosylated and sialylated gp75 molecules (29, 35, 40, 45, 52; Murray and Kabat, J. Biol. Chem., in press). It appears likely that only a minor proportion of the gp55 molecules (component 4) contain extensively processed oligosacchrides. Additional studies are in progress to test these ideas.

Based on these considerations, we suggest that most of the intracellular gp55 may accumulate (perhaps by precipitation) within the endoplasmic reticulum of erythroleukemia cells. A similar phenomenon has been demonstrated for the Z variant of α1-antitrypsin which accumulates in the liver rough endoplasmic reticulum and contains an excess of mannose residues and an absence of galactose and sialic acid (21). The intracellular abundance of gp55 raises the possibility that it might interfere with endoplasmic reticulum secretory functions or with its processing of normal membrane constituents. In addition, it is possible that some gp55 may be on the erythroleukemia cell surface and that it is not readily detectable by the lactoperoxidase-catalyzed iodination procedure. Conceivably, the gp55 in these sites could cause malignant changes in the infected cells.

Our results indicate that there may be a correlation between the amount of gp55 synthesis in nonproducer cell lines and the extent of Friend disease caused by virus rescued from the cell lines by F-MuLV superinfection. In particular, the 2B1 cell line was markedly deficient but above background in both of these activities (Table 1, and Fig. 3 and 4). In addition, the 3E1 and 3F1 cell lines produced very little gp55 and their released viruses caused only slight histologically observed spleen changes that were not clearly diagnosable as Friend disease. On the contrary, all of the cell lines with substantial amounts of gp55 released virus that caused extensive erythroleukemia. One possible explanation for this correlation is that the cell lines differ in extent of transcription of their integrated F-SFFV proviruses and that transcription limits both gp55 synthesis and F-SFFV titer. An F-SFFV provirus might be transcribed poorly either because it is integrated into a region of heterochromatin or because it is a variant. It is relevant that Troxler et al. (46) also observed different amounts of F-SFFV release from different nonproducer cell lines rescued with F-MuLV. Furthermore, different nonproducer cell lines infected with cloned defective avian retroviruses also express the virus-encoded proteins to differing extents (22).

There is evidence that the F-SFFV genome was formed by a recombination event between F-MuLV and a xenotropic virus within or very near to their *env* genes (46, 47). Our peptide map results which show structural similarity between gp55 and gp75 are consistent with this idea. However, gp55 also has distinct tryptic peptides that are absent from gp75, and it may contain amino acid sequences related to the envelope glycoprotein of xenotropic viruses or to other cellular proteins. We have only examined the few methionine-containing tryptic peptides in

the glycoproteins, and our evidence therefore only implies a small extent of amino acid sequence identity between gp55 and gp75.

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