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Analysis of Intracellular Feline Leukemia Virus Proteins

I. Identification of a 60,000-Dalton Precursor of Feline Leukemia Virus p30[1]

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The synthesis and release of feline leukemia virus p30 was studied using a permanently infected feline thymus tumor cell line. Disrupted cells were divided into two subcellular fractions, a cytoplasmic extract (CE) representing cellular material soluble in 0.5% NP-40 and a particulate fraction (PF) insoluble in 0.5% NP-40, but soluble in 0.2% deoxycholate and 0.5% NP-40. Intracellular feline leukemia virus p30 was isolated from infected cells by immune precipitation with antiserum to p30 and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the precipitated proteins. Cells labeled for 3 h with [^{35}S]methionine contained equal amounts of p30 in both the CE and the PF. p30 synthesis was estimated to be 0.8% of the total host cell protein synthesis. Immune precipitates from cells pulse labeled for 2.5 min contained a labeled 60,000-dalton polypeptide (Pp60) in the PF and a polypeptide in the CE that comigrated with feline leukemia virus p30 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When cells were chased after a pulse label, there was a rapid loss of Pp60 in the PF and an accumulation of p30 in the CE within 30 min followed by distribution of p30 in both the PF and the CE. Estimation of intracellular and extracellular p30 levels during a 0.5- to 24-h chase period suggested that most of the newly synthesized p30 was incorporated into extracellular virus. Tryptic peptide analysis of labeled Pp60 and p30 demonstrated the presence of 13 of 15 p30 peptides within the Pp60 molecule. The tryptic peptide analysis in concert with the pulse-chase labeling data provides strong evidence that Pp60 is a precursor of p30.

The polypeptide composition of both the avian and mammalian oncornaviruses has been thoroughly studied in the past several years (13, 14, 17, 18, 25). The oncornaviruses contain five to seven major structural proteins with molecular weights ranging from 10,000 to 85,000 (5). Although the polypeptide composition of the oncornaviruses has been rigorously studied, information concerning the synthesis and processing of these polypeptides has appeared only recently (1, 10, 23, 24, 31, 33, 34).

Evidence obtained from picornavirus-, paramxovirus-, and reovirus-infected cells indicates that nononcogenic RNA virus mRNA is translated from a single initiation site (4). The mRNA of these viruses is well characterized (3, 6, 36). All three types of nononcogenic RNA virus mRNA are translated into polypeptides that correspond in size with the viral mRNA (6, 21, 22). In poliovirus-infected cells, the initial translation product is a large precursor polypeptide, which is subsequently cleaved to yield mature virion polypeptides (21).

Oncornaviruses contain a high-molecular-weight genome composed of 28-35S subunits with a molecular weight of approximately 3×10^6 (2, 7, 9). These RNA subunits contain 3' poly(A) sequences (7, 20, 27). The apparent ability of these subunits to serve as mRNA in in vitro protein-synthesizing systems (24, 30, 35), combined with the presence on polyribosomes of viral-specific RNA with a molecular weight similar to that of genomic subunits (12, 16, 32), suggests that oncornavirus mRNA is very similar to genomic subunits. If oncornavirus mRNA is translated in a manner similar to nononcogenic RNA virus mRNA, then one would expect an initial translation product of about 300,000 daltons.

Attempts to isolate the initial translation product of oncornavirus protein synthesis have been directed to in vitro protein-synthesizing systems and immunoprecipitation of viral polypeptides from infected cells. Various in vitro protein-synthesizing systems have been used

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with limited success (8, 30, 35). Recently, however, polypeptides (molecular weights of 140,000 to 185,000 and 50,000 to 75,000) have been synthesized using Rauscher leukemia virus (RLV) genomic RNA in a cell-free protein-synthesizing system (24), and 75,000- to 80,000-dalton polypeptides have been synthesized in response to added 30–40S RNA of Rous sarcoma virus (35). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of immune precipitates from avian myeloblastosis virus (AMV) (10, 33, 34)- and RLV (1, 31)-infected cells provides evidence for a 76,000-dalton precursor in the former and 200,000-, 80,000-, and 65,000-dalton precursor polypeptides in the latter. Although precursor polypeptides have been isolated, evidence for a 300,000-dalton precursor polypeptide is lacking.

The work reported here was undertaken (i) to determine whether a precursor polypeptide of feline leukemia virus (FeLV) p30 existed and (ii) to monitor the incorporation of intracellular p30 into extracellular virus. Data are presented that demonstrate a 60,000-dalton precursor polypeptide (Pp60) of FeLV p30 and suggest that most of the newly synthesized intracellular p30 is incorporated into extracellular FeLV.

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MATERIALS AND METHODS

Source of cells and virus. The permanently infected feline thymus tumor cell suspension (F-422) was used throughout these experiments. This cell line produces the Rickard strain of FeLV and was propagated as previously described (7).

Radioactive labeling of cells and FeLV. Labeled intracellular protein and extracellular FeLV were obtained from cells incubated for 3 or 20 h, respectively, with either [^{35}S]methionine, ^{3}H-amino acid mixture, or ^{14}C-amino acid mixture (New England Nuclear Corp.). All labeling was done at a starting cell density of 2×10^6 cell/ml with 1 μCi of isotope per 10^6 cells. Labeling with ^{3}H- or ^{14}C-amino acid mixtures was done in medium containing 10% of the normal supplement of amino acids. [^{35}S]methionine labeling was done in growth medium containing 5% of the normal supplement of methionine.

Pulse-chase labeling was done with cells previously incubated in methionine-deficient or amino acid-deficient growth media for 45 min to deplete the amino acid pools. The cells were then labeled for 2.5 min with [^{35}S]methionine or ^{14}C-amino acid mixture (1 μCi/10^6 cells) at a cell density of 50×10^6 cells/ml. The pulse was terminated by placing the labeled cells on frozen media containing 10 times the normal concentration of methionine or amino acids (chase medium), adding 20 volumes of cold chase medium, and collecting the cells by centrifugation.

Cells to be chased were then incubated in warm chase medium for various times at a cell density of 10^6 cells/ml.

Purification of virus. Cells were removed from the growth medium by centrifugation at 1,000 rpm for 5 min in an International PR-6 centrifuge. The growth medium was further clarified by centrifugation at 10,000 rpm for 10 min in a Sorvall GSA rotor. Clarified medium was then overlaid onto a discontinuous gradient consisting of 5 ml of 40% sucrose (wt/wt) in TNE buffer (0.01 M Tris-0.1 M NaCl-0.001 M EDTA, pH 7.5) and 5 ml of 20% sucrose (wt/wt) in TNE. The virus was banded on the 40% sucrose layer by centrifugation at 25,000 rpm for 1.5 h in an SW27 rotor (Beckman). The banded virus was collected, diluted with an equal volume of TNE buffer, and pelleted by centrifugation at 25,000 rpm for 1.5 h in an SW27 rotor. The viral pellet was resuspended in sample buffer or lysis buffer (see below and Fig. 1) for SDS-PAGE and detergent disruption, respectively.

Preparation of subcellular fractions. Cells were collected by centrifugation, washed in Hanks balanced salt solution, resuspended in lysis buffer (0.5% NP-40-0.15 M NaCl-0.01 M Tris, pH 7.4), vortexed for 20 s, and then incubated for 5 min at 4°C. The disrupted cells were then centrifuged at 2,400 rpm for 5 min in an International PR-6 centrifuge. The supernatant was removed and centrifuged at $100,000 \times g$ for 1 h in an SW50.1 rotor (Beckman). The $100,000 \times g$ supernatant (cytoplasmic extract) was carefully removed and the pellet was resuspended in lysis buffer containing 0.2% deoxycholate. The cytoplasmic extract (CE) was also made 0.2% deoxycholate in one experiment (see Fig. 6). Both the resuspended pellet and CE were rapidly freeze-thawed eight times. The solubilized pellet and the CE were then centrifuged at $100,000 \times g$ for 1 h in an SW50.1 rotor. The supernatant from the solubilized pellet was termed the particulate fraction (PF) or NP-40-insoluble fraction, whereas the CE was also termed the NP-40-soluble fraction.

Preparation of antisera. Antiserum to p30 was prepared as previously described (17). Antisera to bovine serum albumin (BSA) was obtained from E. Sanders (Michigan State Univ., East Lansing).

Immunodiffusion analysis. Double diffusion was performed, using 2% Noble agar (Difco), as previously described (17).

Immune precipitation. Antiserum used for immune precipitation was clarified by centrifugation at $100,000 \times g$ for 0.5 h in an SW50.1 rotor. Clarified antiserum was added to subcellular fractions or disrupted virus and incubated for 30 min at 37°C and then overnight at 4°C. Immune precipitates were collected by layering the incubation mixture over 1 ml of 5% sucrose (wt/wt) in lysis buffer, followed by centrifugation at 2,000 rpm for 20 min. The immune precipitates were resuspended in 0.5 ml of lysis buffer, layered over 5% sucrose, and centrifuged.

This was repeated one additional time. The final precipitate was solubilized for SDS-PAGE as described below, or trichloroacetic acid-precipitable radioactivity was assayed as previously described (17).

SDS-PAGE. Electrophoresis in the presence of 1% SDS was done using a 9% polyacrylamide gel similar to that described by Fairbanks et al. (11). Samples were solubilized in sample buffer (0.01 M Tris-hydrochloride–5 mM EDTA–1% SDS–2% mercaptoethanol) and heated for 3 min at 100°C. Electrophoresis was performed at 70 V for 3 h. The gels were fractionated and assayed for radioactivity as previously described, (17), using 3a70B scintillation cocktail (Research Products International Corp., Elk Grove Village, Ill.).

Tryptic peptide analysis. Immune precipitates from cells pulse labeled with ^{3}H-amino acids were electrophoresed in the presence of 1% SDS. The gels were fractionated into 2-mm slices as described above, and the polypeptides, were eluted with 0.4 ml of 0.1% SDS at 37°C for 24 h. Small portions of each fraction were assayed for radioactivity to locate labeled polypeptides. ^{3}H-amino acid-labeled FeLV was prepared and electrophoresed, and p30 was eluted in a similar manner.

The eluted polypeptides plus 1 mg of BSA as carrier were precipitated with 15% trichloroacetic acid and 1 volume of ethanol. The precipitated protein was centrifuged and the pellet was washed four times with ethanol and once with ether. The final pellet was dried under a stream of nitrogen.

The precipitated protein was oxidized as described by Hirs (19) with 1 ml of performic acid (4.5 ml of formic acid plus 0.5 ml of 30% hydrogen peroxide kept at 25°C for 1.5 h) for 1 h at 4°C. A total of 15 ml of distilled water was added, followed by lyophilization. The lyophilized proteins were resuspended in 15 ml of distilled water and lyophilized again.

The oxidized proteins were resuspended in 3 ml of 0.15 M NH_4HCO_3 containing 300 μg of tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemical Corp.) and 10 μl of toluene and then incubated for 4 h at 37°C. An additional 300 μg of TPCK-treated trypsin was added and digestion was continued for 15 h at 37°C. The digested polypeptides were lyophilized, resuspended in 3 ml of distilled water, and lyophilized again. The digested peptides were stored at −76°C.

Cation-exchange chromatography of the tryptic peptides was done by a modification of the technique of Schroeder (28), using a high-pressure column of type P chromobeads (Technicon) maintained at 52.5°C. The tryptic peptides were suspended in 1.5 ml of pH 3.1 buffer (16 ml of pyridine and 278 ml of acetic acid per liter) and then centrifuged at 1,000 rpm for 5 min to remove insoluble cores. The peptides were loaded onto the column under pressure developed from a 30-ml disposable syringe and tight-fitting tygon tubing. The peptides were eluted with a linear gradient of 300 ml of pH 3.1 buffer and 300 ml of pH 5.0 buffer (161 ml of pyridine and 143 ml of acetic acid per liter) at a flow rate of 30 ml/h. Fractions (3 ml) were collected, evaporated at 60°C, and radioactively assayed with 10 ml of 3a70B scintillation cocktail.

RESULTS

Immune precipitation of FeLV p30 from disrupted virus. [^{35}S]methionine-labeled FeLV was prepared and electrophoresed in the presence of 1% SDS. The polypeptide profile (Fig.

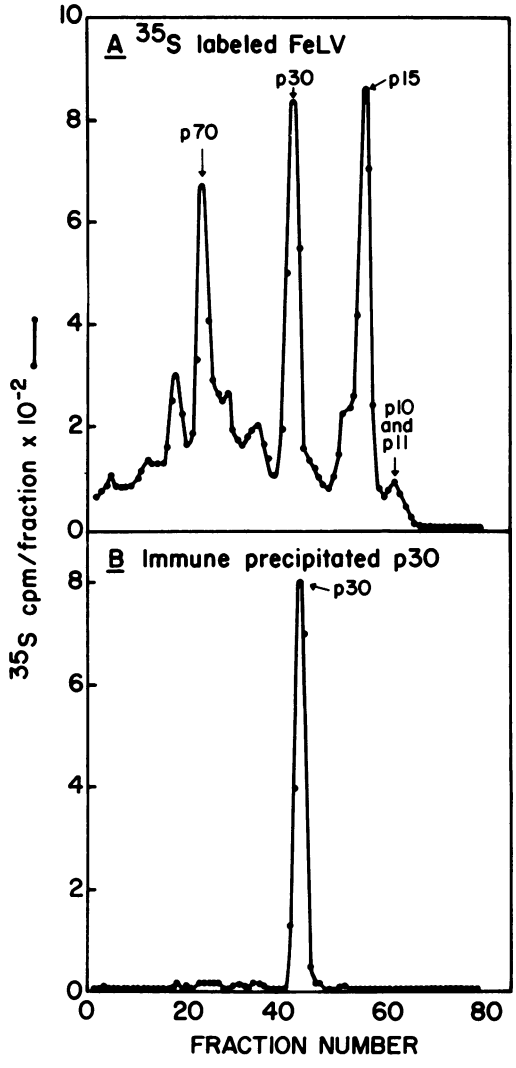

FIG. 1. *SDS-PAGE of immune precipitated p30 from NP-40-disrupted FeLV. ^{35}S-labeled FeLV was prepared from 100 × 10^6 cells incubated with 100 μCi of [^{35}S]methionine for 24 h in 50 ml of growth medium. The virus was purified as described in Materials and Methods. (A) FeLV (20,000 cpm) was resuspended in sample buffer and electrophoresed; (B) FeLV (30,000 cpm) was resuspended in lysis buffer, incubated for 0.5 h at 37°C, and then rapidly freeze-thawed 15 times. The disrupted virus was incubated with 200 μl of anti-p30, and an immune precipitate was collected, resuspended in sample buffer, and electrophoresed, as described in Materials and Methods.*

1A) obtained was similar to that seen with ^{3}H-amino acid-labeled FeLV (17; see Fig. 4). There was, however, little methionine label in the p10 and p11 position of the profile. A shoulder on the high-molecular-weight side of the p15 peak was routinely seen and may correspond to the previously reported p21 of FeLV (17). The majority of label was distributed among p15, p30, and p70.

To demonstrate the specificity of anti-p30, [^{35}S]methionine-labeled FeLV was disrupted with 0.5% NP-40 and 15 rapid freeze-thaw cycles and then immune precipitated. This disruption procedure solubilizes all of the major structural proteins, except p70 (Okasinski and Velicer, manuscript in preparation). SDS-PAGE of the immune precipitate (Fig. 1B) demonstrated a single polypeptide, which migrated at the position of FeLV p30. Immune precipitation of ^{14}C-amino acid-labeled FeLV yielded similar results (data not shown). The data indicated that antiserum to p30 was monospecific with respect to FeLV structural proteins. Control experiments using 5 μg of BSA and 200 μl of anti-BSA showed virtually no precipitation of labeled viral proteins, indicating little or no nonspecific trapping.

Immunodiffusion of intracellular proteins. A CE and a PF were prepared and examined for the presence of p30 by immunodiffusion with anti-p30. Both the CE and the PF were positive for p30, as judged by the presence of a line of identity with disrupted FeLV (Fig. 2A). This antiserum had previously been shown to be monospecific with respect to FeLV proteins in both immune precipitation (Fig. 1B) and immunodiffusion (17). Antiserum to BSA was used in similar immunodiffusion experiments, with no precipitin lines evident (data not shown).

Estimation of the level of intracellular p30 synthesis. To estimate the percentage of host cell protein synthesis directed toward synthesis of p30, a PF and a CE from long-term labeled cells (200×10^6) were each divided into equal portions (counts per minute per portion) and incubated with increasing amounts of anti-p30. The immune precipitable counts per minute in each portion of the CE and PF are expressed as the percentage of total counts per minute (counts per minute in the CE portion and counts per minute in the PF portion). The CE contained 90% of the total counts per minute in this experiment (data not shown). Maximal immune precipitation occurred with 50 μl of antiserum (Fig. 3), which was equivalent to 400 μl of anti-p30 to maximally immune precipitate intracellular p30 from the CE or PF of 100×10^6 cells. The data in Fig. 3 indicated that approximately 0.8% of the total host cell protein synthesis was directed toward production of FeLV p30. The data further suggested that intracellular p30 was equally distributed between the CE (an NP-40-soluble form) and the PF (an NP-40-insoluble form) and indicated a 10-fold enrichment of intracellular p30 in the PF relative to the total counts per minute present in this fraction. Nonspecific precipitation was determined from a parallel experiment employing 5 μg of BSA per portion and increasing anti-BSA. To-

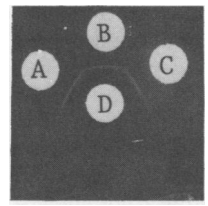

FIG. 2. *Immunodiffusion of the CE, PF, and NP-40-disrupted FeLV with anti-p30. Wells A, B, and C contained CE, NP-40-disrupted FeLV, and PF, respectively. Well D contained anti-p30. The CE and PF were prepared from 100×10^6 cells disrupted with 0.6 ml of lysis buffer, as described in Materials and Methods. NP-40-disrupted FeLV was prepared from unlabeled virus as described in the legend to Fig. 1.*

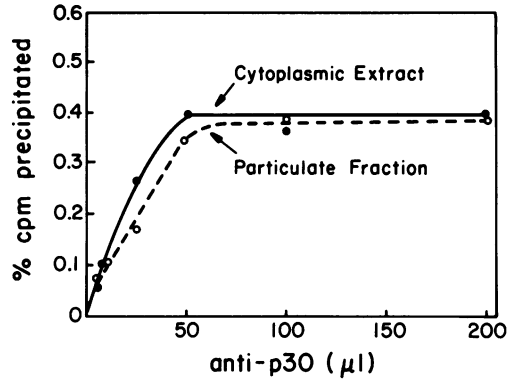

FIG. 3. *Maximal immune precipitation of intracellular p30. A total of 250×10^6 cells were labeled for 3 h with 250 μCi of ^{3}H-amino acid mixture in 125 ml of growth medium, and a CE and a PF were prepared as described in Materials and Methods. Each subcellular fraction was divided into six aliquots (counts per minute per aliquot), and 5 μg of unlabeled NP-40-disrupted virus (prepared as described in the legend to Fig. 1) was added. The aliquots were incubated with either 5, 10, 25, 50, 100, or 200 μl of anti-p30, and immune precipitates were collected as described in Materials and Methods. The immune precipitates were resuspended in 1% SDS and the radioactivity was assayed. The counts per minute of immune precipitates from each aliquot are expressed as the percentage of total counts per minute (counts per minute of a CE aliquot plus counts per minute of a PF aliquot).*

tal nonspecific precipitation was less than 5% of the anti-p30 immune precipitable counts per minute. The total counts per minute (counts per minute in CE plus counts per minute in PF) in this experiment represented greater than 95% of the total trichloroacetic acid-precipitable counts per minute incorporated during a 3-h labeling period (data not shown) and indicated that the cell fractionation procedure allowed examination of greater than 95% of the total protein content of these cells.

SDS-PAGE of intracellular p30 immune precipitates from long-term labeled cells. SDS-PAGE of immune precipitates from the CE and PF of long-term labeled cells routinely yielded a labeled polypeptide that comigrated with FeLV p30 (Fig. 4A and C). In addition, two very small peaks (a and b) are consistently seen in both experimental and control (Fig. 4B and D) profiles. Two polypeptides that migrate slower than polypeptide a were consistently seen in p30 immune precipitates from the CE. The nature of these polypeptides is unknown, but they may represent host polypeptides noncovalently linked to intracellular viral p30. Noncovalent association of host polypeptides with intracellular oncornavirus polypeptides has been reported in Rous sarcoma virus transformed hamster cells (15).

Immune precipitation and SDS-PAGE of intracellular p30 from pulse-chase-labeled cells. Pulse-chase labeling in concert with SDS-PAGE of immune precipitates was done to determine whether a high-molecular-weight precursor of p30 exists and to monitor the release of intracellular labeled p30 into extracellular virus. Cytoplasmic extracts and particulate fractions were prepared from pulse-labeled and pulse-chase-labeled cells followed by immune precipitation with anti-p30. Phenyl methyl sulfonyl fluoride (Sigma Chemical Co.) was added to the lysis buffer to prevent proteolytic cleavage during preparation of subcellular fractions.

When cells were pulse labeled for 2.5 min, the immune precipitate from the PF (Fig. 5A) contained a single polypeptide with a molecular weight of 60,000 (Pp60), whereas the immune precipitate from the CE (Fig. 6A) contained a

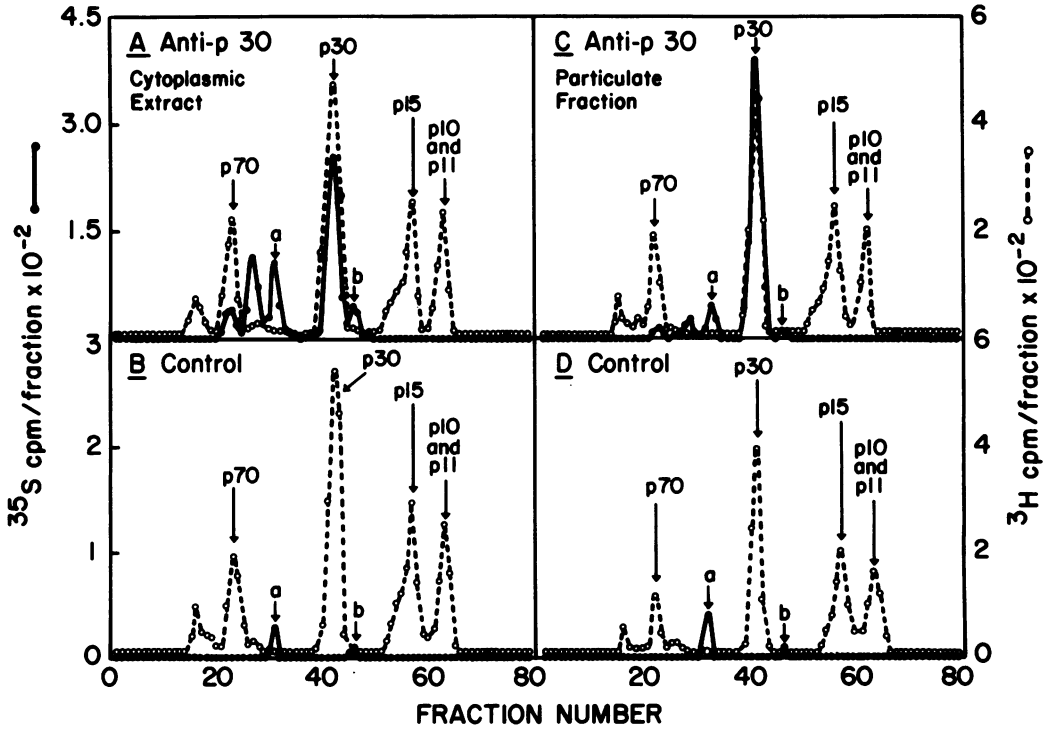

FIG. 4. *SDS-PAGE of immune precipitates from the CE and the PF of long-term-labeled cells. A CE and PF were prepared from 100 × 10⁶ cells labeled for 3 h with 100 μCi of [³⁵S]methionine in 50 ml of growth medium, as described in Materials and Methods. The CE and the PF were each divided into two equal portions (counts per minute per portion) and incubated with either 200 μl of anti-p30 or with 5 μg of BSA and 200 μl of anti-BSA. The immune precipitates were collected and coelectrophoresed with ³H-amino acid-labeled FeLV as described in Materials and Methods. (A) CE and anti-p30; (B) PF and anti-p30; (C) CE with 5 μg of BSA and anti-BSA; (D) PF with 5 μg of BSA and anti-BSA.*

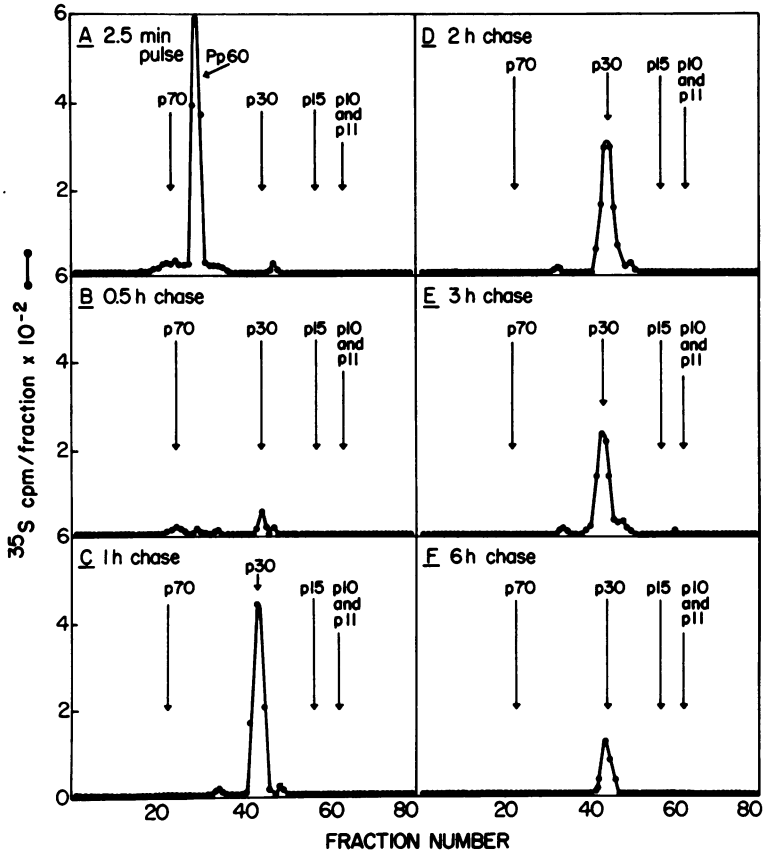

FIG. 5. *SDS-PAGE of immune precipitates from PFs of pulse-chase-labeled cells. A total of 700 × 10⁶ cells were pulse labeled for 2.5 min with 1 mCi of [³⁵S]methionine and then divided into seven aliquots, one of which was lysed immediately, whereas six were chased for periods of 0.5, 1, 2, 3, 6, and 24 h (the cells in each chase aliquot were counted and adjusted to contain 100 × 10⁶ cells). A PF fraction was prepared from both pulse-labeled and pulse-chase-labeled cells, using lysis buffer containing 300 μg of phenyl methyl sulfonyl fluoride per ml, as described in Materials and Methods. NP-40-disrupted FeLV (5 μg) was added to each PF followed by 500 μl of anti-p30. Immune precipitates were collected and coelectrophoresed with ³H-amino acid-labeled FeLV as described in Materials and Methods. Arrows indicate positions of nonglycosylated ³H-amino acid-labeled FeLV polypeptides. (A) PF from cells pulse labeled for 2.5 min; (B to F) PFs from cells chased for 0.5 to 6 h.*

single polypeptide that comigrated with FeLV p30. Control experiments employing BSA and anti-BSA yielded a polypeptide profile (data not shown) identical to that seen with long-term-labeled subcellular fractions (Fig. 4B and D). SDS-PAGE of immune precipitates from cells chased for 0.5 h yielded quite different profiles. The PF contained only low levels of a polypeptide that comigrated with FeLV p30 (Fig. 5B), whereas the CE contained a 30,000-dalton polypeptide in quantities much greater than seen at the end of the pulse (Fig. 6B).

The PF and CE from cells chased for 1 h contained immune precipitable p30 (Fig. 5C and 6C). The loss of labeled p30 seen in the CE during the 0.5- to 1-h chase interval could be accounted for by the appearance of labeled p30 present at 1 h in the PF (Fig. 5C) and by the labeled p30 recovered as extracellular FeLV at 1 h (Fig. 7).

Immune precipitates of cells chased for 1, 2, and 3 h contained p30 in both the PF (Fig. 5C to E) and the CE (Fig. 6C to E). The level of immune precipitable p30 in the PF decreased during this 2-h chase period, whereas the level of p30 in the CE remained relatively constant (several experiments indicated a slight loss of immune precipitable p30 in the CE). The level of immune precipitable p30 in the PF and the CE at the 6-h interval (Fig. 5F and 6F) was relatively equal and both were reduced as compared with the 3-h chase interval. No labeled

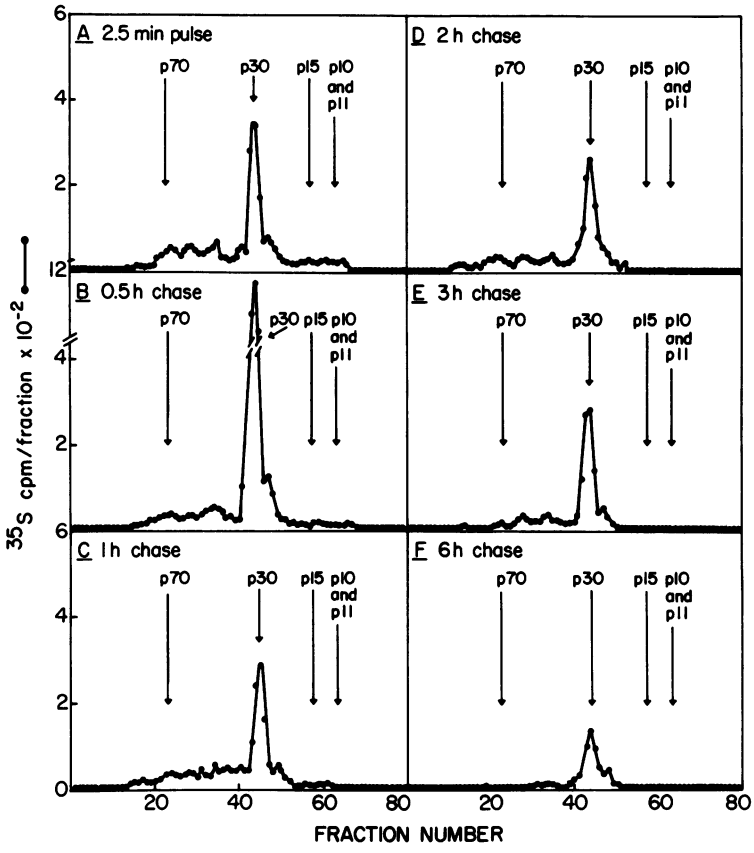

FIG. 6. *SDS-PAGE of immune precipitates from CEs of pulse-chase-labeled cells. CEs were prepared from the same 700 × 10⁶ cells labeled as described in the legend to Fig. 5, using lysis buffer containing 300 μg of phenyl methyl sulfonyl fluoride per ml, as described in Materials and Methods. Deoxycholate was added to a final concentration of 0.2%. NP-40-disrupted FeLV (5 μg) was added to each CE followed by 500 μl of anti-p30. Immune precipitates were collected and coelectrophoresed with ³H-amino acid-labeled FeLV, as described in Materials and Methods. Arrows indicate positions of nonglycosylated ³H-amino acid-labeled FeLV polypeptides. (A) CE from cells pulse labeled for 2.5 min; (B to F) CEs from cells chased for 0.5 to 0.6 h.*

p30 could be recovered at the 24-h chase time (data not shown). Deoxycholate was added to the cytoplasmic extracts (NP-40-soluble fraction) in this experiment (Fig. 6) to demonstrate that Pp60 is found only in the PF under identical detergent conditions. Identical results to those presented in Fig. 6 were obtained in the absence of deoxycholate (data not shown).

To determine whether labeled extracellular FeLV p30 was contaminating the subcellular fractions, a mixing experiment was done employing purified labeled FeLV and unlabeled cells. When 50,000 cpm of purified [^{35}S]-methionine-labeled FeLV was incubated with 100 × 10^6 cells prior to fractionation, no ^{35}S label could be recovered in the subcellular fractions (data not shown). The absence of [^{35}S]methionine-labeled intracellular p30 in the subcellar fractions after a 24-h chase also argued against extracellular contamination of the CE and PF. The influence of labeled p30 associated with newly assembled FeLV in the process of budding from the surface of these cells could not be determined.

Analysis of intracellular and extracellular p30 levels during pulse-chase labeling of cells. The levels of intracellular and extracellular p30 were monitored during a 0.5- to 24-h chase period after a 2.5-min pulse (Fig. 7) to follow the incorporation of intracellular p30 into extracellular FeLV. Extracellular p30 levels were determined by collecting FeLV from the chase media in the previous experiment (Fig. 5 and 6). Unlabeled carrier virus (100 μg) was added to each chase portion (0.5, 1, 2, 3, 6, and 24 h), and the virus was purified and electrophoresed in the presence of SDS. The amount of [^{35}S]-methionine-labeled p30 was determined from

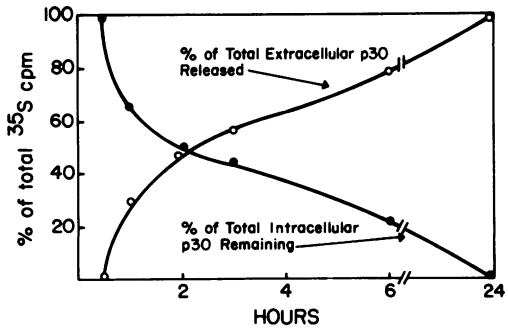

FIG. 7. *Analysis of intracellular and extracellular p30 levels during a 0.5- to 24-h chase period after a 2.5-min pulse. The levels of p30 are expressed as the percentage of total intracellular or extracellular p30. Total intracellular p30 is the level of p30 found in both the CE and PF of cells chased for 0.5 h (determined from Fig. 5 and 6). Total extracellular p30 is the entire amount of virion-associated FeLV p30 released during a 0.5- to 24-h chase after a 2.5-min pulse (determined from SDS-PAGE profiles of labeled FeLV released during this experiment). Symbols: ●, percentage of total labeled immune precipitable intracellular p30 found in both the PF and the CE; ○, percentage of total labeled extracellular FeLV p30.*

the resulting electropherograms (electropherograms not shown). The 24-h chase portion provided the total extracellular FeLV p30 value, whereas all remaining values were represented as a percentage of the total extracellular FeLV p30 (Fig. 7). All values for extracellular FeLV were corrected for virus released during the first 0.5 h of each chase period by subtracting the amount of labeled p30 appearing in FeLV at the 0.5-h time point. This analysis, therefore, examined the appearance of labeled extracellular FeLV p30, using the 0.5-h chase time as a starting point and the 24-h chase time as the total.

Intracellular p30 levels during the 0.5- to 24-h chase period were obtained from the data shown in Fig. 5B to F and 6B to F. The 0.5-h chase time provided the total (100%) intracellular level of p30 in this analysis. The counts per minute comigrating with viral p30 from both the CE and PF were used to determine the level of intracellular p30 at each chase time. The level of p30 at each chase time is represented as the percentage of total intracellular p30.

An analysis of the data presented in Fig. 7 indicated that at a time (approximately 2.0 h) when 50% of the total extracellular viral p30 appeared, approximately 50% of the total intracellular p30 was no longer detectable. The data suggested that the intracellular p30 obtained in these experiments was indeed a precursor of extracellular FeLV.

Tryptic peptide analysis. High-pressure, cation-exchange chromatography of tryptic peptides was employed to determine whether labeled peptides of FeLV p30 were also found in Pp60. ^{3}H-amino acid-labeled Pp60 was used for this analysis to insure labeling of all peptides. The tryptic peptide map (Fig. 8) contained 25 ^{3}H-labeled peptides present in Pp60, whereas ^{3}H-labeled p30 contained 15 peptides. Of the 15 peptides present in ^{3}H-labeled p30, 13 are also present in Pp60. These chromatograms of labeled tryptic peptides indicated that p30 is found within the Pp60 molecule.

DISCUSSION

Evidence from tryptic peptide analysis and pulse-chase labeling demonstrates that Pp60 is a rapidly cleaved precursor of FeLV p30. An examination of intracellular and extracellular p30 levels during pulse-chase labeling experiments suggests that newly synthesized p30 is subsequently distributed into an NP-40-soluble and -insoluble form and indicates that most of the intracellular p30 is assembled into extracellular virus.

Results of SDS-PAGE of immune precipitates from FeLV-infected cells demonstrated the presence of intracellular p30 in both an NP-40-soluble (CE) and -insoluble (PF) form (Fig. 4). Solubilization of p30 from the PF by the use of 0.2% deoxycholate suggests a membrane association; however, further experimentation will be required to confirm this point. A similar distribution of intracellular oncornavirus proteins between a membrane fraction and a cytoplasmic extract has been reported for murine sarcoma-leukemia virus-infected rat cells (29). Pp60 appears to be limited to the PF and is only observable upon double-detergent treatment (0.5% NP-40 and 0.2% deoxycholate). This inferred membrane association of an oncornavirus precursor polypeptide has been observed by other workers. AMV-infected primary chick fibroblasts synthesize a 76,000-dalton precursor polypeptide, whose cleavage in vitro can be inhibited by membrane-dissociating agents (34). Van Zaane et al. (31) have also described two membrane-associated precursor polypeptides in JLS-V9 cells infected with RLV.

The results of pulse-chase labeling experiments (Fig. 5 and 6) indicate that Pp60 is rapidly cleaved to form a large pool of NP-40-soluble p30 (CE), which subsequently becomes distributed between the CE and the PF. The presence of Pp60 in the PF and p30 in the CE at the end of a 2.5-min pulse could also suggest that

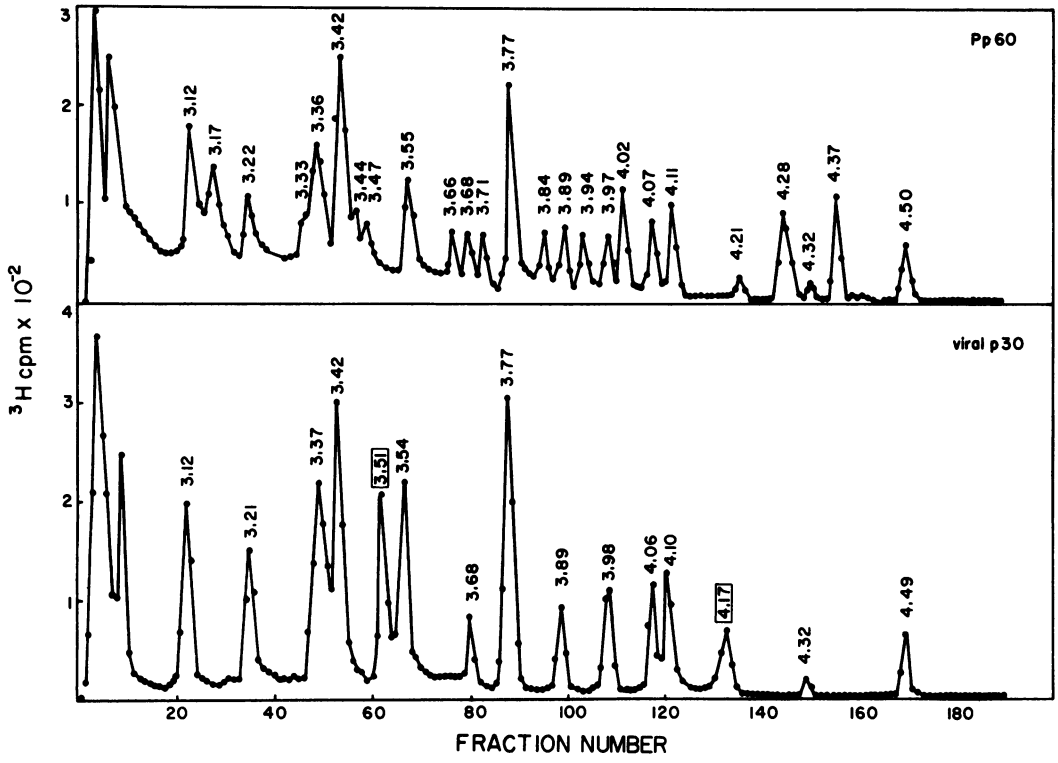

FIG. 8. *Tryptic peptide analysis of Pp60 and FeLV p30 eluted from SDS-polyacrylamide gels. Labeled Pp60 was immunoprecipitated from the PF of 500 × 10^6 cells pulse labeled for 2.5 min with 500 μCi of ^{3}H-labeled amino acid mixture. The immune precipitate was electrophoresed in parallel with ^{3}H-amino acid-labeled FeLV, as described in Materials and Methods. The labeled polypeptides were eluted from the gels and tryptic peptides were prepared as described in Materials and Methods. The recovery for the entire procedure was 70 to 80%. Various peptide peaks are identified by pH of elution determined from reading pH values with a PHM 26 expanded-scale pH meter (Radiometer, Copenhagen, Denmark).* □, *Elution pH values of p30 tryptic peptides absent in Pp60.*

p30 is synthesized both as part of a large precursor and as a mature virion polypeptide. Preliminary pulse-chase labeling experiments employing a general protease inhibitor (manuscript in preparation) suggest that inhibition of Pp60 cleavage is associated with a decrease of labeled immune precipitable p30 at the end of a 2.5-min pulse, which would argue against synthesis of p30 as a mature virion polypeptide.

The relatively rapid loss of intracellular p30 from the PF during the 1- to 3-h chase period, combined with a slow loss of p30 from the CE during this interval, suggests that extracellular FeLV p30 may arise from an NP-40-insoluble form of intracellular p30. The data (Fig. 5 and 6), however, do not rule out the possibility that NP-40-soluble p30 is directly assembled into extracellular FeLV. The well-documented cell surface assembly of the oncornaviruses seems to favor a membrane association of viral proteins during assembly and could be taken as supportive of our interpretation of these results.

The existence of two intracellular pools of p30, differing in solubility in NP-40 and apparently in the kinetics of incorporation into extracellular virus, raises several possibilities. For example, the appearance of an initial large pool of labeled p30 in the CE after pulse labeling (Fig. 6) may suggest further posttranslational processing of intracellular p30 undetectable in these experiments. The slow release of p30 from the CE into extracellular FeLV may suggest some functional role for p30 within these cells or may reflect host regulation of intracellular p30 levels.

The analysis of intracellular and extracellular p30 levels presented in Fig. 7 indicates that most of the immune precipitable p30 synthesized and processed during a 2.5-min pulse and 30-min chase is subsequently assembled into extracellular virus. The 30-min chase time was

chosen as the starting point for this analysis because it provided the highest level of labeled intracellular p30 among the times examined. An earlier starting time was not possible due to the simultaneous cleavage of Pp60 and the appearance of p30. Approximately 5% of the total extracellular viral p30 is released during a 30-min chase of pulse-labeled cells (data not shown), which indicates that the initial level of intracellular p30 is at least 5% higher than was determined in this analysis.

Tryptic peptide analysis of Pp60 and p30 demonstrates that Pp60 contains p30 (Fig. 8). Using a similar experimental approach, AMV-infected cells have been shown to contain a 76,000-dalton precursor polypeptide (10, 33, 34). Precursor polypeptides have also been reported in RLV-infected cells by two different groups of workers (23, 31). One group (23) originally reported the existence of 140,000-, 65,000-, and 50,000-dalton precursors of RLV p30 in JLS-V16 cells. More recently the same group detected the existence of an approximately 200,000-, as well as 80,000- and 65,000-, dalton precursor of RLV p30 in the same cell system, using a different SDS-PAGE system and the analysis of a limited number of methionine-labeled tryptic peptides (1). A second group, using JLS-V9 cells, could only detect 72,000- and 65,000-dalton precursors of RLV p30. Although a final judgment must await tryptic peptide analysis of RLV precursors identified by both groups, preferably with larger numbers of peptides than can be labeled with methionine as pointed out by Arcement et al. (1), their results may reflect host cell influence on precursor processing. Recently, Oskarsson et al. (26) have suggested that a 60,000-dalton polypeptide at the FeLV pseudotype of Maloney sarcoma virus may be an uncleaved precursor of Maloney sarcoma virus FeLV p30. Although the evidence from avian, murine, and feline oncornavirus is consistent in demonstrating precursor polypeptides within infected cells, the processing of these precursors ranges from the very rapid rate reported here to an aberrant cleavage resulting in the incorporation of large amounts of a possible uncleaved precursor into assembled virions (26). These differences in processing indicate that it may be essential to investigate not only both avian and mammalian oncornavirus precursor polypeptides but also the effects of various host cells upon precursor processing.

If oncornavirus mRNA is equivalent to the 3 $\times 10^6$-dalton viral genome subunits (12, 16, 31) and translated from a single initiation site, one would expect a precursor polypeptide with a molecular weight of approximately 300,000. The failure to detect such a giant precursor polypeptide in our experiments may be due to several factors: (i) the p30 antigenic determinant of such a large polypeptide may be inaccessible to antibody; (ii) Pp60 may be a cleavage product of a nascent polypeptide; (iii) antibody prepared against p30 purified by gel filtration in guanidine hydrochloride may not contain antibody binding sites to all native p30 antigenic determinants, thus lowering the efficiency of immune precipitation; (iv) the hypothesized 300,000-dalton precursor may not exist.

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