SYNTHESIS AND POST-TRANSLATIONAL PROCESSING OF VIRUS-RELATED PROTEINS IN CULTURED FRIEND ERYTHROLEUKEMIA CELLS

by

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I dedicate this work to my late friend, Peter Goldstein, who first showed me that I had a mind worth cultivating, and to my wife, Patricia, who has given me the strength to do it.

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INTRODUCTION

Origin of Friend Virus

In 1957, Charlotte Friend, intrigued by the observation by electron microscopy of arrays of uniform, round particles in Ehrlich ascites tumor cells, attempted to detect biologically active viral agents in association with these cells (1). A cell-free extract of Ehrlich ascites tumor cells was inoculated into 30 newborn Swiss mice which then seemed to remain healthy for 14 months. At that time the animals were sacrificed and six were found to have enlarged spleens and livers. A cell suspension from an enlarged spleen was inoculated into adult Swiss mice and rapidly produced enlargement of both liver and spleen in the recipients. A cell-free extract of one of these enlarged spleens inoculated into adult Swiss mice was capable of producing palpably enlarged spleens in two to three weeks and death of all infected animals within 100 days. The physical and biological characteristics of the agent contained in the cell-free extracts indicated that it was a virus. Subsequent pathologic investigations (2) revealed that the hepatic and splenic enlargement were due to a massive proliferation of large mononuclear cells then referred to as "reticulum cells". Accompanying this solid tissue proliferation was an increase in nucleated red cell precursors, lymphocytes, and primitive mononuclear cells in the peripheral blood. This pathologic entity came to be known as Friend disease and the agent which produced it as the Friend virus.

The Nature of Friend Disease

The most striking feature of the disease induced by Friend virus was the rapidity with which it developed. Widespread proliferation of neoplastic cells in the spleen was detectable as early as three days following virus inoculation and many infected animals died of splenic rupture within 10 to 30 days (2). Subsequent studies have demonstrated that spleen cells become committed to transformation (i.e., acquire the potential for autonomous growth when transplanted intravenously into non-irradiated histocompatible mice) within three hours after infection with Friend virus *in vivo* (3). Although the early pathologic studies of Friend disease referred to the neoplastic elements as "reticulum cells", the target cells for Friend virus transformation are in fact erythroid precursors, specifically the erythropoietin-responsive stem cells (4, 5). Infected cells acquire the capacity to undergo erythroid differentiation, at least to the erythroblast stage, in the absence of erythropoietin (6, 7). Although the early pathologic descriptions of Friend disease centered on the spleen, which undergoes such dramatic enlargement, cells susceptible to virus transformation are present elsewhere (e.g., in the liver and bone marrow) and Friend disease can be induced in splenectomized mice (8).

Numerous cultured cell lines of neoplastic erythroid cells have been derived from the spleens of mice infected with Friend virus (9, 10). Further differentiation of these cells along the erythroid pathway, including synthesis of large amounts of normal adult mouse hemoglobin, can be induced by dimethyl sulfoxide (DMSO) (10, 11).

Isolation of a Lymphatic Leukemia Virus (LLV) From Friend Virus

Friend virus was eventually found to produce typical Friend disease in a number of mouse species (12) and attempts were made to produce Friend disease in rats. The virus was indeed pathogenic in rats; however, the disease produced was quite different from that seen in mice (13). Rats inoculated as newborns developed lymphocytic lymphomas involving the thymus, spleen, liver, bone marrow and lymph nodes with eventual generalized dissemination to the kidneys, lungs and other organs. In addition, the peripheral blood had the appearance of lymphocytic leukemia. Cell-free extracts of lymphomatous organs produced similar lymphomas in rats, but induced typical Friend disease when mice were inoculated. A major advance in understanding this phenomenon was the discovery by Dawson et al. (14) that, following serial cell-free passages of Friend virus in rats, a virus was obtained which induced lymphocytic lymphomas in almost all mice inoculated, only an occasional animal acquiring typical Friend disease. This agent was termed the "lymphatic leukemia virus" (LLV); immunologic characterization revealed it to be closely related antigenically to the original Friend virus from which it was isolated (15). Viral agents capable of producing lymphocytic lymphomas in mice were also isolated from Friend virus using the technique of endpoint dilution (16-19) and by inoculating Friend virus into C57BL mice which are resistant to the development of Friend disease (19). The viruses isolated by all of these techniques were closely related antigenically (19).

Nature of the Disease Produced by LLV

The pathologic features of the disease induced by LLV in mice were quite distinct from those caused by Friend virus. The disease had a markedly slower course; significant splenomegaly did not occur until 10 weeks following inoculation of newborn Swiss mice (19). The disease was characterized by thymic, splenic and lymph node enlargement due to a proliferation of malignant lymphocytes (14, 19). Affected animals had a marked lymphocytosis of the peripheral blood. The neoplastic lymphocytes induced by LLV were recently identified as T cells (20).

Relation Between Friend Virus and Lymphatic Leukemia Virus

The fact that LLV could be isolated from Friend virus by endpoint dilution without resort to passage through Friend disease-resistant animals suggested that, as originally isolated by Charlotte Friend, the Friend virus was a mixture of viral agents capable of producing at least two distinct diseases. A puzzling aspect of the early studies was that, although LLV could be isolated from the crude Friend virus free from any agent capable of producing Friend disease in mice, no one was successful in isolating the Friend disease-producing agent free of contaminating LLV (15, 16, 19). Interestingly, it was the analysis of the genetic regulation of host susceptibility to LLV infection which first suggested a resolution of this dilemma and helped to elucidate the relationship between the various components of the Friend virus complex.

The pathologic response of mice to the various murine leukemia viruses (MuLV), of which LLV is one, is controlled by a genetic locus,

Fv-1, at which there are two codominant alleles (21). The Fv-1^b allele confers resistance to a class of MuLV which are known as N-tropic; Fv-1^n confers resistance to the so-called B-tropic viruses. It is important to note in this regard that N and B tropism are not invariant characteristics of particular strains of MuLV. By forced passage through resistant animals, the tropism of a virus may be altered, a process known as adaptation. (21, 22).

Steeves and Eckner (22) investigated the relation of LLV to other components of the Friend virus complex using the phenomenon of host restriction together with a quantitative assay for Friend virus in which mice are inoculated intravenously (iv) with various dilutions of virus and macroscopic foci of neoplastic erythroblasts are enumerated on the surface of the spleen nine days later (23). They found that when Friend virus which had been maintained by serial passage in Swiss mice (which have the genotype Fv-1^n/Fv-1^n) was inoculated iv into Swiss mice the relation between virus dilution and spleen focus formation (a measure of the number of spleen cells productively infected) was linear. This relation is termed a "one-hit" dose-response and indicates that a single virus particle is required for infection. When the Friend virus which had been maintained in Swiss mice was inoculated iv into Balb/c mice (which are Fv-1^b/Fv-1^b and thus relatively resistant to infection by the LLV maintained in Swiss mice), the dose-response curve was of the "two-hit" type, suggesting that more than one virus particle was required for infection. Addition of a great excess of purified LLV along with the

Swiss mouse-derived Friend virus restored the "one-hit" dose response curve. These results suggest that spleen focus formation (and, by extrapolation, induction of Friend disease) depends on infection by two types of virus particles, the LLV and a second agent called the spleen focus-forming virus (SFFV). The data also indicate that the SFFV is defective and requires that the LLV act as a "helper virus" for productive infection.

More conclusive evidence of the defectiveness of SFFV has come from study of nonproducer cell culture lines which contain only the SFFV genome (24, 25, 26). These cell lines were produced by infection of cells with complete Friend virus followed by the intentional (26) or unintentional (27) selection of cells containing only the SFFV genome. Upon addition of pure LLV to such cells, virus capable of inducing Friend disease was produced. Rescue of SFFV could also be accomplished by addition of MuLV's other than the Friend LLV (24, 25). That these helper viruses performed necessary viral functions for the SFFV and did not merely induce its production was indicated by the fact that the Friend virus "pseudotypes" formed in each case had the host range characteristics (tropism) of the helper MuLV used for rescue (28).

Nature of the Defectiveness of SFFV

Although the viral functions specified by the SFFV genome have not been studied in detail, some things are known about the nature of its defectiveness. It is known that SFFV is not defective in functions required for transformation of cells. This fact is implied by the

existence (see above) of malignant tumor cell lines containing SFFV but not LLV (27). Further experiments utilizing the host restriction phenomenon indicated conclusively that LLV functions are not required for spleen cell transformation. Inoculation of Friend virus complex (SFFV and LLV) into mice resistant to the LLV markedly reduced the production of spleen foci and infectious virus and produced a "two-hit" dose-response curve for these phenomena (as described above) (22, 29). It was possible, however, to independently assay for cell transformation by inoculating spleen cell suspensions from infected animals into heavily irradiated, syngeneic mice and observing the formation of tumor cell colonies in the depleted spleens of these animals. Such experiments indicated that the number of spleen cells transformed was dependent on SFFV titer alone, regardless of whether the infected animal was resistant to the accompanying LLV (29). Thus the SFFV is independently capable of transforming susceptible cells.

An indication of one aspect of the defectiveness of SFFV came from a study of neutralization by various MuLV-specific antisera of the infectivity of artificial Friend virus pseudotypes formed by combining SFFV with various helper MuLV's. The Friend virus pseudotypes acquired the neutralization characteristics of the MuLV used as helper, indicating that the virus-specific envelope proteins are specified by the MuLV genome (30).

Recent work has indicated that the properties of N and B tropism may be related to characteristics of the major virion core protein, p30

(31). Since Friend virus pseudotypes acquire the host range of their helper virus, SFFV may be defective for the genetic material which specifies p30 as well. Studies of non-producer cell lines containing only the SFFV genome have indicated that they lack the ability to produce the viral enzyme reverse transcriptase as well as the viral structural proteins p30, p12 and gp70 (see below) (26).

Protein Components of Murine Leukemia Viruses

The protein components of murine leukemia viruses* have been examined in considerable detail (33) and their essential characteristics are uniform. In general, virions contain five major "structural" proteins (34). One of these, a glycoprotein, is associated with the viral envelope; the other four are elements of the viral core, which also contains the viral RNA genome. In addition, virus particles contain an RNA-dependent DNA polymerase, also known as reverse transcriptase (35).

The major envelope glycoprotein of the murine leukemia viruses has an apparent molecular weight of about 70,000 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis systems and has been termed gp70 (36). In many cases there appear to be two envelope glycoproteins of

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*Although for historical reasons the designation LLV is used above to indicate the murine leukemia virus derived from Friend virus, henceforth a uniform nomenclature, slightly modified from that of Steeves (32) shall be employed. All murine leukemia viruses shall be referred to as MuLV followed where necessary by a suffix indicating the specific strain (e.g., Friend [-F], Rauscher [-R], Moloney [-M]. SFFV shall refer only to the defective spleen focus-forming component of either Friend (SFFV-F) or Rauscher (SFFV-R) viruses. The complex of SFFV-F plus MuLV-F shall be referred to by the words "Friend virus".

very similar molecular weight, which have been called gp69/71 (37). These glycoproteins appear to play a major role in viral infectivity; antiserum to purified gp70 will neutralize MuLV infectivity and addition of purified gp70 to cultures of cells susceptible to MuLV seriously interferes with viral infectivity, presumably by blocking a site on the cellular membrane (38).

Many preparations of murine leukemia virus also contain a lower molecular weight glycoprotein (39) which has been called gp45. A detailed analysis of this species by Marquardt and Oroszlan (40) suggests that it has a molecular weight of 48,000 and a polypeptide chain identical to that of gp70 with only 5% as much carbohydrate. The functional significance of gp45 is not known.

The core proteins of the MuLV comprise species of 30,000 (p30), 15,000 (p15), 12,000 (p12), and 10,000 (p10) molecular weight (41, 42). The 30,000 dalton component is overwhelmingly the most abundant by mass and is probably primarily a structural element, forming the viral core shell. This protein may, however, have additional functions related to virus replication; Hopkins, Schindler and Hynes (31) have recently shown a correlation between alterations in p30 electrophoretic mobility and changes in the MuLV host range or tropism.

Both p12 and p10 are associated with the viral RNA genome (42, 43). The former, p12, is a phosphoprotein which binds specifically to viral RNA from murine leukemia viruses, but not to similar viral RNA molecules isolated from avian or primate leukemia viruses (42). Molecules of p12

isolated from MuLV virions show varying degrees of phosphorylation, a factor which evidently regulates the extent but not the specificity of the p12-viral RNA interaction (44). p10 binds to single-stranded nucleic acids in a relatively nonspecific fashion, showing essentially the same affinity for single-stranded calf thymus DNA, heterologous viral RNA and homologous viral RNA (43).

The reverse transcriptase or RNA-dependent DNA polymerase is the only enzyme known to be coded by the MuLV genome (45) and is considered to be unique to the RNA tumor viruses or oncornaviruses, the viral class to which the murine leukemia viruses belong (46). The reverse transcriptase is essential to the replicative cycle of the virus (45); by forming a DNA transcript of the viral RNA genome it allows the viral genome to be integrated into the host cell genetic material, a step which is essential for viral replication (47) and probably also for malignant transformation.

Since SFFV virions have not been isolated free of MuLV particles, a rigorous analysis of the protein components of SFFV has not been conducted. In early studies, SFFV-F and MuLV-F were found to be very similar antigenically (15). Thus it is likely that the two types of virus contain similar proteins, but a more detailed investigation of SFFV protein components would be desirable.

RNA Components of SFFV and MuLV

The major biochemical difference between Friend SFFV and MuLV lies in the size and composition of the virion RNA. All MuLV species which

have been studied have a major native RNA component with sedimentation coefficient of 60-70S (48-50). Following denaturation by heat or DMSO, the 60-70S component dissociates to form subunits of approximately 35S. Oligonucleotide mapping of RNA from MuLV-M has shown that these subunits are identical (51). The 35S MuLV RNA appears to contain the viral genetic information and has been used to direct synthesis of virus-specific polypeptides in cell-free translation systems (52-54).

Friend virus likewise has a native high molecular weight RNA sedimenting between 60 and 70S; however, on heat denaturation, subunits of both 30S and 35S are formed (49, 50). In most samples, the 30S component is much more abundant. Since 35S subunits are produced by heat denaturation of RNA from non-defective MuLV particles, the 30S RNA species is considered to represent the monomeric genome of defective SFFV (49). Recent studies of Friend virus RNA by gel electrophoresis and electron microscopy have confirmed the existence of the two monomeric species described above and have detected an additional RNA component of smaller size which is present in amounts approximately equal to the 30S SFFV RNA (55). This additional RNA may represent the genome of an endogenous virus thought to contaminate the cell cultures used to produce the Friend virus. More important, however, the electron microscopic studies demonstrated that the RNA subunits do associate as dimers and that almost all dimers are composed of two RNA subunits of the same size. Thus, there are probably very few virions which contain both LLV and SFFV genetic information. This conclusion was confirmed by a molecular

hybridization analysis of the high molecular weight (60-70S) peak of Friend virus RNA isolated by sucrose density gradient centrifugation (26). Using complementary DNA probes capable of distinguishing the MuLV and SFFV genomes, it was found that the apparently homogeneous RNA peak was in fact composed of two partially separated components, a heavier species which hybridized with the MuLV probe and a lighter species which hybridized with the SFFV-specific probe; there was no peak of intermediate molecular size hybridizing with both probes.

The molecular characteristics of the MuLV and SFFV genomes have been further distinguished by detailed molecular hybridization analysis (26). Approximately 50% of the SFFV-F genome is homologous with the genome of MuLV-F; these are termed MuLV-specific sequences. Of the remaining genetic material (SFFV-specific sequences), about half is homologous to the genomes of several xenotropic MuLV's. Although the SFFV-F is biologically similar to the murine sarcoma viruses (MuSV) in the rapidity with which it transforms susceptible cells, the SFFV-F specific sequences are not homologous to the genomes of three MuSV strains (Kirsten, Harvey, Moloney) which were tested. SFFV-F specific sequences do, however, have a high degree of homology to the genome of Rauscher SFFV, which produces a similar erythroblastosis in mice (56). The inferences drawn from these results are: 1) the SFFV-F genome probably arose by a recombination between MuLV-F and an endogenous xenotropic viral genome, and 2) the sequences responsible for the transforming function of SFFV are different from those which provide that

function for the MuSV's, but similar to those of Rauscher SFFV. The latter conclusion seems reasonable when one considers the different cell types transformed by the two classes of viruses: erythroid precursors in the case of the SFFV's, fibroblasts in the case of MuSV's (32).

Synthesis and Processing of MuLV Proteins in Infected Cells

The formation of the structural proteins of the Friend and Rauscher MuLV's in infected fibroblasts has lately been studied by many investigators (57-65). Data from different laboratories are consistent and indicate that the viral proteins are initially synthesized as high molecular weight precursor polypeptides which are processed by proteolysis and glycosylation to form the mature virion proteins. The pathways leading to the major envelope glycoproteins, gp69/71, and the major virion core protein, p30, have been most clearly delineated.

A pair of glycoproteins of approximately 90,000 daltons appear to be the direct precursors to the envelope glycoproteins (60, 61, 63-65). Analysis of the labeling kinetics of these precursors suggests that they are primary gene products (Evans, L., and Kabat, D., unpublished observations); however, in the presence of 2-deoxyglucose, an inhibitor of glycosylation, a 70,000 dalton protein precipitable with anti-gp69/71 accumulates in infected cells (64). This protein may in fact represent the translation product of the envelope glycoprotein genome; and the normally rapid appearance of the 90,000 dalton precursor may be due to glycosylation of the 70,000 dalton protein either nascently or immediately following release by the ribosome.

Rapidly labeled polypeptides which are antigenically related to p30 have been observed with molecular weights of approximately 65,000, 75,000 and slightly over 200,000 (58, 59, 62-65). The latter was resolved into two species of 220,000 and 230,000 daltons in one study (65). Analysis of the labelling kinetics of these four rapidly labelled p30-related proteins suggests that all four are primary gene products and that there are no precursor-product relations among them (65). (This conclusion is supported by the recent findings that translation of 35S MuLV virion RNA in a reticulocyte cell-free system yields protein products of 65,000, 75,000 and 200,000 daltons [Philipson, L., and Baltimore, D., personal communication to D. Kabat, May, 1977].) Following a prominent lag period the p30-related 75,000 dalton precursor appears to be glycosylated to form a 93,000 dalton component (65). Although the 65,000 dalton protein may be an immediate precursor to p30, the final steps of the processing pathway are not known.

Formation of Virus-Specific Proteins in Friend Leukemia Cells

There has been a single report describing viral protein synthesis and processing in cells infected with Friend virus (i.e., SFFV and MuLV) (66). A single p30-related precursor of 65,000 daltons was observed. This appeared to be processed directly to p30 and, interestingly, this processing seemed to be blocked in a Friend cell line deficient in virus production.

Examination of gp70-related processing revealed a species of 80,000 daltons which comigrated with the gp70 precursor from MuLV-R infected

fibroblasts and which appeared to be the direct precursor to gp70. In addition, a major anti-gp70 precipitable species of about 55,000 daltons was present. Although this protein was described as becoming more "diffuse" and "heterogeneous" with time, it was not obviously processed to form any other species and was not detected in virions. All three of these anti-gp70 precipitable proteins appeared to be glycosylated; however, the 80,000 dalton species was only minimally so.

Thesis Objective

As described above, the pathologic expressions of the SFFV and MuLV components of Friend virus are markedly different. The SFFV is capable of rapidly inducing malignant transformation in erythroid precursors, while the MuLV produces lymphoid neoplasms following a long latent period. The cause of this difference in pathologic expression is unknown; however, it is presumably related to the SFFV-specific portion of the viral genome. As a result of a preliminary analysis of virus-specific protein processing in Friend leukemia cells in our laboratory, a previously unrecognized major gp70-related protein of 50,000 daltons was detected. (This result was subsequently confirmed by the study [66] described above.) Since it is known that gp70 and other virus-specific glycoproteins are found on the membranes of the MuLV-infected cells (67, 68) and it is recognized that alterations in membrane glycoproteins are commonly associated with malignant transformation (69), a further study of virus-specific protein processing in Friend leukemia cells seemed to hold the possibility of explaining some of the differences in biological

activity between SFFV and LLV.

MATERIALS AND METHODS

Cells and Cell Culture Techniques

Eveline (EII) cells, a fibroblast cell line which produces large quantities of MuLV-F and negligible amounts of SFFV (70), were obtained from D. Bolognesi, Duke University Medical Center. The cells were grown as suspension cultures in Dulbecco's modified Eagle's medium (Gibco) supplemented with 0.62 gm/l $NaHCO_3$, 10% fetal calf serum (FCS) (Gibco) which had been heated to 56 C for 30 minutes, 100 units/ml penicillin and 1 mg/ml streptomycin (Penicillin-Streptomycin Solution; Gibco), and 100 µg/ml gentamicin (Schering). Cultures of 60 ml were grown in 250 ml plastic Erlenmeyer flasks (Corning) at 37 C, oscillating on a New Brunswick gyrotory shaker at 90 rpm. The cultures were transferred at 2 to 3 day intervals, maintaining the cell concentration between 7×10^5 and 2.5×10^6 cells/ml.

The Friend leukemia cell line F4-6, originally derived from the spleen of a DBA/2 mouse infected with Friend virus (71) was obtained from W. Ostertag. This cell line has been shown to produce both MuLV and SFFV (72). The cells were maintained as suspension cultures in tissue culture flasks in Eagle's basal medium (Gibco) supplemented to four times the specified amount of L-glutamine, twice the specified amounts of other amino acids, and twice the specified amount of vitamins (L-glutamine [100X], BME amino acids solution [50x], BME vitamins solution [100x]; Gibco). The medium also contained 100 units/ml penicillin,

1 mg/ml streptomycin, 4 gm/l $NaHCO_3$ and 12.5% FCS. Cells were grown at 37 C in 5% CO_2 and maintained between 5 x 10^5 and 2 x 10^6 cells/ml.

Sc-1 cells, a fibroblast cell line originally obtained from a feral mouse embryo (73), were obtained from J. Weaver at the Cell Culture Laboratory, School of Public Health, University of California, Berkeley. They were grown as monolayer cultures in McCoy's 5a medium (as modified by Iwakata and Grace) (Gibco) supplemented by 10% heat-treated FCS, 100 units/ml penicillin, 1 mg/ml streptomycin and 2.5 µg/ml Fungizone (Antibiotic-Antimycotic Solution; Gibco) and 100 µg/ml gentamicin. Inoculums of 5 ml of medium containing 5 x 10^4 cells in 25 cm^2 tissue culture flasks (Falcon) were incubated at 37 C in 5% CO_2. The cells were transferred when confluent (usually after 7 days) in the following manner. Each cell monolayer was washed twice with 0.7 ml of 0.05% trypsin-0.02% EDTA (Trypsin-EDTA Solution; Gibco), then incubated with 0.7 ml of trypsin-EDTA until the cells were dispersed (about 2 minutes). Finally, 2.8 ml of supplemented McCoy's 5a medium were added, the cells were counted in a hemocytometer, and appropriate numbers were inoculated into new flasks.

Infection of Sc-1 cells with virus from F4-6 cells was accomplished in the following manner. A 25 cm^2 tissue culture flask was inoculated with 2 x 10^5 cells in 5 ml of supplemented McCoy's 5a medium and incubated for 24 hours. The medium was then withdrawn and replaced by 2 ml of medium which had been taken from a culture of F4-6 cells growing rapidly at a concentration of 1.0 x 10^6 cells/ml, centrifuged for 10

minutes at 10,000 rpm in a Sorvall SS-34 rotor and adjusted to 8 μg/ml Polybrene (Aldrich). Following incubation for 2 hours at 37 C, 3 ml of supplemented McCoy's 5a was added and the incubation was continued for 2 days. The medium was then decanted and the cells were transferred in the usual fashion.

S^+L^- cells were provided by P. Fischinger, National Institutes of Health. The cells were grown as monolayer cultures in 25 cm^2 tissue culture flasks in supplemented McCoy's 5a medium as described above for Sc-1 cells, except that an additional 3 ml of medium was added to each 25 cm^2 flask after 4 days of incubation.

Antisera

Goat antisera to gp69/71 and p30 purified from MuLV-R were supplied by J. Gruber, National Cancer Institute. Goat antiserum to p12 isolated from MuLV-R was donated by S. Tronick, National Cancer Institute.

Assay of Virus--SFFV

The titer of SFFV was determined by the method of Axelrad and Steeves (23). Culture medium was cleared of cells by centrifugation at 4000 rpm for 10 minutes, then at 10,000 rpm for 10 minutes in a Sorvall SS-34 rotor. Female Swiss mice (6-8 weeks old) were inoculated intravenously (tail vein) with 0.5 ml of either full strength medium or a 1:4 or 1:24 dilution of medium in Dulbecco's PBS. For each sample, at least three mice were injected with each dilution. After 9 days the mice were sacrificed and the spleens were removed and fixed in Bouin's solution (0.015% picric acid, 9.25% formaledhyde, 5% acetic acid). Macroscopic

white foci on the surface of the spleen measuring greater than 0.5 mm in diameter were counted. Results are reported as foci/10^6 cells in the original cell culture and are considered to be proportional to the number of infectious SFFV particles present (22, 23).

Assay of Virus--MuLV

The titer of MuLV was measured using a variation of the S^+L^- method of Bassin et al. (74). 2 x 10^5 S^+L^- cells were placed in 25 cm^2 tissue culture flasks in 5 ml of supplemented McCoy's 5a medium. After 24 hours of incubation in 5% CO_2 at 37 C, the medium was removed and replaced with 1 ml of medium containing 25 µg/ml of DEAE-dextran (Sigma) and incubated for 30 minutes at 37 C. The dextran solution was decanted, a 0.5 ml virus sample was added, and the flasks were incubated for another 30 minutes. Five milliliters of fresh medium was then added and the flasks were incubated, tightly closed, at 37 C. After 3 days an additional 3 ml of medium was added. Foci were counted 6 to 7 days after sample application.

Labelling of Cells

For labelling with [^{35}S]-L-methionine, Eveline cells (1.9 x 10^6 cells/ml) or F4-6 cells (1.1 x 10^6 cells/ml) were sedimented by low speed centrifugation (setting of 3 on an IEC Clinical Centrifuge, Model CL) for 5 minutes at 37 C, washed once with methionine-free MEM (Gibco, Selectamine Kit) containing 10% dialyzed FCS, again sedimented, and resuspended in methionine-free MEM containing 10% dialyzed FCS and [^{35}S]-L-methionine (New England Nuclear) at a concentration of 17 µCi/ml.

The cells were then incubated at 37 C for the times indicated in the text (the "pulse" period). In some cases, cells were sedimented after labelling for 30 minutes, resuspended in the appropriate complete medium and incubated at 37 C for a further "chase" period. Samples taken at various times were immediately cooled in an ice-water bath and pelleted by centrifugation at 2000 rpm for 5 minutes at 4 C in an IEC PR-J centrifuge in a 269 rotor. Cell pellets were then extracted (either before or after storage at -70 C) for 20 minutes at 4 C 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 [Sigma]; 0.5% sodium desoxycholate; 0.1% sodium dodecyl sulfate [SDS] [Matheson, Coleman and Bell]) equal to 20% of the original cell culture sample volume. The cell extracts were centrifuged at 50,000 rpm in a Beckman 65 rotor and the pellet was discarded.

For labelling with [^{14}C]-D-glucosamine, F4-6 cells (1.1 x 10^6 cells/ml) were sedimented by low speed centrifugation (as above) and resuspended in medium containing Dulbecco's PBS (Gibco), BME amino acids and BME vitamins (Gibco) at four times the standard concentrations, 0.25 gm/l D-glucose, 0.62 gm/l $NaHCO_3$, 100 units/ml penicillin, 1 mg/ml streptomycin, and 3.3 µCi/ml [^{14}C]-D-glucosamine (Amersham-Searle). The cells were incubated for 24 hours at 37 C, then pelleted and extracted as described above.

For labelling with [^{3}H]-D-glucosamine (New England Nuclear), the compound was added directly to a culture of F4-6 cells growing rapidly in complete medium to a concentration of 17 µCi/ml. Following incubation at 37 C for 7 hours the cells were pelleted and extracted as above.

Monolayer cultures of Sc-1 cells infected with virus from F4-6 cells were labelled in 75 cm^2 tissue culture flasks (Falcon) which had reached 50-60% confluency. Each monolayer was washed twice with methionine-free MEM containing 10% dialyzed FCS, then incubated for 30 minutes in 3 ml of methionine-free MEM containing 10% dialyzed FCS and [^{35}S]-L-methionine (15 µCi/ml). Some flasks were further incubated with 10 ml supplemented McCoy's 5a medium for either 30 or 120 minutes. Monolayers were then immediately drained of all medium and frozen at -70 C. They were later thawed at 4 C and extracted by covering each monolayer with 3 ml immune buffer A for 20 minutes at 4 C. The extract was spun at 50,000 rpm in a Beckman 65 rotor and the pellet was discarded.

Immune Precipitation

For primary immune precipitation, the cell extract was diluted with a volume of immune buffer B (immune buffer A plus 20 mg/ml of bovine serum albumin and 50 µg/ml poly-L-lysine) equal to the volume of the cell extract plus the volume of antiserum to be used. Immune serum was added and the mixture was incubated overnight at 4 C. The precipitates were collected by centrifugation at 2900 rpm for 20 minutes in an IEC PR-J centrifuge with a 269 head. The precipitates were washed three times with 0.5 ml immune buffer A and twice with 0.5 ml immune buffer C (immune buffer A plus 0.5 M NaCl); the precipitates were resuspended at each step by pipetting and were resedimented by centrifugation at 2900 rpm for 10 minutes in the 269 rotor. The precipitates were then resuspended in 0.3 ml immune buffer C, layered over 0.5 ml 20% sucrose in

immune buffer C and spun at 2900 rpm for 20 minutes in the 269 rotor. Finally, the precipitates were washed three times in 10 mM Na_2HPO_4, pH 7.2; all but approximately 15 µl of supernatant were removed from each pellet following the final wash and the precipitates were dissolved in electrophoresis sample buffer as described below.

For secondary immune precipitation, the cell extract, immune buffer B and primary immune serum were combined as described above and incubated for 3 hours at 4 C. Rabbit anti-goat gamma globulin (RAGS) (Pacific Biologicals) was then added in an amount determined to achieve maximal precipitation of radioactivity. The final mixture was incubated overnight and the precipitate was processed as described above.

One Dimensional Polyacrylamide Gel Electrophoresis

Acrylamide and N,N'-methylenebisacrylamide (Eastman Organic Chemicals) were recrystallized before use. Acrylamide was dissolved in $CHCl_3$ at 50 C, filtered and crystallized at -20 C overnight. The crystals were collected by suction filtration, washed with heptane and dried by prolonged air suction. Methylenebisacrylamide (BIS) was dissolved in acetone at 50 C and recrystallized at -20 C. The crystals were collected by suction filtration.

One-dimensional slab electrophoresis was carried out by a modification of the procedure of Laemmli (75). 10% polyacrylamide slab gels were made in 0.1875 M Tris, pH 8.8, containing 0.1% SDS and 8 M urea (Mallinckrodt, analytical grade) as follows: 3.75 ml of 1.5 M Tris, pH 8.8, and 0.3 ml of 10% SDS were added to 10 ml of a 30% acrylamide

solution containing 0.8% BIS. 15 gm of urea were added and the solution was warmed to 37 C until the urea was dissolved. The total volume was adjusted to 30 ml with water and the solution was allowed to cool to room temperature. 0.15 ml of 10% ammonium persulfate was added, followed by 0.01 ml of N,N,N',N'-tetramethylethylenediamine (TEMED) (Eastman Organic Chemicals). The solution was mixed and pipetted into a gel mold consisting of two glass plates separated by lucite spacers as described by Reid and Bieleski (76). The gel was overlaid with water and allowed to polymerize for at least 30 minutes. 5% stacking gels were made in 0.0625 M Tris, pH 6.8, containing 0.1% SDS and 5.5 M urea as follows: 1.25 ml of 0.5 M Tris, pH 6.8, 0.1 ml of 10% SDS and 6.8 ml of 8 M urea were added to 1.67 ml of a 30% acrylamide solution containing 0.8% BIS. 0.1 ml of 10% ammonium persulfate was added, followed by 0.005 ml of TEMED. The solution was mixed and layered over the separating gel. A sample-wall template was inserted into the stacking gel solution and the solution was sprayed with a fine mist of water. The gel was allowed to polymerize for at least 1 hour. Samples were dissolved by mixing with an equal volume of 0.1 M Tris-HCl, pH 6.8, 2% SDS, 1% 2-mercaptoethanol, 40% glycerol and 8 M urea, and heating to 100 C for 90 seconds. The samples were applied to the gel, which was placed between electrode chambers containing 0.05 M Tris, pH 8.3, 0.38 M glycine, and 0.1% SDS. A potential of 100 V was applied across the gel and electrophoresis was continued until the tracking dye (bromphenol blue added to the upper electrode chamber) had reached the bottom of the gel (usually about 4.5

hours). The gels were then fixed overnight in 12.5% trichloroacetic acid (TCA), washed twice for 60 minutes in 500 ml DMSO and finally equilibrated for 3 hours in 100 ml of a 20% solution of 2,5-diphenyloxazole (PPO) (New England Nuclear) in DMSO, following the procedure of Bonner and Laskey (77). The gels were then placed in 500 ml of water for at least one hour, after which they were placed on Whatman 3 MM paper and dried using the apparatus described by Fairbanks (78). The dried gels were overlaid with Kodak X-Omat medical x-ray film and exposed at -70 C. The film was developed using standard techniques.

When it was desired to stain gels for protein, they were soaked in a solution of 0.2% Coomassie brilliant blue in 10% acetic acid, 20% methanol for 10 to 12 hours following fixation in TCA. Unbound stain was removed by immersion in 10% acetic acid, 20% methanol for 24 hours using several changes of solution. After this procedure, gels could be impregnated with PPO as described above and the stained protein bands remained visible.

Two-Dimensional Polyacrylamide Gel Electrophoresis

Two-dimensional electrophoresis was performed by the method of O'Farrell (79) utilizing separation by isoelectric focusing in the first dimension and by molecular weight (using SDS-polyacrylamide gel electrophoresis) in the second dimension. The procedure used was identical to that of O'Farrell except for 1) substitution of 9 M for 9.5 M urea in the first dimension sample buffer and gel; 2) replacement of the Ampholines specified by a mixture (LKB) composed of those with the following

pH ranges--3-10, 9-11, 4-6 and 5-7, in the ratio 14:2:1:1 (total Ampholine concentration was not altered); and, 3) use of a second dimension separating gel composed of a gradient from 10% to 20% acrylamide in place of the homogeneous gels specified.

Preparation of [^{35}S]-L-Methionine Labelled Virus From F4-6 Cells

F4-6 cells (1.0 x 10^6 cells/ml) were labelled for 90 minutes in methionine-free MEM containing 10% dialyzed FCS and 12.5 µCi/ml [^{35}S]-L-methionine (New England Nuclear) as described above. The cells were then incubated for 16 hours in complete medium. The cells were pelleted at 4000 rpm for 10 minutes in the SS-34 rotor and the medium was further clarified by centrifugation at 10,000 rpm for 10 minutes. The virus was pelleted by centrifugation at 28,000 rpm for 60 minutes in a Beckman 30 rotor. The viral pellets were resuspended in a small amount of TSE (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.4), layered onto a linear density gradient from 15% to 60% sucrose in TSE and centrifuged for 3 hours at 25,000 rpm in a Beckman SW27 rotor. The bottom of the tube was punctured and 1.5 ml fractions collected. Aliquots of 0.05 ml from each fraction were mixed with 5 ml of Aquasol (New England Nuclear) and radioactivity was counted on a Packard Liquid Scintillation Spectrometer. The density of selected fractions was determined by weighing 0.1 ml aliquots on a Mettler balance. Fractions were selected as indicated in the text, pooled, diluted with TSE and pelleted by centrifugation at 25,000 rpm for 60 minutes in a Beckman SW27 rotor.

RESULTS

Virus-Related Proteins in F4-6 Cells

Figure 1 compares the virus-specific proteins precipitated by antisera to gp69/71, p30 and p12 from labelled F4-6 and Eveline cells. The gp69/71-specific protein patterns of the two cell types are clearly quite different. In cells labelled for 30 minutes, proteins of 92,000, 75,000 and 55,000 daltons are precipitated by anti-gp69/71 from F4-6 cells (Sample A); the Eveline cells contain only the pair of proteins of approximately 90,000 daltons, which have been identified as the gp69/71 precursors (65, 70), and a trace of gp69/71 (Sample B). A very high molecular weight protein is also precipitated from both F4-6 and Eveline cells by gp69/71. Previous work with EII cells (65) has shown this to be a nonspecifically precipitated cellular contaminant; the same will be demonstrated for F4-6 cells below.

Immune precipitations with anti-p30 demonstrate the presence of five p30-related polyproteins in F4-6 cells, 220,000, 91,000, 74,000, 68,000 and 65,000 daltons (Sample D), compared with four, 220,000, 93,000, 75,000 and 65,000 daltons, in EII cells (Sample E). The immune precipitations with anti-p12 show that in F4-6 cells (Sample F), as in EII cells (Sample G), the p12-related proteins are identical in molecular weight to the p30-related proteins.

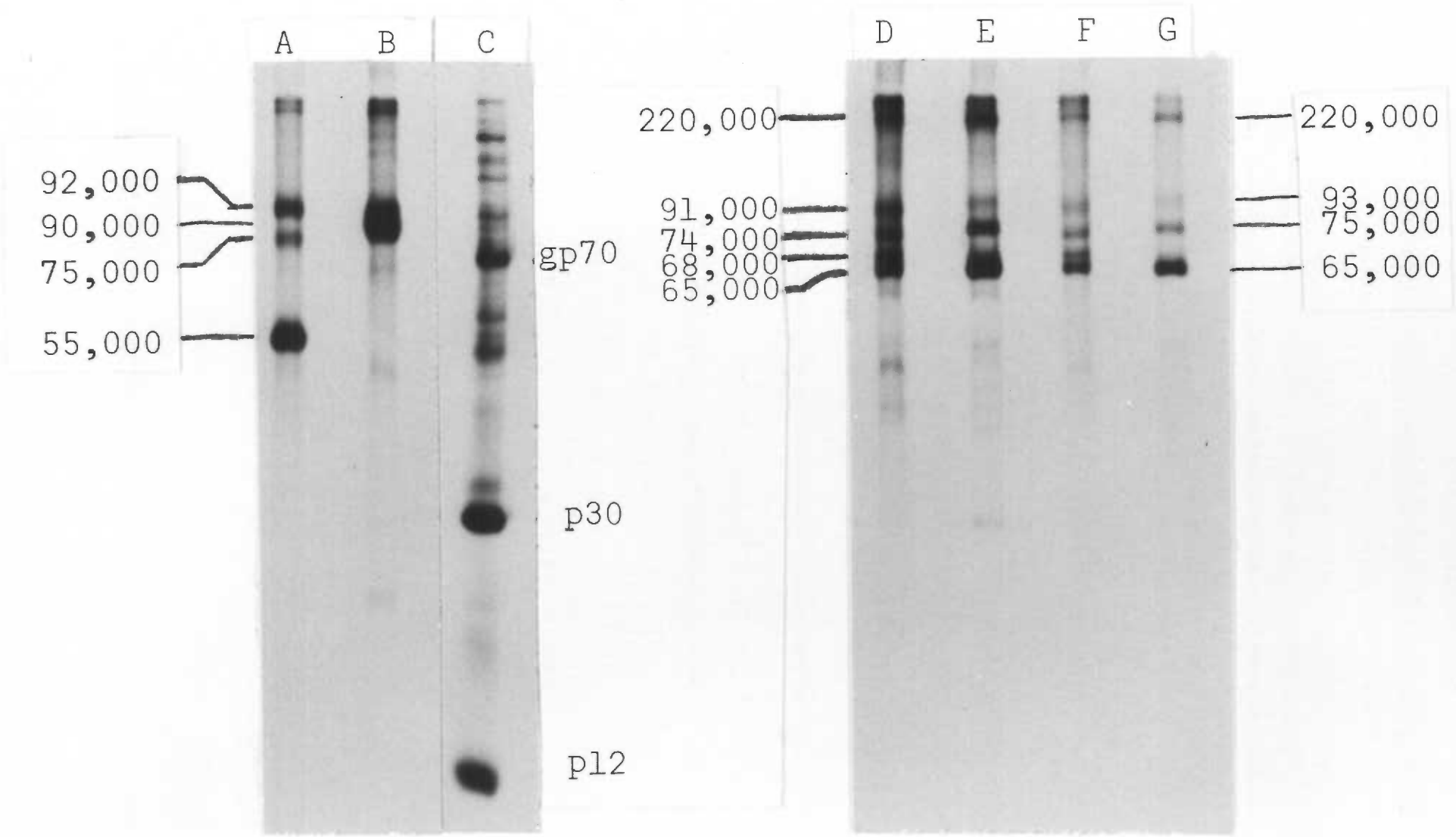
Figure 1

Comparison of [^{35}S]-L-methionine labelled virus-related proteins from F4-6 and Eveline cells.

F4-6 and Eveline cells were labelled for 30 minutes with [^{35}S]-L-methionine, the cells were extracted, the virus-related proteins were separated by primary immune precipitation, and the immune precipitates were analyzed by one dimensional polyacrylamide gel electrophoresis in the presence of 0.1% SDS and 8 M urea. The samples are as follows:

A. F4-6 cell extract precipitated with anti-gp 69/71;
B. EII cell extract precipitated with anti-gp69/71;
C. [^{35}S]-L-methionine labelled virus produced by EII cells (not immune precipitated);
D. F4-6 cell extract precipitated with anti-p30;
E. EII cell extract precipitated with anti-p30;
F. F4-6 cell extract precipitated with anti-p12;
G. EII cell extract precipitated with anti-p12.

The approximate molecular weights of the EII virus-related proteins are those determined in reference 65. Approximate molecular weights of the virus-related proteins from F4-6 cells were determined by comparison with the migration of the EII proteins, bovine serum albumin (69,000 daltons), ovalbumin (45,000 daltons) and chymotrypsinogen A (25,000 daltons). The values to the left of sample D refer to samples D and F; those to the right of sample G refer to samples E and G.


A
B
C
D
E
F
G
92,000
90,000
75,000
55,000
gp70
p30
p12
220,000
91,000
74,000
68,000
65,000
220,000
93,000
75,000
65,000

Characterization of Virus from F4-6 Cells

Figure 2 shows a characterization by equilibrium density gradient centrifugation of the material in the cell-free culture medium of [^{35}S]-L-methionine labelled F4-6 cells. A major peak of radioactivity is seen which includes the density region typically associated with murine leukemia viruses (1.16 gm/cm). Fractions in this region were pooled and the material in them was sedimented and analyzed by polyacrylamide gel electrophoresis (Figure 3). The overall protein pattern of this material (sample C) is quite similar to that of virus from Eveline cells (samples A and B), although some significant differences exist. The F4-6 virus contains [^{35}S]-L-methionine labelled proteins which comigrate with Eveline p30 and p12. There is no band corresponding to Eveline gp70; however, there is a component of relatively low abundance in F4-6 virus which comigrates with the 75,000 dalton protein precipitated from F4-6 cells by anti-gp69/71. The F4-6 virus also contains a protein which comigrates with the 55,000 dalton protein precipitated from F4-6 cells by anti-gp69/71, and which is at least as abundant in the F4-6 virion as the 75,000 dalton protein. Although proteins of approximately 55,000 daltons are present in Eveline virus (and are glycoproteins as indicated by [^{3}H]-D-glucosamine labelling), they are notably less abundant than gp70.

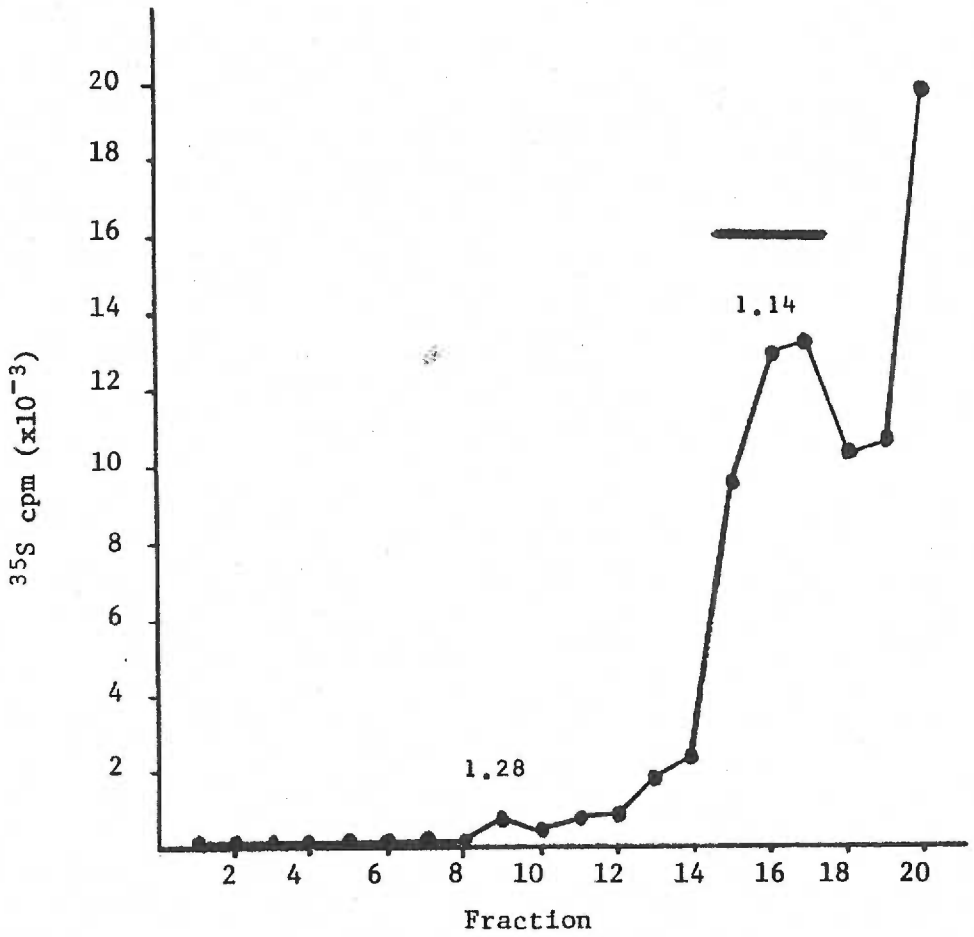
Synthesis and Processing of gp69/71-Related Proteins in F4-6 Cells

Figure 3 also shows a pulse-chase analysis of the synthesis and processing of proteins precipitated from F4-6 cells by anti-gp69/71

Figure 2

Analysis of [^{35}S]-L-methionine labelled virus from F4-6 cells by equilibrium density gradient centrifugation.

F4-6 cells were labelled with [^{35}S]-L-methionine for two hours, then incubated overnight in complete medium. The medium was cleared of cells by centrifugation and virus was pelleted by ultracentrifugation. The virus pellet was suspended in TSE and layered over a 35 ml linear density gradient from 15% to 60% sucrose in TSE. This was centrifuged at 25,000 rpm for 3 hours in a Beckman SW27 rotor. Fractions of 1.5 ml were collected through a hole punched in the bottom of the tube and an aliquot of each was counted for radioactivity. The densities of fractions 9 and 16 were measured by weighing 0.1 ml portions. The portion of the gradient under the bar was pooled, diluted with TSE and the virus in it and pelleted and analyzed in Figure 3.


20
18
16
14
12
10
8
6
4
2
^{35}S cpm ($x10^{-3}$)
1.14
1.28
2
4
6
8
10
12
14
16
18
20
Fraction

(samples D-I). As shown above, three proteins are precipitated; all three are labelled within five minutes. The 75,000 dalton component appears to be labelled more slowly than the other two during the pulse period and it persists into the chase period longer than the 92,000 dalton protein. These data suggest that the 92,000 and 75,000 dalton proteins may be related in a precursor-product manner as are the 90,000 dalton proteins and gp69/71 in Eveline cells (65). The 55,000 dalton protein seems to persist undiminished throughout the 2-hour chase period.

A comparison of samples J and K of Figure 3 dmonstrates that the very high molecular weight protein seen in immune precipitates is nonspecifically precipitated when non-immune goat serum is substituted for antiserum to viral protein. Thus, it is probably a cellular protein precipitated because of a high affinity for antigen-antibody complexes.

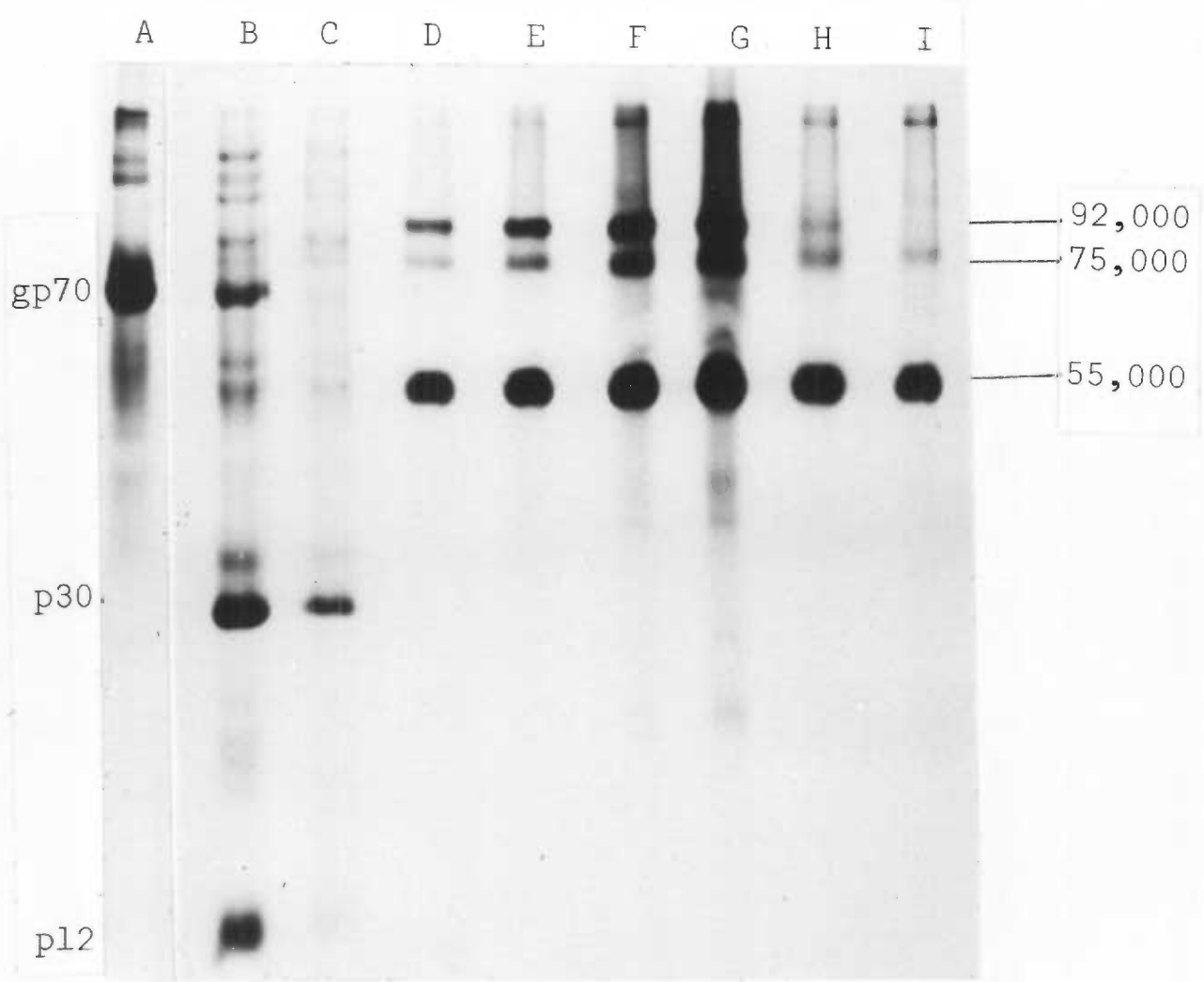
Virus-Related Glycoproteins in F4-6 Cells

Figure 4 presents an analysis of virus-related glycoproteins present in F4-6 cells labelled with radioactive glucosamine for either 7 hours or 24 hours. In material labelled for 7 hours (samples A-D), it can be seen that the 92,000 and 75,000 gp69/71-related proteins are glycosylated. The 75,000 dalton component is heavily labelled and appears much more diffuse in 7-hour glucosamine-labelled cells (sample A) than in cells labelled with [^{35}S]-L-methionine for 30 minutes (sample B). The 55,000 dalton component is not labelled with [^{3}H]-D-glucosamine, but a closely migrating 52,000 dalton protein is heavily labelled. This is more clearly shown by two-dimensional electrophoresis below.

Figure 3

Comparison of virus produced by F4-6 and EII cells. Pulse-chase analysis of gp69/71-related proteins in F4-6 cells. Control immune precipitation of F4-6 cell extract.

[^{35}S]-L-methionine labelled virus from F4-6 cells was prepared as described in Materials and Methods and Figure 2. Eveline cells were used in the same fashion to produce virus labelled with [^{35}S]-L-methionine or [^{3}H]-D-glucosamine. F4-6 cells were also pulse labelled with [^{35}S]-L-methionine for 30 minutes, then incubated in complete medium for two hours (the chase period). Cell extracts were analyzed by secondary immune precipitation and polyacrylamide gel electrophoresis as described in Figure 1. Virus samples (not immune precipitated) are as follows: A) [^{3}H]-D-glucosamine labelled EII virus; B) [^{35}S]-L-methionine labelled EII virus; C) [^{35}S]-L-methionine labelled F4-6 virus. Samples D through I are the following F4-6 pulse-chase cell extracts precipitated with anti-gp69/71: D) 5 minute pulse; E) 10 minute pulse; F) 20 minute pulse; G) 30 minute pulse; H) 30 minute pulse followed by 30 minute chase; I) 30 minute pulse followed by two hour chase. Sample J is an extract of F4-6 cells labelled for 20 minutes with [^{35}S]-L-methionine and precipitated with anti-gp69/71; K is the same extract precipitated using non-immune goat serum. Samples J and K are from a different gel than A through I. Approximate molecular weights of virus-related proteins, as determined in Figure 1, are indicated.


A
B
C
D
E
F
G
H
I
gp70
p30
p12
92,000
75,000
55,000

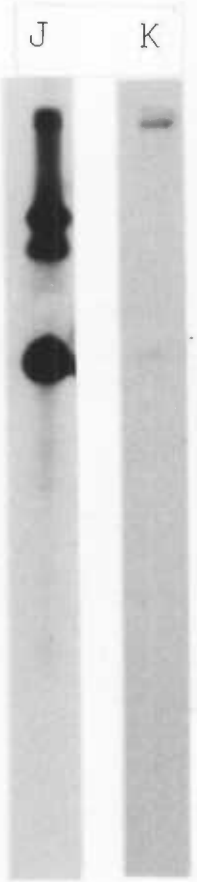
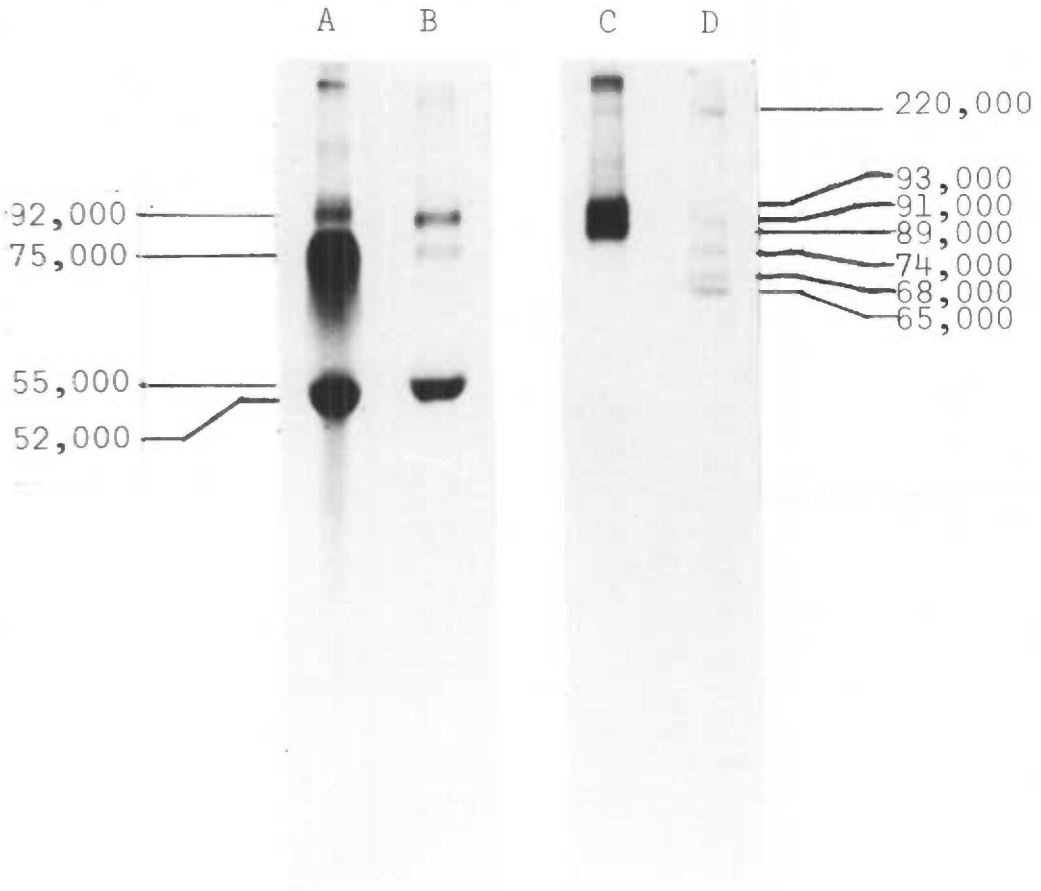
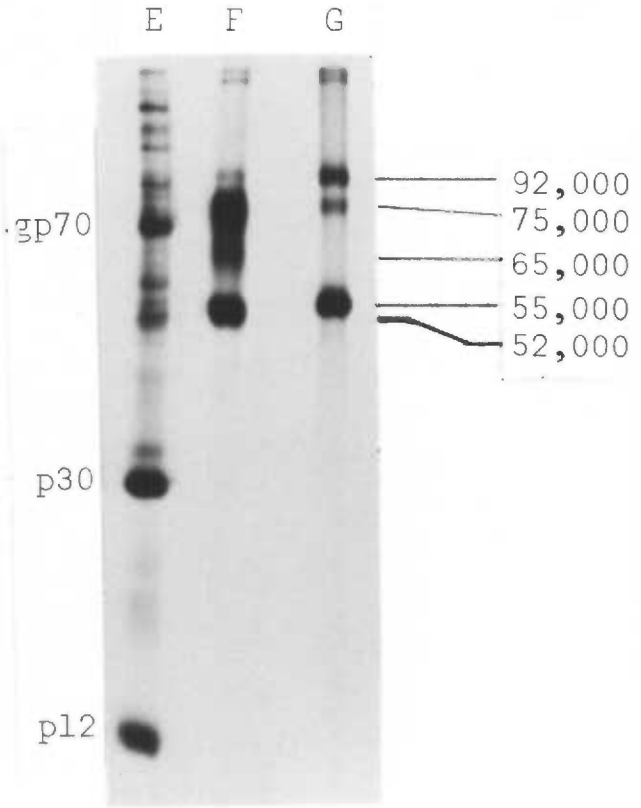

J
K

Figure 4

Radioactive glucosamine-labelled virus-related proteins of F4-6 cells.

Extracts of F4-6 cells labelled for 24 hours with [^{14}C]-D-glucosamine, for 7 hours with [^{3}H]-D-glucosamine, or for 30 minutes with [^{35}S]-L-methionine and analyzed by secondary immune precipitation and polyacrylamide gel electrophoresis as in Figure 1. Samples are as follows: A) extract of F4-6 cells labelled for 7 hours with [^{3}H]-D-glucosamine, precipitated with anti-gp69/71; B) extract of F4-6 cells labelled for 30 minutes with [^{35}S]-L-methionine, precipitated with anti-gp69/71; C) same extract as A, precipitated with anti-p30; D) same extract as B, precipitated with anti-p30; E) [^{35}S]-L-methionine labelled virus produced by Eveline cells; F) extract of F4-6 cells labelled for 24 hours with [^{14}C]-D-glucosamine, precipitated with anti-gp69/71; G) same as sample B. Samples A-D and samples E-G are from two different gels. Approximate molecular weights of virus-related proteins, as determined in Figure 1, are indicated.


A
B
C
D
92,000
75,000
55,000
52,000
220,000
93,000
91,000
89,000
74,000
68,000
65,000


E
F
G
gp70
p30
p12
92,000
75,000
65,000
55,000
52,000

After 24 hours of labelling with [^{14}C]-D-glucosamine (sample F), the 92,000 and 52,000 dalton gp69/71-related proteins are both heavily labelled. In addition, there is a diffuse band of labelling in the vicinity of the 75,000 dalton protein and a diffuse band of approximately 65,000 daltons.

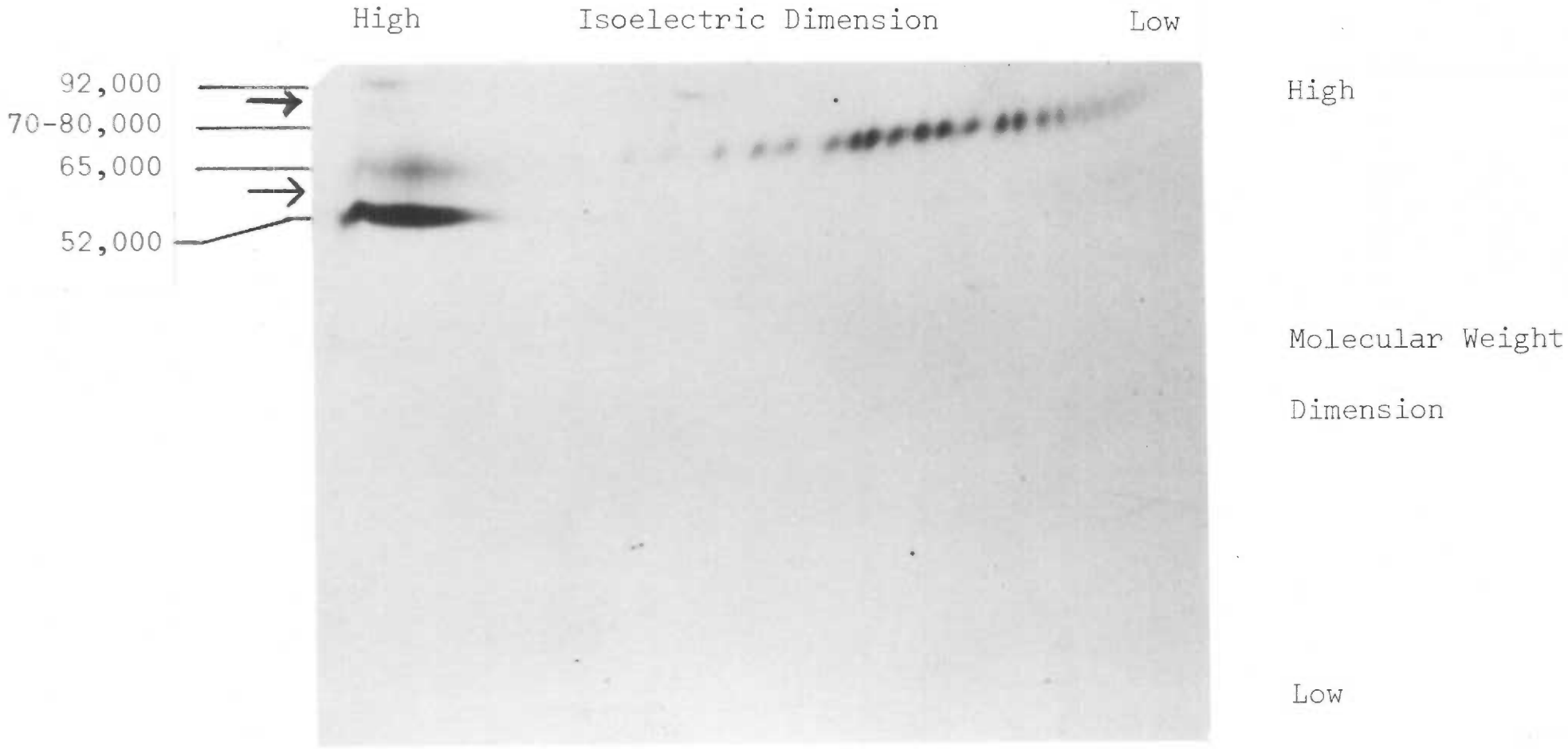
Analysis of p30-related glycoproteins in the 7-hour labelled cells (sample C) reveals two major bands, one slightly larger than the 91,000 dalton protein and one slightly smaller; these are designated 93,000 and 89,000 daltons. In addition, the 220,000 dalton p30-related protein is slightly labelled with [^{3}H]-D-glucosamine.

An analysis by two dimensional polyacrylamide gel electrophoresis of the gp69/71-related labelled with [^{14}C]-D-glucosamine for 24 hours is shown in Figure 5. The sample applied was the same immune precipitate seen as sample F in Figure 4. It can be seen that the 92,000 dalton, 65,000 dalton and 52,000 dalton proteins are relatively homogeneous. The diffuse nature of the band in the 70-80,000 dalton region is seen to be the result of the presence of a large number of discrete protein species with simultaneously decreasing isoelectric point and increasing apparent molecular weight. A very similar heterogeneity of the gp69/71 of virus from Eveline cells disappears following treatment with neuraminidase (Murray, M., and Kabat, D., unpublished observations), indicating that it is due to the presence of varying numbers of terminal sialic acid residues on the carbohydrate side chains of the glycoprotein. An anti-gp69/71 immune precipitate of F4-6 cells labelled for 30 minutes

Figure 5

Analysis by two dimensional polyacrylamide gel electrophoresis of gp-69/71 related proteins from F4-6 cells labelled with [^{14}C]-D-glucosamine.

An extract of F4-6 cells labelled for 24 hours with [^{14}C]-D-glucosamine was precipitated with anti-gp69/71 and analyzed by two dimensional polyacrylamide gel electrophoresis as described in Materials and Methods. Approximate molecular weights of virus-related proteins are indicated. The upper and lower arrows indicate the migration positions of the 75,000 dalton and 55,000 dalton [^{35}S]-L-methionine labelled proteins, respectively, on a similar two dimensional polyacrylamide gel.


High
Isoelectric Dimension
Low
92,000
70-80,000
65,000
52,000
High
Molecular Weight
Dimension
Low

with [^{35}S]-L-methionine (identical to sample G, Figure 4) was also analyzed by two-dimensional electrophoresis. The 30-minute [^{35}S]-L-methionine labelled 92,000 dalton protein comigrated with the 24-hour [^{14}C]-D-glucosamine labelled 92,000 dalton component in both dimensions. The 30-minute [^{35}S]-L-methionine labelled 75,000 dalton component migrated in the position indicated by the upper arrow in Figure 5 with the same isoelectric profile as the 92,000 dalton component. The 55,000 dalton [^{35}S]-L-methionine labelled protein migrated in the position indicated by the lower arrow in Figure 5 and had the same isoelectric profile as the 52,000 dalton [^{3}H]-D-glucosamine-labelled protein; there was no evidence that the 55,000 dalton protein is labelled with [^{3}H]-D-glucosamine.

Virus Production by Sc-1 Fibroblasts Infected by Virus from F4-6 Cells

Sc-1 mouse fibroblasts were infected with virus from F4-6 cells (MuLV-F and SFFV-F) as described in the Materials and Methods. The infected cells were morphologically identical to uninfected Sc-1 cells. To demonstrate that the cells had been infected by both elements of the Friend virus complex, rapidly growing infected cells were assayed for both MuLV and SFFV activity; the results are shown in Table 1. It can be seen that the Sc-1 cells produce large amounts of infectious MuLV and SFFV. Although the ratio of SFFV to MuLV production is much higher for the F4-6 cells than the Friend virus-infected Sc-1 cells, the difference is mostly due to a much greater MuLV production per cell by the latter; the difference in SFFV production per cell by the two cell lines is

Table 1

VIRUS PRODUCTION BY CELL LINES INFECTED WITH FRIEND VIRUS

Cell Line	MuLV[a]	SFFV[a]
F4-6[b]	39,700	843
Friend virus-infected Sc-1	937,500	117

[a]Medium from rapidly growing cell cultures was assayed for MuLV and SFFV titers as described in Materials and Methods. Results are expressed as the number of foci produced by a volume of culture medium containing 10^6 cells.

[b]Values for F4-6 cells are from reference 72.

small; it is smaller, in fact, than the differences in SFFV production between some Friend leukemia cell lines (72).

Virus-Related Proteins in Friend Virus-Infected Sc-1 Cells

Figure 6 presents a comparison of virus-related proteins in F4-6 cells and Friend virus-infected Sc-1 cells. Although the same three gp69/71 proteins are present in the Sc-1 cells (samples B-D) as in F4-6 cells (sample E), the post-translational processing of these proteins differs slightly. While the 75,000 dalton protein is prominent in F4-6 cells labelled for 30 minutes, it is barely apparent in the Sc-1 cells under the same conditions. It does appear during the chase period, however, as the 92,000 dalton component disappears. Thus, as suggested above for F4-6 cells, it seems likely that the 92,000 dalton protein is processed, probably by proteolysis, to form the 75,000 dalton component. As in F4-6 cells, the 55,000 component appears undiminished in the 2-hour chase sample.

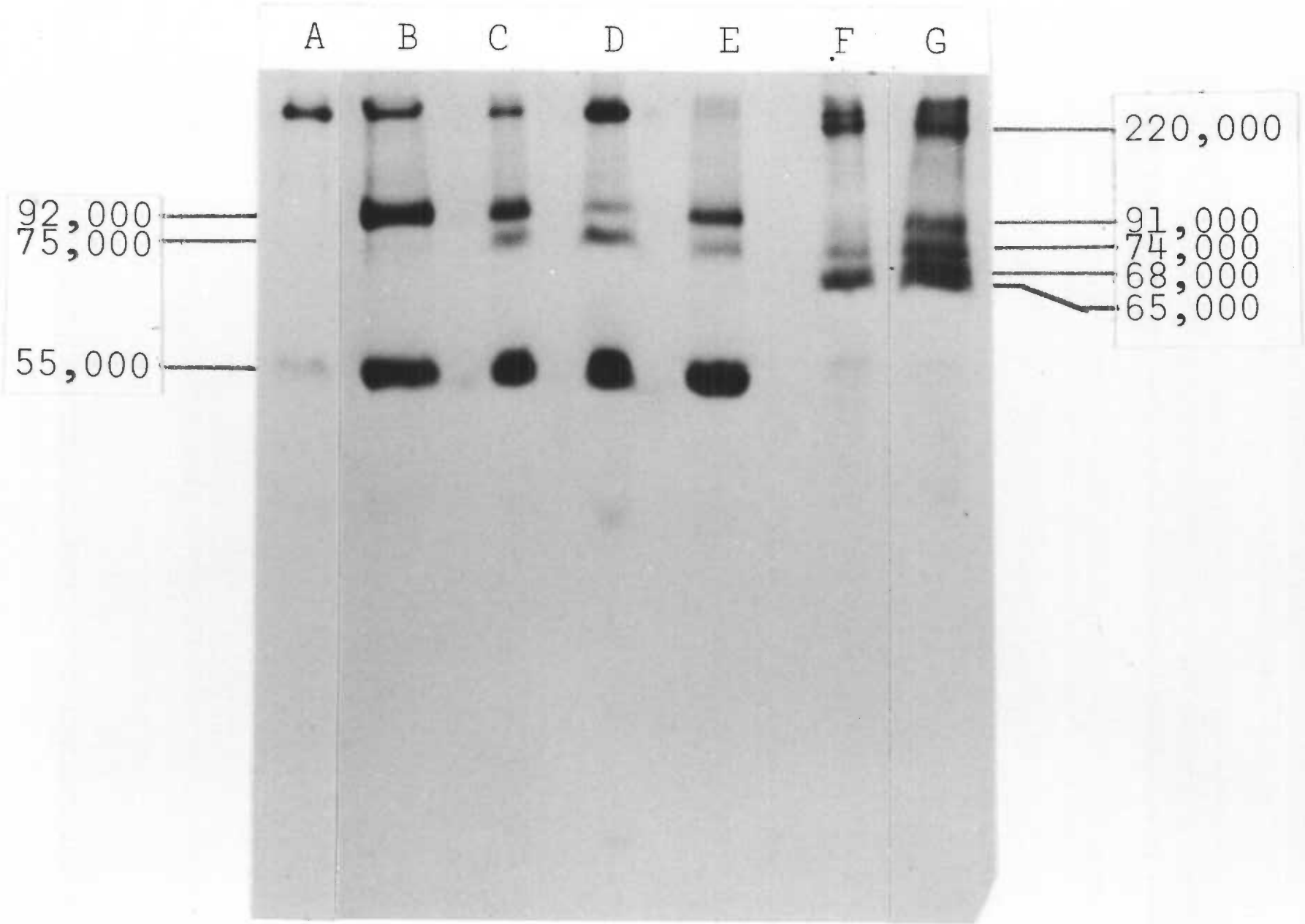
There are likewise dissimilarities between the p30-related proteins in F4-6 cells and Friend virus-infected Sc-1 cells (samples F and G). The 68,000 dalton component is not present in the infected Sc-1 cell line. Also, the 91,000 dalton protein is much less abundant in 30-minute pulse labelled Sc-1 cells than in similarly labelled F4-6 cells.

The precipitation of labelled infected Sc-1 cells with non-immune goat serum (sample A) shows that in these cells, as in F4-6 cells, the very high molecular weight protein in immune precipitates is nonspecifically precipitated and thus not virus related.

Figure 6

Virus-related proteins in Sc-1 fibroblasts infected with Friend virus.

Sc-1 cells were infected with virus from F4-6 cells, labelled for 30 minutes with [^{35}S]-L-methionine and chased for 30 minutes or 2 hours in complete medium. Secondary immune precipitates of cell extracts were analyzed by polyacrylamide gel electrophoresis as in Figure 1. Samples are as follows: A) 30 minute pulse labelled Friend virus-infected Sc-1 cells, precipitated with non-immune goat serum; B) same sample as A, precipitated with anti-gp69/71; C) Friend virus-infected Sc-1 cells labelled for 30 minutes and chased for 30 minutes, precipitated with anti-gp69/71; D) Friend virus-infected Sc-1 cells labelled for 30 minutes and chased for 2 hours, precipitated with anti-gp69/71; E) F4-6 cells labelled for 30 minutes, precipitated with anti-gp69/71; F) Friend virus-infected Sc-1 cells labelled for 30 minutes, precipitated with anti-p30; G) F4-6 cells labelled for 30 minutes, precipitated with anti-p30. Approximate molecular weights of virus-related proteins, as determined in Figure 1, are indicated.


A
B
C
D
E
F
G
92,000
75,000
55,000
220,000
91,000
74,000
68,000
65,000

DISCUSSION

Synthesis and Processing of Proteins Related Antigenically to MuLV gp69/71

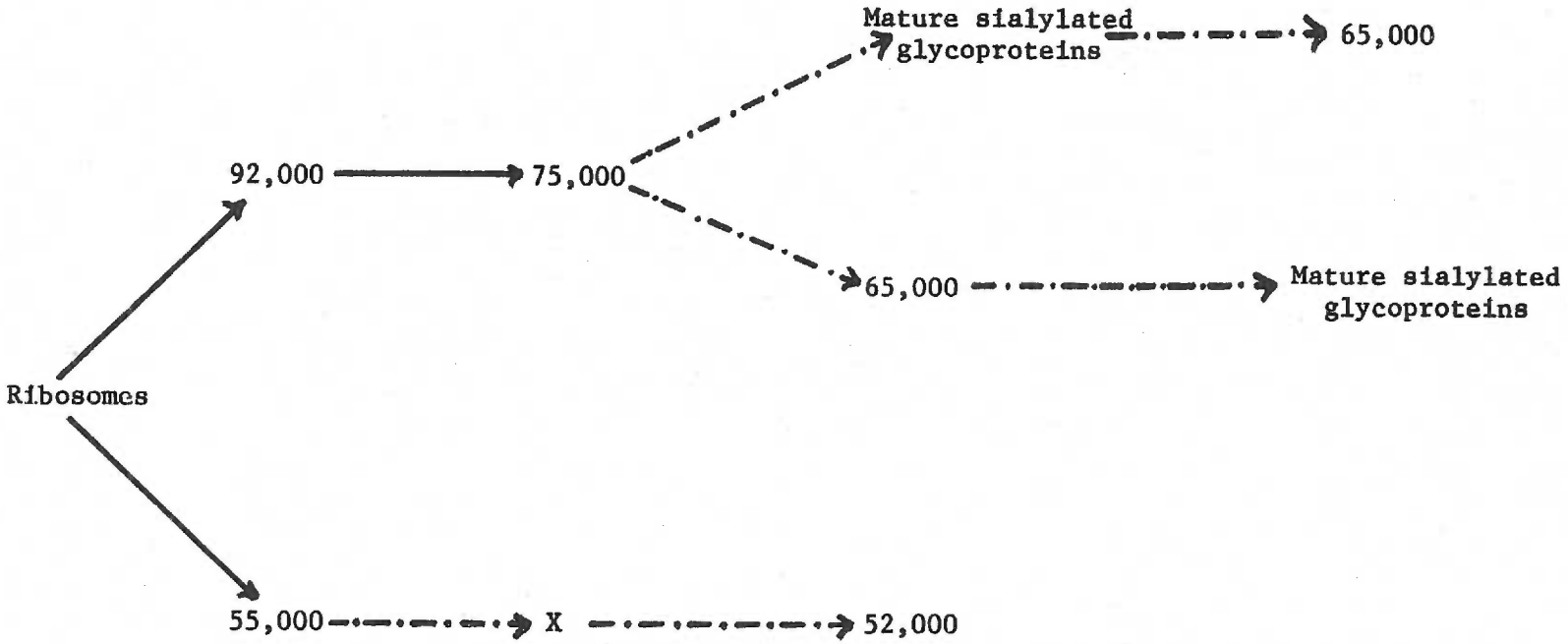
The data presented above permit several conclusions (Figure 7) regarding processing of Friend virus proteins related antigenically to MuLV gp69/71. Antiserum to gp69/71 precipitates three proteins from F4-6 cells, two of which (92,000 daltons and 55,000 daltons) appear to have the kinetic properties of primary translational products. The 92,000 dalton protein can be labelled with [^{3}H]-D-glucosamine (Figure 4); thus it is glycosylated either nascently while attached to the ribosome or very rapidly following release. The labelling kinetics in F4-6 cells of the 75,000 dalton gp69/71-related protein suggest that it is derived by post-translational processing of the 92,000 dalton component (Figure 3); this conclusion is even more strongly suggested by the labelling kinetics of the two proteins in Sc-1 cells infected with F4-6 virus (Figure 6). The nature of the process by which the 92,000 dalton protein is converted to the 75,000 dalton component is open to speculation. Since these two proteins have identical isoelectric profiles in the two-dimensional polyacrylamide gel electrophoresis system, it seems more likely that the conversion of the 92,000 dalton to the 75,000 dalton protein is due to removal of neutral carbohydrate than that it results from proteolytic cleavage. A relatively small change in the size of a carbohydrate side chain would be sufficient to produce a change in apparent molecular weight from 92,000 to 75,000 in the SDS-polyacrylamide gel electrophoresis system (40).

Figure 7

Proposed pathway for processing of gp69/71-related proteins in F4-6 cells.

Numbers refer to apparent molecular weights of the various proteins, as determined in Figure 1. Solid arrows indicate processes for which substantial evidence is presented. Broken arrows indicate processing steps which are largely speculative.

Two alternative pathways for the processing of the 75,000 dalton glycoprotein to the mature sialylated glycoproteins are presented. "X" refers to a hypothetical, unidentified intermediate in the processing of the 55,000 dalton protein.


Mature sialylated glycoproteins
65,000
92,000
75,000
65,000
Mature sialylated glycoproteins
Ribosomes
55,000
X
52,000

Following the relatively rapid formation of the 75,000 dalton protein, there appears to be further slow processing of this component by glycosylation to form a diffuse band between 70,000 and 80,000 daltons on one-dimensional polyacrylamide gels (Figure 4). Examination by two-dimensional polyacrylamide gel electrophoresis of gp69/71-related proteins from F4-6 cells labelled for 24 hours with [^{14}C]-D-glucosamine (Figure 5) reveals that the diffuse 70-80,000 band is composed of numerous discrete species which simultaneously show slightly increasing apparent molecular weight and decreasing isoelectric point (Figure 5). The envelope glycoproteins (gp69/71) of virus from Eveline cells have been shown to have a similar heterogeneity which disappears following treatment with neuraminidase (Murray, M., and Kabat, D., unpublished observations). Thus, it is likely that the isoelectric point heterogeneity in both systems is due to variation in the number of terminal sialic acid residues on the carbohydrate portions of the molecule. Whether in F4-6 cells sialic acid is added directly to the 75,000 dalton component or whether sialylation occurs following an additional processing step is not clear. The [^{14}C]-D-glucosamine labelled cells contain a 65,000 dalton glycoprotein with a slightly lower isoelectric point than the 92,000 and 75,000 dalton proteins. A line extrapolated back through the spots representing the sialylated species would intersect this 65,000 dalton protein. This suggests that the 65,000 dalton protein may be the species to which sialic acid is added; the 65,000 dalton component could be formed from the 75,000 dalton protein by proteolysis and/or partial

removal of carbohydrate. Alternatively, sialic acid could be added directly to the 75,000 dalton protein and the 65,000 dalton protein could be produced by degradation of the sialylated glycoproteins.

The relation of the 52,000 dalton glycoprotein to the rapidly labelled gp69/71-related species is not clear. Two-dimensional gel electrophoresis demonstrates that the rapidly labelled 55,000 dalton component has the same isoelectric profile as the 52,000 dalton protein. The 55,000 dalton protein is not glycosylated, however, and its conversion to the 52,000 dalton glycoprotein would necessitate both proteolysis and glycosylation, all while maintaining essentially the same isoelectric characteristics. Although such complex processing may seem unlikely, the fact that the 55,000 dalton and 52,000 dalton species are seen in Friend leukemia cell lines (Figure 1 and reference 66), but not in any of the other murine leukemia virus-infected cells which have been examined, suggests that they are related in some way.

The question of which proteins in F4-6 virus result from the above processing scheme is not resolved, since viral protein immune precipitation data are not available. F4-6 virus does contain, however, a protein which comigrates with the 75,000 dalton gp69/71-related protein (Figure 3). Although it is unlikely that this virion protein is identical to the discrete 75,000 dalton precursor, it is quite possible that it is a composite of a number of the sialylated species (Figure 5) which are most abundant in the 75,000 dalton region. Proteins migrating in the 50-55,000 dalton region are also present in F4-6 virus, suggesting that the 52,000 dalton glycoprotein may also be incorporated into virions.

Processing of p30-Related Proteins

Although the processing of high molecular weight p30-related proteins was not examined in this study, the p30-related proteins in Friend virus-infected cells were identified and inferences regarding processing can be drawn by comparing the precursor species in Friend virus-infected cells with those in Eveline cells, for which the p30-related processing pathway is known (65). Except for slight differences in molecular weights, the pattern of p30-related proteins in Friend virus-infected Sc-1 fibroblasts (Figure 6) corresponds well with that in Eveline cells (Figure 1), which are also derived from fibroblasts. Presumably the processing of these proteins in the two cell lines is also similar. In F4-6 cells, an additional p30-related protein of 68,000 daltons is present which does not correspond to any of the normal p30 precursors of EII cells. It is probably similar, however, to a 70,000 dalton p30-related protein which appears in EII cells when they are labelled in the presence of 1 mM $ZnCl_2$ (Dresler S., and Kabat, D., unpublished observations).

Comparison of Viral Protein Processing in F4-6 and Eveline Cells

General Features

The processing of gp69/71-related proteins in F4-6 cells, which are infected with SFFV-F and MuLV (70), differs significantly from that seen in Eveline cells, which are essentially infected with MuLV alone (70, 80); the 55,000 dalton protein precipitable from F4-6 cells by anti-gp69/71 is not present in Eveline cells at all (Figure 1). Furthermore,

the major glycoprotein precursors in F4-6 cells have molecular weights of 92,000 and 75,000 compared with 90,000 and 69,000/71,000 in EII cells. In addition, while all of the gp69/71-related proteins of Eveline cells occur as doublets of very similar molecular weights (65, 70), the proteins in F4-6 cells are all single species. There are some similarities between the two cell lines, however, in the general pattern of processing of gp69/71-related proteins. In both cases a glycosylated primary translational product in the 90,000 dalton range is processed to form a glycoprotein in the 70,000 dalton range, which is then processed to form multiple discrete species which differ due to the extent of terminal sialylation of the carbohydrate side chains. The possible causes of these differences and similarities in processing will be discussed below.

Cellular Factors

In comparing the virus-related protein processing in F4-6 and EII cells it is desirable first to consider whether any processing differences are due to differences between the cell types infected; F4-6 cells are erythroid and Eveline cells are derived from mouse embryo fibroblasts. For this reason, virus-related protein processing was examined in Sc-1 mouse fibroblasts infected with virus produced by F4-6 cells. As regards the gp69/71-related proteins, only a minor kinetic difference was noted: the 92,000 dalton protein was processed to form the 75,000 dalton protein slightly less rapidly in the Sc-1 cells than in F4-6 cells. The 55,000 dalton protein was equally prominent in F4-6 cells and Sc-1 cells infected with F4-6 virus.

As noted above, there is a single major difference between the p30-related proteins in F4-6 cells and Sc-1 cells infected with F4-6 virus: a 68,000 dalton protein which is present in the former is not detected in the latter. The data do not exclude the presence of the 68,000 dalton protein in infected Sc-1 cells; it is probably merely turned over very rapidly and not detectable by the methods used here.

Viral Factors

As shown above, the major differences between F4-6 and Eveline cell processing, i.e. the presence of the 55,000 dalton gp69/71-related protein and the differences in molecular weight of other gp69/71 and p30-related precursors, are not related to cellular factors. Differences in the viruses infecting the two cell types are probably the cause of the differences in virus-related protein synthesis and processing. Since the SFFV component of Friend virus alone does not seem to be capable of directing the synthesis of either p30 or gp69/71-related proteins (26), the basic synthesis and processing of such proteins in F4-6 cells should be attributable to MuLV-F, the same virus which is said to infect Eveline cells (80). Differences between the processing in Eveline and F4-6 cells could be attributed to either: 1) an alteration of MuLV-F protein processing caused by the presence of the SFFV genome, or 2) a difference in the MuLV genomes present in F4-6 and Eveline cells. The latter implies either that the MuLV in Eveline cells is not truly the Friend MuLV, or that the F4-6 cells may have acquired a different helper MuLV, perhaps by activation of an endogenous virus,

during prolonged maintenance of the cells in culture. It is also possible that F4-6 cells contain two MuLV's, one only slightly different from that in EII cells and one, the source of the 55,000 and 52,000 dalton proteins, which could be an activated endogenous virus. These possibilities can be assessed to a degree with information already available. Virus-related protein processing has been examined in two Friend leukemia cell lines, F4-6 and Friend 745, isolated and maintained independently by different investigators in two different geographic locations (72). Processing in these two was found to be identical to one another (Sherton, C. C., and Dresler, S., unpublished observations) and to that reported for a third Friend leukemia cell line (66). Thus, if the differences between F4-6 and Eveline cell processing result from the acquisition by F4-6 cells of another helper MuLV, this event has occurred in several Friend leukemia cell lines maintained in separate locations. The possibility that an endogenous virus has been activated in at least one Friend leukemia cell line, FSD-1 (from which F4-6 was cloned), is suggested by the finding that those cells produce three types of virions differentiated by the size classes of RNA they contain (55). Two contain genomes with the sizes commonly assigned to MuLV and SFFV, but a third, with a genome slightly smaller than that of SFFV, was also present. The latter could represent an endogenous virus replicating in the Friend leukemia cells. This situation will be further investigated by analyzing viral protein processing in cells infected with the Friend virus originally isolated by Charlotte Friend to determine whether

they have processing similar to that of existing Friend leukemia cell lines.

Differences between processing in F4-6 and EII cells resulting from the influence of the SFFV genome on processing of MuLV proteins will be investigated using cells infected with MuLV cloned from F4-6 virus and thus free of SFFV. A comparison of processing in such cells with that in F4-6 cells will reveal any influence of SFFV.

There are several possible ways in which SFFV could alter MuLV gene expression. Most simply, an SFFV gene product could directly influence processing of the high molecular weight precursors coded by the MuLV genome, e.g., by proteolysis or glycosylation. The SFFV genome might indrectly alter MuLV processing by inducing or repressing cellular enzymes which act on MuLV protein precursors. Such a situation has been identified in another tumor virus system: avian sarcoma virus induced a cellular sialyl transferase in transformed cells which alters the carbohydrate composition of virus-related proteins (81). A third fascinating possibility is that the presence of the MuLV genome in F4-6 cells could permit expression of SFFV genes which are otherwise repressed. It would be difficult to distinguish this latter possibility from the situations described earlier in which SFFV alters MuLV expression.

SUMMARY AND CONCLUSIONS

The synthesis and post-translational processing of virus-related proteins were studied in F4-6 Friend leukemia cells, which are infected with both the defective Friend spleen focus-forming virus and a helper murine leukemia virus. Two apparent primary translational products related antigenically to the MuLV envelope glycoproteins gp69/71 were identified, a glycoprotein of 92,000 daltons and a protein of 55,000 daltons. The 92,000 dalton glycoprotein seems to be processed to a 75,000 dalton glycoprotein which is then slowly processed to form multiple glycoproteins of similar molecular weight and different isoelectric points, which appear to differ mainly in the number of terminal sialic acid residues on their carbohydrate side chains. The 55,000 dalton protein appears to be processed very slowly to form a 52,000 dalton glycoprotein. The synthesis and processing of gp69/71-related proteins is essentially identical in Sc-1 mouse fibroblasts infected with virus from F4-6 cells.

Proteins precipitable by antiserum to the major MuLV core protein, p30, were also examined. In F4-6 cells there are p30-related proteins of 91,000 daltons, 74,000 daltons, 68,000 daltons and 65,000 daltons. In Sc-1 cells infected with F4-6 virus, all except the 68,000 dalton protein are present.

Comparison of the synthesis and processing of gp69/71-related proteins in F4-6 cells with that in Eveline cells, which are generally thought to be infected with the same MuLV as that found in Friend leukemia cells, revealed several significant differences. The 55,000

dalton protein found in F4-6 cells is not present in Eveline cells at all and there are differences in the molecular weights of most of the other virus-related proteins. There are several possible explanations for the differences between F4-6 and Eveline cells: 1) the SFFV-F genome alters the expression of the MuLV genome in F4-6 cells; 2) the MuLV's in F4-6 and Eveline cells are actually not identical; or 3) two MuLV's are expressed in F4-6 cells, one which is very similar to that in Eveline cells and another which is dissimilar, perhaps an endogenous virus.

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