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Synthesis and Glycosylation of Polyprotein Precursors to the Internal Core Proteins of Friend Murine Leukemia Virus

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Synthesis and post-translational processing of murine leukemia virus proteins were analyzed in a murine cell line (Eveline) that produces large amounts of Friend lymphatic leukemia virus. Immunoprecipitation of L-[^{35}S]methionine-labeled cell extracts demonstrated that several different virus-specific proteins antigenically related to the virion core (*gag*) proteins p12 and p30 become radioactive within 1 min of labeling and exhibit labeling kinetics characteristic of primary translation products. The most abundant of these were proteins with molecular weights of 75,000 and 65,000. There were, in addition, two large glycosylated polyproteins with apparent molecular weights of 220,000 and 230,000, which were precipitated by antisera to p30 or p12 but not by antiserum to the major envelope glycoproteins gp69/71. Several lines of evidence, including labeling with D-[^{3}H]glucosamine and binding to insolubilized lectins, suggested that the 75,000-dalton internal core polyprotein is slowly processed to form a glycoprotein with an apparent molecular weight of 93,000. On the contrary, the 65,000-dalton protein appeared to be an immediate precursor to the virion core proteins. Its processing can involve intermediates containing p30 and p12 antigens with molecular weights of 50,000 and 40,000; however, the latter did not appear to be obligatory intermediates. The detection of the 40,000-dalton protein suggested that the genes for p30 and p12 are adjacent on the viral genome. These results indicated that there are several pathways of synthesis and post-translational processing of polyprotein precursors to the *gag* proteins and that several of these polyproteins are glycosylated. A comparison of *gag* precursor processing in rapidly growing, slowly growing, and stationary cells indicated that different pathways are favored under different conditions of cell growth. Our analysis of envelope glycoprotein synthesis has confirmed the existence of two rapidly labeled 90,000-dalton glycoproteins, which appear to be precursors to the envelope glycoproteins gp69/71.

Several recent reports have indicated that Rauscher murine leukemia virus (MuLV-R) proteins are formed by intracellular processing of higher-molecular-weight precursors (1, 2, 7, 13, 19, 23). A 60,000- to 65,000-dalton precursor to the major virion core protein, p30, has been identified (1, 7, 19, 21, 23). In addition, van Zaane et al. (23) have detected a 72,000-dalton precursor in cells treated with an arginine analog, and Famulari et al. (7) have described p30 precursors of 65,000 and 75,000 daltons. Arlinghaus' group (1, 2, 13) has identified p30-containing proteins of approximately 65,000 and 80,000 daltons as well as two closely migrating proteins of approximately 200,000 daltons, which are antigenically related to p30 as well as to reverse transcriptase. Two closely migrating glycoproteins of approximately 90,000 daltons have been described as precursors to the envelope glycoproteins gp69/71 (7, 13, 19).

A model has recently been proposed in which the 200,000- and 90,000-dalton MuLV precursors are derived by nascent cleavage of a translation product of a large mRNA representing the entire viral genome (2). In this scheme, the 90,000-dalton protein, presumably glycosylated nascently or very soon after nascent cleavage, is processed to form the envelope glycoproteins, whereas the 200,000-dalton precursor is processed to form the viral DNA polymerase and the lower-molecular-weight virion core proteins by a series of proteolytic cleavages. The 80,000- and 65,000-dalton proteins are proposed as intermediates in this cleavage pathway.

We have studied MuLV protein synthesis in Eveline cells, which produce large amounts of the Friend lymphatic leukemia virus (MuLV-F). Our results indicate that the post-translational processing of MuLV-specific proteins is more complex than previously believed. In particular,

the major precursors to the virion core proteins are involved in several different processing pathways rather than as sequential intermediates in a single pathway; and their processing has been found to involve glycosylation as well as proteolysis. Furthermore, different processing routes appear to be favored under different conditions of cellular growth.

MATERIALS AND METHODS

Cells and virus. STU mouse cells chronically infected with MuLV-F (Eveline cells) (5, 14, 18) were kindly provided by D. Bolognesi, Duke University Medical Center. These cells produce large amounts of MuLV-F and negligible amounts of spleen focus-forming virus (L. Evans, unpublished observations). The abbreviations used for the viruses are those proposed by Steeves (22). The cells were grown as suspension cultures in Dulbecco modified Eagle medium (dry powdered medium, Grand Island Biological Co. [GIBCO]) containing 10% fetal calf serum (GIBCO or Flow Laboratories). Sixty-milliliter cultures growing in 250-ml Erlenmeyer flasks (Corning) were passaged at 2- to 3-day intervals. The cell concentration was maintained between 6×10^5 and 3×10^6 cells/ml.

Antisera. MuLV-F for the production of antiserum was obtained from the medium of logarithmically growing Eveline cells. Cells were removed by centrifugation at 3,000 rpm for 10 min in a Sorvall GSA rotor at 4°C, and the supernatant was further clarified by centrifugation at 10,000 rpm for 10 min. Virus was collected by centrifuging 25-ml portions of the medium on discontinuous density gradients composed of 5 ml of 60% sucrose and 7 ml of 15% sucrose in TSE (0.1 M NaCl–0.01 M Tris-hydrochloride [pH 7.4]–0.001 M EDTA) in a Beckman SW27.1 rotor for 90 min at 25,000 rpm. The virus was removed from the surface of the 60% sucrose layer, diluted with 3 volumes of TSE, and layered onto linear gradients of 15 to 60% sucrose in TSE. The virus was purified isopycnically by centrifugation for 3 h at 25,000 rpm, and the band at 1.15 to 1.16 g/cm^3 was collected, diluted in TSE buffer, and pelleted by centrifugation at 25,000 rpm for 90 min. Purified MuLV-F was suspended in 2% Triton X-100 (Sigma) at a concentration of 1 mg of protein per ml and agitated in ice water for 20 min. An equal volume of diethyl ether was added, and the mixture was shaken for an additional 30 min. The mixture was centrifuged at 300 rpm in a Sorvall SS-34 rotor at 4°C for 10 min, and the aqueous layer was withdrawn. One milligram of this disrupted virus was mixed with an equal volume of Freund complete adjuvant and injected intramuscularly into a male New Zealand white rabbit. Intravenous booster injections of 1 mg of disrupted virus were administered at 2- to 3-week intervals, and serum was collected by cardiac puncture 7 to 10 days after each injection. Each milliliter of immune serum was adsorbed for 4 h at room temperature with approximately 300 mg of mouse protein obtained from various tissues and serum and insolubilized according to the procedure of Avrameas and Ternynck (3).

Goat antiserum directed against MuLV-R p12 was kindly donated by S. Tronick, Viral Carcinogenesis Branch, National Cancer Institute. Goat antisera against Rauscher gp69/71 and p30 were generously supplied by J. Gruber, National Cancer Institute, National Institutes of Health. These three antisera had radioimmunoassay titers of 50,000, 80,000, and 50,000, respectively, against their corresponding antigens. The monospecificity of these sera against single virus proteins was shown by precipitation of the appropriate individual proteins from preparations of [^{3}H]leucine-labeled MuLV-F, which had been disrupted with immune buffer A (see below). In addition, as shown in Results, the antiserum against gp69/71 did not precipitate any polyproteins containing p30 or p12 antigens, and the antisera against p30 and p12 did not precipitate the gp69/71-containing precursors. The latter precursors are known to contain p12E and p15E antigens (13, 23). Furthermore, it is shown in Results that the antiserum against p12 does not precipitate p30 from cellular extracts. Rabbit anti-goat immunoglobulin was obtained from Pacific Biologicals (Richmond, Calif.). Nonimmune goat serum was a gift from J. Metcalfe, University of Oregon Health Sciences Center.

Labeling and extraction of cells. Cells were sedimented by low-speed centrifugation at 37°C and resuspended in methionine-free minimal essential medium (GIBCO). After a 5-min incubation at 37°C, the cells were sedimented again and resuspended in methionine-free minimal essential medium containing 10% dialyzed fetal calf serum and L-[^{35}S]methionine (New England Nuclear Corp.), as indicated in the figure legends. Where indicated, pulse-labeled cells were chased by the addition of a 500-fold excess of unlabeled methionine to the culture. For labeling of carbohydrate, D-[^{3}H]glucosamine (New England Nuclear Corp.; 20.7 Ci/mmol) was added directly to a growing culture at a concentration of 17 μCi/ml. Samples of the cultures were taken at various times, immediately cooled to 0°C, and pelleted by low-speed centrifugation at 4°C. The cell pellets were extracted for 15 min 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-100–0.5% sodium deoxycholate–0.1% sodium dodecyl sulfate [SDS]) equal to 20% of the volume of the cell culture sample. The extracts were centrifuged at 200,000 × *g* for 20 min in a Beckman 65 rotor, and the pellet was discarded. The supernatant contained 96 to 99% of the radioactivity incorporated into the cells.

Affinity chromatogrphy of cell extracts on insolubilized lectins. Pellets containing cells that had been labeled for 60 min with L-[^{35}S]methionine as described above were extracted with lysis buffer for lectin binding (40 mM Tris-hydrochloride [pH 7.4]–150 mM NaCl–5 mM $MgCl_2$–6 mM 2-mercaptoethanol–0.5% Nonidet P-40–0.5% sodium deoxycholate) for 20 min at 4°C. The volume of the extract was adjusted to 20% of the volume of the original cell culture, and the extracts were centrifuged at 200,000 × *g* for 20 min in a Beckman 65 rotor.

The cell extracts (1.1-ml samples) were applied to 2-ml columns containing concanavalin A-Sepharose (ConA) (Sigma), *Lens culinaris* hemagglutinin-agarose (LCH) (Miles), or *Ricinus communis* agglutinin-

agarose (RCA_1) (Miles), which had been prewashed with 1 M $MgCl_2$, then with water, and finally with the lysis buffer minus sodium deoxycholate. The cell extracts were incubated on the columns at room temperature for 1.5 h to allow glycoprotein binding. Nonabsorbed proteins were then thoroughly eluted with the lysis buffer lacking sodium deoxycholate. The glycoproteins were eluted with the same solution containing either 0.3 M α-methyl-D-mannoside (for ConA and LCH) or 0.3 M D-galactose (for RCA_1). Virus-specific proteins were precipitated from the glycoprotein fraction by secondary immune precipitation as described below.

Immune precipitation procedures. Cell extracts were diluted in a volume of immune buffer B (immune buffer A containing 20 mg of bovine serum albumin [fraction V] per ml [Calbiochem] and 50 μg of poly L-lysine per ml [Sigma type 1] [molecular weight, ~75,000]) equal to 50% of the final incubation volume. For primary immune precipitations, antiserum containing 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS was added, and the mixture was incubated overnight at 4°C. The precipitates were collected by centrifugation through 20% sucrose in immune buffer C (immune buffer A containing 0.5 M NaCl), washed three times with immune buffer A and three times with immune buffer C, and centrifuged again through 20% sucrose in immune buffer C. The precipitates were then resuspended in 0.01 M Na_2HPO_4 (pH 7.6)–0.15 M NaCl and pelleted. The final precipitate was drained and dissolved in electrophoresis sample buffer as described below. For secondary immune precipitations the primary antiserum was added to the cell extract after the addition of immune buffer B, as above, and incubated for 1 h. Rabbit anti-goat immunoglobulin was added in an amount determined to achieve maximum precipitation of radioactivity. The final mixture was incubated overnight at 4°C, and the precipitates were processed as described above.

SDS-urea polyacrylamide gel electrophoresis. Slab gel electrophoresis was carried out by a modification of the procedure of Laemmli (10). 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 2 min 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. After electrophoresis, the gels were fixed in 12.5% trichloroacetic acid for at least 1 h and processed for fluorography according to the procedure of Bonner and Laskey (6). The gels were dried and overlaid with Kodak X-Omat medical X-ray film and exposed at −70°C. Where indicated, the films were exposed for varying times and scanned with a Transidyne General integrating densitometer. Bands that were proportional to the exposure time were quantitated for each exposure, and curves were constructed by comparing band intensities of adjacent samples on the gels.

Molecular weights of virus-specific proteins were estimated by comparison to rabbit myosin (molecular weight, 212,000), nonreduced bovine serum albumin (135,000), [^{14}C]ADP ribosylated elongation factor 2 (100,000), bovine serum albumin (69,000), ovalbumin (48,000), creatine kinase (41,000), pancreatic DNase (32,000), chymotrypsinogen (24,000), and lysozyme (14,000).

Determination of infectivity and radioactive MuLV during cellular growth. Stationary-phase Eveline cells were diluted into fresh growth medium containing L-[^{14}C]leucine (New England Nuclear Corp.) in the amounts indicated in the figure legends. Samples taken subsequently were assayed for MuLV infectivity as described previously (20). For determination of radioactive virus release, samples of the medium were cleared of cells by low-speed centrifugation and layered onto tubes containing 25-ml linear gradients from 15 to 60% sucrose in TSE overlaid with 10 ml of 15% sucrose in TSE. These were centrifuged for 4 h at 27,000 rpm in a Beckman SW27.1 rotor, and the virus banding at a density of 1.15 to 1.17 g/cm^3 was precipitated by adjusting the solution to 20% trichloroacetic acid. The precipitated material was filtered on membrane filters (0.45-μm pore size; Millipore Corp.), and radioactivity was counted in a Nuclear-Chicago low-background gas flow counter.

RESULTS

Pulse-labeled virus-specific proteins in Eveline cells. A cell extract made after L-[^{35}S]methionine incorporation for 10 min was precipitated with antisera made to purified gp69/71, p30, and p12 and compared with the precipitate obtained with antiserum made to whole disrupted MuLV-F. Anti-MuLV-F precipitated two approximately 90,000-dalton proteins and also 75,000- and 65,000-dalton proteins (Fig. 1). Antiserum to the envelope glycoprotein gp69/71 precipitated the two 90,000-dalton proteins, whereas antisera to the *gag* core proteins p30 and p12 precipitated the 65,000- and 75,000-dalton proteins. In addition, the latter antisera precipitated two large polyproteins with estimated molecular weights of 220,000 and 230,000, which were not precipitated by the antiserum to gp69/71.

Pulse-chase analysis of p30 precursors. Figure 2 shows a pulse-chase experiment using monospecific antiserum to purified p30. Three large proteins were resolved in the 220,000-, 230,000-, and 245,000-dalton size range. The largest of these proteins became labeled to a significant extent only after a long lag of approximately 30 min, and it continued to accumulate radioactivity throughout the cold chase with nonradioactive methionine. As will be shown below, this large 245,000-dalton protein is a cellular protein that binds nonspecifically to antigen-antibody complexes. In addition, a late labeling protein of approximately 93,000 daltons

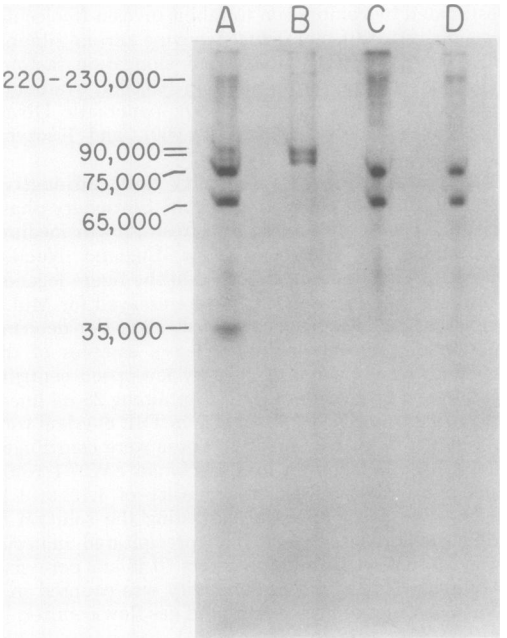

FIG. 1. *Precipitation of 10-min pulse-labeled cells with monospecific antisera. Eveline II cells (1.4 × 10^6 cells/ml) were pulse-labeled with 50 μCi of L-[^{35}S]-methionine per ml for 10 min. Samples of the cell extract were precipitated with antisera to whole disrupted virus, gp69/71, p30, and p12 by primary antiserum precipitation. The precipitates were subjected to electrophoresis in 10% polyacrylamide slab gels containing 8 M urea and 0.1% SDS as described in the text. The samples applied to the gel were cell extracts precipitated with: (A) antiserum to whole disrupted MuLV-F; (B) antiserum to gp69/71; (C) antiserum to p30; (D) antiserum to p12. Approximate molecular weights of the virus-specific proteins are indicated at the left of the autoradiogram. The 35,000-dalton protein was not reproducibly observed in our immunoprecipitates and has not been characterized.*

was specifically precipitated by the anti-p30 serum. In contrast to the two gp69/71 precursors, which were detectable after a 10-min pulse, the approximately 93,000-dalton component was not detected at this time (also see Fig. 1). The labeling kinetics of the 93,000-dalton protein suggest that it is an intermediate in virus protein processing and are consistent with the possibility that it is derived from the 75,000-dalton protein. Experiments described below support this proposal.

Analysis of many different pulse-chase experiments with p30 antiserum suggests that the 220,000- and 230,000-dalton polyproteins are not obligatory precursors to the smaller p30-containing proteins. If the large polyproteins, which occur in relatively low concentrations, were obligatory precursors to the 75,000- and 65,000-dalton proteins, they would be expected to turn over much more rapidly. Yet they were not rapidly labeled to a steady-state level, and they did not rapidly lose radioactivity during a cold chase with nonradioactive methionine. Furthermore, the labeling kinetics of the 75,000- and 65,000-dalton proteins also suggest that these proteins are not related in a simple precursor-product manner. Figure 3 shows a quantitative analysis of the labeling of the 220,000- and 230,000-dalton polyproteins and of the 75,000- and 65,000-dalton proteins during very brief periods of incorporation of L-[^{35}S]methionine. The 75,000- and 65,000-dalton polypeptides, as well as the large polyproteins, were detected after labeling for only 1 min, and they each accumulated radioactivity without any lag phase.

Glycosylation of MuLV-specific proteins. Figure 4 shows an immunological and electrophoretic characterization of the L-[^{35}S]methionine-labeled glycoproteins, which had been purified by affinity chromatography on a column of RCA_1 agglutinin covalently coupled to agarose. Glycoproteins with apparent molecular weights of 93,000 and 85,000 are precipitated by antiserum against p30 (Fig. 4A). The 85,000-dalton glycoprotein is only 10 to 20% as abundant as the 93,000-dalton glycoprotein. Identical results were obtained with the LCH and ConA lectins (data not shown). In addition, we have reproducibly observed that both the 220,000- and 230,000-dalton polyproteins, which precipitate with p30 antiserum, are adsorbed to the lectin columns and are eluted with the specific sugars used. In agreement with previous reports (7, 13, 19), the 90,000-dalton precursors of gp69/71 and the fully processed gp69/71 molecules are also glycoproteins.

Figure 5 shows an electrophoretic comparison of the D-[^{3}H]glucosamine-labeled and L-[^{35}S]methionine-labeled cellular proteins, which were precipitated by the antisera to p30 and gp69/71. The results show clearly that the 93,000-dalton component precipitated by anti-p30 serum is glycosylated. This antiserum also precipitated an 85,000-dalton glycoprotein, which was present in this experiment in a relatively low concentration. In addition, the 90,000-dalton precursors of gp69/71 and the fully processed gp69/71 molecules were labeled with the radioactive glucosamine.

Figure 5 also shows control secondary precipitations of L-[^{35}S]methionine- and D-[^{3}H]glucosamine-labeled cell extracts, using nonimmune goat serum in place of viral protein-specific antiserum. As mentioned above, the 245,000-dalton, methionine-labeled protein was precipitated

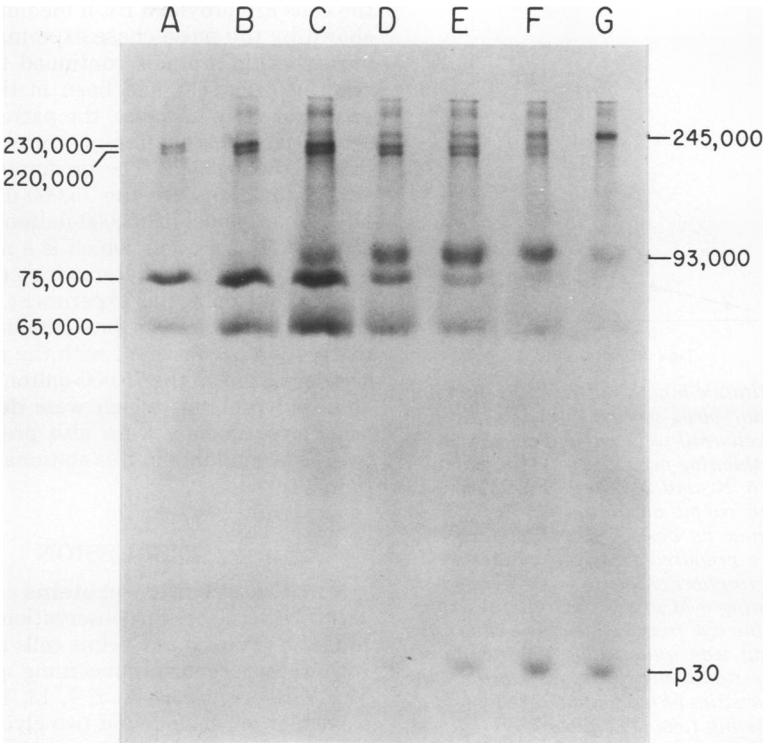

FIG. 2. *Pulse-chase analysis of p30-specific proteins in Eveline II cells. Eveline cells (1.5 × 10⁶ cells/ml) were pulse-labeled with 50 μCi of L-[³⁵S]methionine per ml for 30 min and chased by the addition of unlabeled methionine for 2 h. Samples taken during the experiment were precipitated with antisera to p30 by secondary immune precipitation and subjected to electrophoresis in 10% polyacrylamide gels containing 8 M urea and 0.1% SDS as described in the text. The samples were subjected to electrophoresis for twice the time required for the dye front to migrate to the length end of the gel. The samples correspond to immune precipitates obtained from cells after a: (A) 10-min pulse; (B) 20-min pulse; (C) 30-min pulse; (D) 30-min pulse and 15-min chase; (E) 30-min pulse and 30-min chase; (F) 30-min pulse and 60-min chase; (G) 30-min pulse and 120-min chase. The approximate molecular weights of the virus-specific proteins are indicated at the sides of the gel.*

nonspecifically and probably represents a cellular protein with high affinity for antigen antibody complexes. No nonspecifically precipitated D-[³H]glucosamine-labeled proteins were detected.

Multiple processing pathways in MuLV-infected cells. The results described above are consistent with the possibility that there are multiple pathways for processing the MuLV core proteins. Furthermore, different pathways appear to be favored under different conditions of cell growth. Figure 6 shows the results of an experiment in which cells nearing the end of their growth phase were pulse-labeled for 1 h, extracted, and immune precipitated with antisera to gp69/71, p30, and p12. Antiserum to gp69/71 (Fig. 6C) precipitated the two closely migrating 90,000-dalton gp69/71 precursors along with small amounts of the virion proteins gp69/71. Antisera directed against p12 (Fig. 6D) and p30 (Fig. 6E) precipitated the 65,000-dalton protein and two additional proteins of approximately 50,000 and 40,000 daltons. The 50,000- and 40,000-dalton proteins were not detected in any samples of the pulse-chase analysis of p30-related proteins in rapidly growing cells (Fig. 2). Furthermore, the proteins of 75,000 and 93,000 daltons, which were precipitated by antisera to both p30 and p12, were only slightly labeled in this experiment. This result lends further support to the suggestion that the 93,000-dalton protein is produced by a modification of the 75,000-dalton protein. The low amounts of the 75,000- and 93,000-dalton proteins likely reflect a low level of synthesis of the 75,000-dalton protein and consequently a low amount of the 93,000-dalton protein derived from it. It is conceivable that the levels of both the 75,000- and

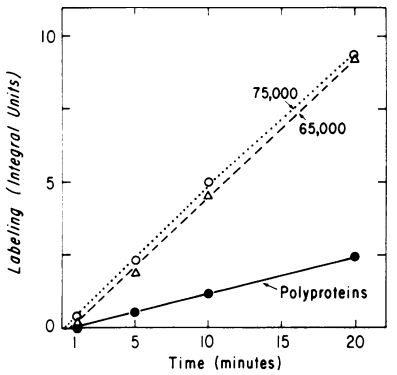

FIG. 3. *Quantitative kinetics of L-[^{35}S]methionine incorporation into virus-specific proteins. Eveline cells (1.4 × 10^6 cells/ml) were pulse-labeled with 50 μCi of L-[^{35}S]methionine per ml for 20 min. Samples were taken at 1, 5, 10, and 20 min and were immune precipitated with rabbit antiserum to MuLV-F by direct precipitation as described in the text. The precipitates were prepared for electrophoresis and subjected to electrophoresis on 10% polyacrylamide slab gels containing 8 M urea. Electrophoresis was continued until the dye front reached the end of the gel. The labeling was quantitated on the fluorographic films by densitometry as described in the text, and the intensities of the bands were plotted as a function of labeling time. The plots represent: (●) the high-molecular-weight 220,000- plus 230,000-dalton polyproteins; (○) the polypeptide of approximately 75,000 daltons; (△) the polypeptide of approximately 65,000 daltons.*

93,000-dalton components reflect a very rapid turnover of these proteins; however, this seems unlikely considering the stability of the 93,000-dalton component in pulse-chase studies (Fig. 2). In any event, the viral protein processing in this experiment appears to be different than that observed in the experiments with cells in the logarithmic phase of growth.

Several studies have indicated that cultured cells infected with MuLV release infectious virions only during periods of active growth and cease virus production in the G_0 state of proliferative arrest (15, 16, 20). This also seems to be true of Eveline cells. The release of virus ceased when the cells entered the stationary phase of the cellular growth cycle (Fig. 7). Furthermore, the dilution of stationary cells into fresh culture medium was followed within 8 h by a reproducible burst of infectious virus release into the medium. However, this burst of infectivity was not paralleled by a similar burst of radioactive virus release. These results suggest that stationary-phase cells, which were not releasing virus, may nevertheless contain appreciable amounts of virus proteins that are released only when the cells are provided fresh medium. Indeed, as shown by the pulse-chase experiment in Fig. 8, virus-specific proteins continued to be synthesized in cells that had been in the stationary phase for 24 h; however, the pattern of protein synthesis appears different from that observed in the growing cells. The predominant radioactive components are the 75,000-dalton protein and the late-labeling 93,000-dalton protein. The 65,000-dalton protein, which is a major component in logarithmically growing cells, was only slightly labeled in this experiment. The appearance of the prominent late-labeling 93,000-dalton protein is consistent with the proposal that it is derived from the 75,000-dalton protein. The large polyproteins, which were detected in all other experiments, were also present in relatively low amounts in this stationary-phase culture.

DISCUSSION

MuLV-F-specific proteins in Eveline cells. Several of our observations concerning MuLV-F proteins in Eveline cells are consistent with recent reports concerning processing of MuLV-R precursors (1, 2, 7, 13, 19, 21, 23). In particular, we have found two glycoprotein precursors of gp69/71 with apparent molecular weights of approximately 90,000 and *gag*-containing polyproteins with molecular weights of 75,000 and 65,000. In addition, we have observed two large polyproteins with molecular weights of 220,000 and 230,000, which appear similar to the large polyproteins with *gag* and *pol* antigens that have been previously described (1, 2). Large polyproteins containing gp69/71 antigens have also been detected in cells infected with MuLV-R (19) but were not observed in our experiments.

We have also detected a 40,000-dalton protein precipitable with antisera to p30 and p12 in cells near the end of their growth phase. A similar protein has been described by Barbacid et al. (4) in cells infected with MuLV-R. Since we do not detect the 40,000-dalton protein in rapidly growing cells, it does not seem to be an obligatory intermediate in *gag* processing. However, its existence suggests that the genes for p30 and p12 are adjacent on the MuLV-F genome.

Glycosylated polyproteins with p30 and p12 antigens. A major feature of these experiments is the detection of several glycosylated polyproteins containing *gag* antigens. Most abundant of these is a glycoprotein with an apparent molecular weight of 93,000, which is labeled after a long lag phase in pulse-chase experiments (Fig. 2). Since the large 220,000- and 230,000-dalton polyproteins are present in

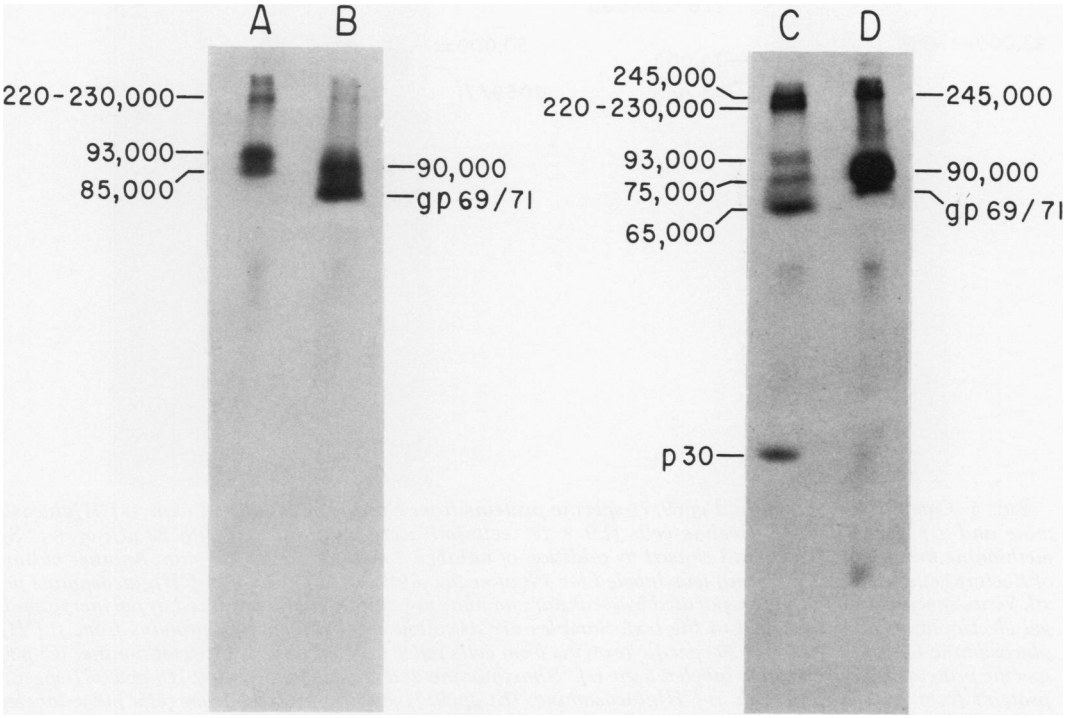

FIG. 4. *Analysis of virus-specific glycoproteins by affinity chromatography on the insolubilized lectin, RCA_I, covalently coupled to agarose. A logarithmically growing cell culture was labeled with 50 μCi of L-[^{35}S]methionine for 60 min, and the cell extract was prepared as described for lectin binding. A portion of the extract was used to isolate the glycoprotein fraction by affinity chromatography. Secondary immune precipitates were subjected to electrophoresis in 10% polyacrylamide slab gels containing 8 M urea and 0.1% SDS. The samples analyzed were as follows: (A) glycoprotein fraction precipitated with antiserum to p30; (B) glycoprotein fraction precipitated with antiserum to gp69/71; (C) whole-cell extract precipitated with antiserum to p30; (D) whole-cell extract precipitated with antiserum to gp69/71.*

insufficient amounts to serve as its precursor, we suggest that it is formed by glycosylation of one of the smaller *gag*-containing proteins (i.e., the 65,000- or 75,000-dalton proteins). Indeed, it seems likely that the 93,000-dalton glycoprotein derives from the 75,000-dalton protein, because variations in the amounts of these two proteins correlate well in cells growing under different conditions. In experiments with cells late in their growth phase, these two proteins are both formed in barely detectable amounts (Fig. 6), whereas in stationary-phase cells they are the major MuLV-specific proteins (Fig. 7). On the contrary, the amount of the 65,000-dalton protein appears to vary independently of the 93,000- and 75,000-dalton proteins. These data are also consistent with the conclusion that the 75,000- and 65,000-dalton proteins are not related in a simple precursor-product manner (see below).

In addition to the 93,000-dalton glycoprotein, our studies suggest that Eveline cells also contain a glycoprotein antigenically related to p30 with an apparent molecular weight of 85,000 (Fig. 4). Furthermore, the 220,000- and 230,000-dalton proteins with p30 and p12 antigens also bind to three different insolubilized lectins and therefore very likely contain some carbohydrate. The amount of carbohydrate in these proteins is possibly small relative to methionine, because they were not appreciably labeled with D-[^{3}H]-glucosamine (Fig. 5). Alternatively, the carbohydrate side chain of these polyproteins may contain only other sugars. Therefore, it appears that there may be four different polyprotein precursors of the internal core *gag* proteins that are glycosylated (molecular weights, 220,000, 230,000, 93,000, and 85,000). Ledbetter et al. (12) have recently described 95,000- and 85,000-dalton glycoproteins with p30 antigens on the surface membrane of leukemic AKR-strain mouse cells, but the synthesis of these glycoproteins was not analyzed. If the *gag*-containing glycoproteins in Eveline cells are similarly located in

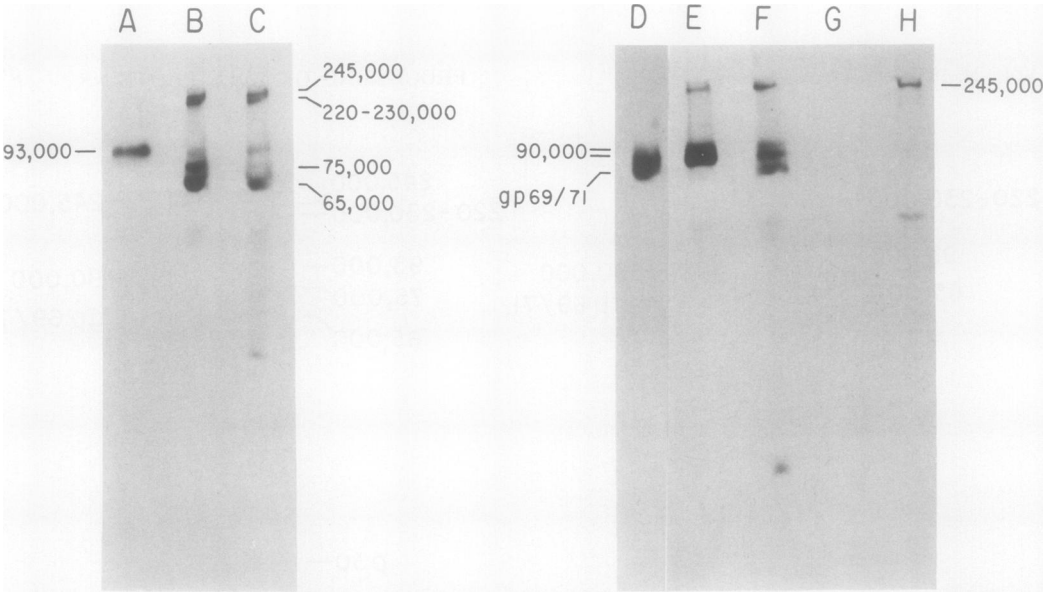

FIG. 5. *Comparison of p30- and gp69/71-specific proteins from Eveline cells labeled with D-[^{3}H]glucosamine and L-[^{35}S]methionine. Eveline cells (1.9 × 10^6 cells/ml) were pulse-labeled with 22 μCi of L-[^{35}S]-methionine per ml for 30 min and chased by addition of unlabeled methionine for 100 min. Another culture of Eveline cells (1.2 × 10^6 cells/ml) was labeled for 7 h after the addition of 17 μCi of D-[^{3}H]glucosamine per ml. Virus-specific proteins were separated by secondary immune precipitation and analyzed by polyacrylamide gel electrophoresis as described in the text. Samples are as follows: (A) p30-specific proteins from D-[^{3}H]-glucosamine-labeled cells; (B) p30-specific proteins from cells pulse-labeled with L-[^{35}S]methionine; (C) p30-specific proteins from cells pulse-labeled with L-[^{35}S]methionine and chased for 100 min; (D) gp69/71-specific proteins from cells labeled with D-[^{3}H]glucosamine; (E) gp69/71-specific proteins from cells pulse-labeled with L-[^{35}S]methionine; (F) gp69/71-specific proteins from cells pulse-labeled with L-[^{35}S]methionine and chased for 100 min; (G) proteins precipitated from D-[^{3}H]glucosamine-labeled cells, using nonimmune goat serum; (H) proteins precipitated from cells pulse-labeled with L-[^{35}S]methionine, using nonimmune goat serum. (Very long autoradiographic exposures demonstrate more clearly D-[^{3}H]glucosamine labeling of the p30-specific glycoprotein of 85,000 daltons as well as slight labeling of the 220,000- to 230,000-dalton polyproteins [A].)*

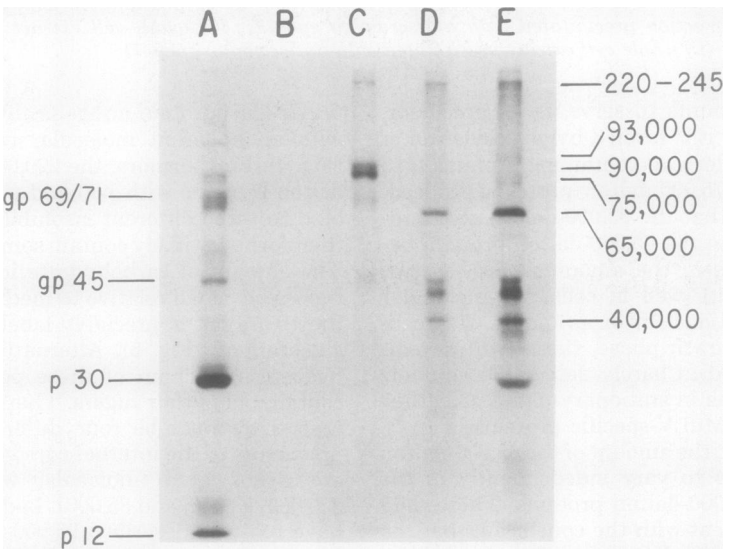

FIG. 6. *Precipitation of gp69/71-, p30-, and p12-specific proteins from a slowly growing culture of Eveline II cells. Eveline II cells growing slowly near to the end of their growth phase (1.8 × 10^6 cells/ml) were pulse-labeled with 10 μCi of L-[^{35}S]methionine for 1 h. Samples of the cell extract were precipitated by primary immune precipitation with nonimmune goat serum and antiserum made to gp69/71, p30, and p12. The immune precipitates were analyzed as described in the legend to Fig. 1. The sample wells contained: (A) L-[^{35}S]methionine-labeled MuLV-F; (B) precipitate obtained with nonimmune serum; (C) precipitate obtained with goat antiserum to gp69/71; (D) precipitate obtained with goat antiserum to p12; (E) precipitate obtained with goat antiserum to p30. The approximate molecular weights of virus-specific proteins are indicated at the right of the autoradiogram.*

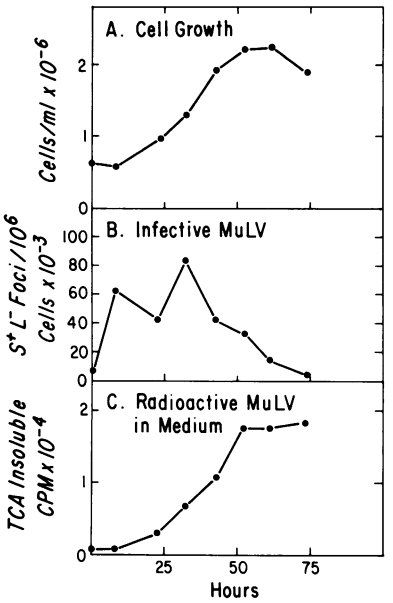

FIG. 7. *Production of infective and radiolabeled virus particles during cellular growth. Eveline II cells in the stationary phase (2.5 × 10⁶ cells/ml) were diluted into fresh medium containing 1.67 μCi of [¹⁴C]leucine per ml. Samples were taken at the indicated times, the cells were counted, and the medium was analyzed for MuLV and radioactive virus particles as described in the text. (A) Cell concentration; (B) concentration of infectious MuLV; (C) radioactive virus in the culture medium.*

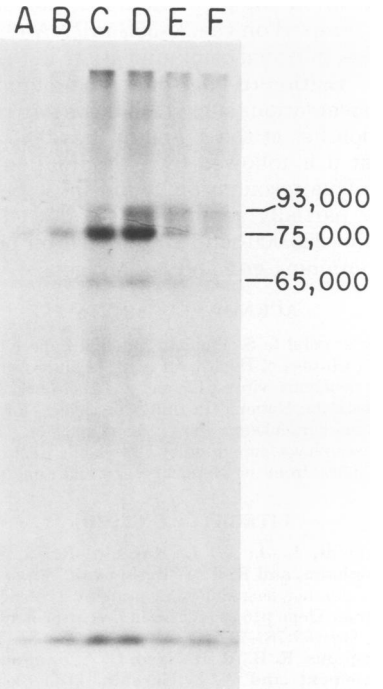

FIG. 8. *Pulse-chase analysis of MuLV-F-specific proteins in stationary-phase cells. An Eveline II cell culture in the stationary phase, which had ceased growing 24 h earlier, was pulse-labeled for 30 min with 50 μCi of L-[³⁵S]methionine per ml. The culture was chased for an additional 1 h by the addition of unlabeled methionine. Samples taken at various times were precipitated by primary immune precipitation with antiserum to MuLV-F and analyzed on slab gels as described in the legend to Fig. 1. The samples applied to the gels were precipitated from samples taken after a: (A) 5-min pulse; (B) 10-min pulse; (C) 20-min pulse; (D) 30-min pulse; (E) 30-min pulse and 30-min chase; (F) 30-min pulse and 60-min chase. The approximate molecular weights of the MuLV-specific proteins are indicated at the right of the autoradiogram.*

the cell membrane, then a part of the virion core protein processing may occur there. In some conditions of cellular growth (Fig. 7), the majority of the *gag* processing could take place in this region of the cell.

Multiple synthetic pathways for MuLV-specific proteins. Previous workers have considered the possibility that the MuLV proteins may derive from a single large precursor via a single pathway of sequential proteolytic cleavages. However, the large 220,000- and 230,000-dalton polyproteins we have detected are present in low amounts, and their turnover rates are too slow to provide a sufficient source for the smaller 65,000- and 75,000-dalton *gag* precursors. In addition, the 75,000- and 65,000-dalton *gag* proteins are both detected after very brief periods of L-[³⁵S]methionine incorporation, and they are both labeled with approximately linear kinetics between 0 and 20 min (Fig. 3). It therefore seems likely that these two proteins are released separately by ribosomes and that they are not related in a precursor-product manner. These considerations, along with our glycosylation data and our studies of processing at different stages of the cellular growth cycle, indicate that ribosomes in MuLV-infected cells release several different *gag*-containing polypeptides (large polyproteins, p65 and p75), which are metabolized along at least partially separate pathways.

Although our results suggest that four or five different MuLV-specific proteins are released directly by ribosomes, present evidence suggests that there may be two major virus-specific mRNA's, having sedimentation coefficients of 30-35S and 20S (8, 9, 11; H. Hanafusa, personal communication). The gp69/71 precursor is apparently synthesized on the 20S mRNA, which derives from the 3′ half of the 30-35S viral RNA,

whereas all of the *gag*-containing polyproteins are synthesized on the 30-35S mRNA (8, 11; H. Hanafusa, personal communication; L. Philipson and D. Baltimore, personal communication). Other observations suggest that the *gag* messenger region lies at the 5′ end of the 30-35S RNA and that it is followed by a termination codon (9, 24). This termination codon must be inefficient or partially suppressed to allow the production of 220,000- and 230,000-dalton polyproteins containing *gag* and *pol* antigens.

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