THE SYNTHESIS OF THE STRUCTURAL PROTEINS OF FELINE LEUKEMIA VIRUS

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Leland F. Velicer

Major professor

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ABSTRACT

THE SYNTHESIS OF THE STRUCTURAL PROTEINS
OF FELINE LEUKEMIA VIRUS

By

Gregory F. Okasinski

The main structural protein of feline leukemia virus (FeLV) is a 30,000 dalton polypeptide termed p30. The synthesis of FeLV p30 was studied using a permanently infected feline thymus tumor cell line (F-422). Intracellular proteins 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 p30 was isolated from these subcellular fractions by immune precipitation with antiserum to FeLV p30 and subsequent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). When cells were labeled for 3 h with ^{35}S methionine, equal amounts of p30 were found in both subcellular fractions. Immune precipitates from pulse labeled cells contained a 60,000 dalton protein (Pp60) in the PF and FeLV p30 in the CE. When pulse labeled cells were chased the label seen at the 60,000 dalton position was rapidly lost while the

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level of radioactivity in the p30 position increased. Examination of the intracellular and extracellular p30 levels during a 0.5-24 h chase period suggested that most of the intracellular p30 was assembled into extracellular virus. Tryptic peptide analysis of Pp60 and viral p30 taken together with the kinetic data provides strong evidence that Pp60 is a precursor of FeLV p30.

The rapid loss of radioactivity from Pp60, following pulse labeling, could be inhibited by the general protease inhibitor, phenyl methyl sulfonyl fluoride (PMSF). This inhibition was found only to occur if PMSF was present during pulse labeling. When cells were grown in the presence of the proline analogue, L-azetidine-2-carboxylic acid, a 70,000 dalton polypeptide (Pp70) was found in addition to Pp60 upon immune precipitation of pulse labeled cells. Intracellular Pp70 and Pp60, and FeLV virion p70, p30, p15, p11, and p10 were subjected to tryptic peptide analysis. Pp70 and virion p70 were identical under this type of analysis and both contained the tryptic peptides of FeLV p30, p15, p11, and p10, while Pp60 lacked those of p11. The results of pactamycin gene ordering experiments indicated that the small structural proteins of FeLV are ordered p11-p15-p10-p30. The data indicates that the small structural proteins of FeLV are synthesized as part of a 70,000 dalton precursor

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polypeptide. A cleavage scheme for the generation of FeLV p70, p30, p15, p11, and p10 from precursor polypeptides is proposed.

THE SYNTHESIS OF THE STRUCTURAL PROTEINS OF FELINE LEUKEMIA VIRUS

By

Gregory F. Okasinski

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DEDICATION

To Chris and Kim

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MANUSCRIPT II - Analysis of Intracellular Feline Leukemia Virus Proteins. II. The Generation of FeLV Structural Proteins from Precursor Polypeptides

INTRODUCTION

Feline leukamia virus (FeLV) contains five nonglycosylated proteins, four of which have been purified by gel filtration in guanidine hydrochloride. The most abundant of these proteins has a molecular weight of 30,000 daltons (p30). Antiserum to p30 previously prepared in this laboratory is monospecific for this particular viral protein.

A permanently infected feline thymus tumor cell suspension was used in these experiments. In these cells synthesis of viral specific proteins occurs in addition to the synthesis of host cell macromolecules. This biological relationship necessitates the availability of a specific immune probe for the isolation of intracellular viral proteins.

The general approach of this work was to employ antiserum to p30 as a specific immune probe and pulse-chase radioactive labeling to provide a population of readily identifiable newly synthesized and mature intracellular proteins. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the resulting immune precipitates would provide evidence concerning the size of molecules carrying

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p30 antigenic determinants in the two populations of intracellular proteins.

The principal objective of this work was to determine if FeLV p30 was synthesized as part of a high molecular weight precursor polypeptide. Just prior to initiating this work, a single report appeared (80) which provided evidence for precursor proteins in avian oncornavirus infected cells. Our experimental approach yielded presumptive precursors of 60,000 and 70,000 daltons. Tryptic peptide analysis was used to confirm the biochemical relationship between the precursor proteins and FeLV p30. Further experiments provided evidence that four of the five nonglycosylated proteins were synthesized as part of a 70,000 dalton precursor.

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LITERATURE REVIEW

Since numerous reviews of oncorvaviruses have appeared during the past few years, this review will concentrate on literature concerning the basic phenomenon investigated, that being post-translational cleavage and processing.

Post-Translational Cleavage of Eukaryotic Proteins

The earliest and best known examples of post-translational cleavage are found in the blood clotting and complement cascade systems. In the blood clotting system the conversion of prothrombin to thrombin proceeds via the action of Stuart factor (extrinsic pathway) or by the action of Hageman factor (intrinsic pathway). Thrombin itself acts enzymatically to convert fibrinogen to fibrin and thus complete clot formation (53). A second important plasma protein, plasma kinin, is generated by post-translational cleavage of inactive precursors. The pharmacologically active kinins are very small peptides (nine amino acid residues) generated by cleavage at basic amino acid residues. The inactive precursors are very

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large polypeptides of greater than 50,000 molecular weight (21, 34).

The complement system is a third example of this ubiquitous phenomenon of extracellular generation of biologically active peptides from inactive precursors. The complement system is composed of nine fragments termed C1-C9 which are generated in a cascading fashion from enzymes within the system. There are at least five known enzymes in the complement system--C1r, C1s, C42, C423, and C3 activator. These all exist as inactive precursors which become activated to act enzymatically on other inactive precursors of the complement cascade (19, 41).

In recent years it has become apparent that there are large precursor like polypeptide forms of many of the small biologically active peptide hormones. These large forms have been isolated in many cases from both the synthesizing cell of origin and from the circulation. Work by Steiner et al. (68) has clearly demonstrated that proinsulin is a precursor of insulin. These workers have obtained the amino acid sequence of bovine proinsulin and found it to be a single polypeptide chain ordered: NH_2-B chain-Arg-Arg-C peptide-Arg-A chain-COOH. Proinsulin is cleaved to yield insulin at the paired basic residues by trypsin like enzymes (68).

The clearest biochemical evidence for biosynthetic precursor-product relationships for peptide hormones

exists in the case of parathyroid hormone (PTH) (18, 35) and glucagon (47, 74). PTH, long recognized as a prime factor in the regulation of calcium metabolism, is a simple polypeptide of 84 amino acid residues (18). Classical biosynthetic experiments employing pulse-chase labeling, immune precipitation, tryptic peptide mapping, and amino acid analysis have been used to demonstrate the synthesis of PTH. The PTH precursor is 109 amino acids long of which 84 are retained in the active hormone (18, 35). Cleavage of the precursor occurs by a trypsin like enzyme at the NH_2- terminus. Glucagon, important as a hyperglycemic agent as well as a stimulator of insulin secretion, also appears to be synthesized as a precursor (proglucagon) (47) whose size and properties are not entirely established. A strong candidate for a possible fragment of proglucagon is an isolated 37 residue peptide from bovine glucagon (74). This peptide contains 29 NH_2-terminal residues which are identical to bovine glucagon with eight additional residues at the COOH- terminus. This molecule is believed to be a biosynthetic intermediate of glucagon (74).

In addition to PTH and glucagon, large prohormone molecules have been identified for both vasopressin (55) and B-melanocyte stimulating hormone (B-MSH) (15). While direct biosynthetic evidence for precursors of these hormones is lacking, strong suggestive evidence exists for them. The evidence for precursors of vasopressin comes

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from labeled amino acid incorporation studies that demonstrate continual incorporation of amino acids into vasopressin long after protein synthesis is inhibited by puromycin (55). Additional evidence indicates that large immunoreactive forms of vasopressin are seen in gel filtration experiments (55). The best evidence for precursors of B-MSH is the isolation of a 90 residue peptide containing the residues of the mature hormone (15).

There is ample evidence for functional proteolytic cleavage of other eukaryotic proteins in addition to those of the small biologically active peptides. The synthesis and assembly of collagen is a well studied example of this phenomenon (7). Collagen is synthesized as a precursor molecule (procollagen) that readily assembles into the triple helical structure of tropocollagen. This molecular conformation can then readily undergo specific extracellular cleavage prior to assembly into collagen fibers (7). Evidence is accumulating which suggests that albumin may be synthesized as a proalbumin moiety containing five additional residues at the albumin NH_2-terminus (76). Immunoglobulin light chains also appear to be synthesized as precursor molecules (59). These precursors contain a leucine rich, hydrophobic, 20 residue peptide extending from the light chain NH_2-terminus.

This brief survey of eukaryotic protein processing serves to illustrate the ubiquitous nature of

post translational cleavage of linear precursor molecules. More examples will probably be found as other systems are investigated. One can conclude that eukaryotic cells utilize post-translational cleavage to generate and control the level of some biologically active proteins. As will be seen in the following sections, the availability of protein processing machinery is a valuable aid for virus replication. This host cell proteolytic machinery allows the infecting virion to maintain genetic simplicity by obviating the need for virion coded protein processing systems.

Post-Translational Cleavage of Picornavirus Proteins

The generation of mature virion proteins from high molecular weight precursor polypeptides is a well documented phenomenon observed in many animal virus infected cells. Post-translational cleavage of virus proteins was first described for poliovirus infected cells (31, 70). Poliovirus is a small "plus" strand RNA virus of the picornavirus group. The virus contains four main structural proteins termed: VP-1, VP-2, VP-3, and VP-4, with molecular weights of 35,000, 28,000, 23,000, and 6,000 respectively (30). The mRNA of poliovirus does not contain a blocked 5'-terminal structure and is identical to the virion RNA in size and base composition and sediments as a 35S molecule in neutral sucrose gradients (27, 48).

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The mRNA of poliovirus has been estimated to code for 2 to 3 x 10^5 daltons of protein (30). If poliovirus mRNA is translated from a single initiation site, then one would expect the initial translation product to be a polyprotein of 2 to 3 x 10^5 daltons.

When poliovirus infected cells are pulse labeled with radioactive amino acids, polypeptides of 34,000, 100-105,000, and 125-130,000 (NCVP-X, NCVP-1½ and NCVP-1, respectively) daltons are detectable by sodium dodecyl sulfate polyacrylamide gel electrophoresis (31, 70). Mature virion structural proteins are unlabeled under these conditions (31, 70). Pulse chase labeling of these cells revealed a rapid loss of label from NCVP-1 and the appearance of label in the viral structural proteins. These experiments strongly suggested a precursor product relationship between NCVP-1 and the structural proteins of poliovirus (30, 31, 70). When cells are grown in the presence of the phenylalanine analog, p-fluorophenylalanine, the loss of label in NCVP-1 can be prevented. The analog also inhibits the incorporation of label into the structural proteins (30). A large poliovirus polyprotein could be detected in cells treated with chymotrypsin inhibitors (38, 72), amino acid analogs (30), or diisopropyl fluorophosphate (30). Under these conditions a polyprotein of greater than 200,000 daltons (NCVP-00) could be isolated (30, 38, 72). Jacobson et al. (30) concluded that this

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polyprotein may represent the entire genome of poliovirus. The initial translation product of poliovirus mRNA translation is believed to be NCVP-00 (30, 38, 72).

This polyprotein is believed to undergo proteolytic cleavage as a growing nascent polypeptide (4). This proteolytic activity has been termed "nascent" cleavage (4). These nascent cleavages result in the formation of NCVP-X, NCVP-1, and NCVP-1½. When monkey kidney cells or HeLa cells are infected with poliovirus, chymotrypsin inhibitors do not prevent nascent cleavage in the former but do in the latter (38). This evidence indicates that at least part of the proteolytic activity is supplied by the host cell.

A combination of pulse chase labeling experiments and tryptic peptide analysis (30, 31, 70) as well as immune precipitation of in vivo and in vitro cleavage products (38) has provided a cleavage scheme for the generation of the structural proteins of poliovirus. The structural proteins (VP-1 - VP-4) are formed by sequential cleavage of NCVP-1 (27, 30, 31, 70). The first cleavage results in the formation of VP-1 and a precursor termed NCVP-3. NCVP-3 is further cleaved to form VP-3 and VP-0. VP-0, a procapsid protein, gives rise to VP-2 and VP-4 in an assembly related cleavage step. The precursors NCVP-X and NCVP-1½ are believed to give rise to the nonstructural proteins responsible for virus replication.

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Encephalomyocarditis virus (EMC), another picornavirus, directs the synthesis of its structural proteins in a manner similar to that described for poliovirus (11, 12). This virus contains five structural proteins termed α, β, γ, σ, and ε with molecular weights of 34,000, 30,000, 25,000, 6,700, and 42,000, respectively (10, 12). The primary translation products of EMC mRNA are three precursor proteins termed A, F, and C with molecular weights of 100,000, 38,000, and 84,000, respectively (12). These proteins are very similar to poliovirus NCVP-1, NCVP-X and NCVP-1½ generated by nascent cleavage of NCVP-00. Like poliovirus, all of the EMC structural proteins are generated by sequential cleavage of one precursor (EMC protein A) (11, 12). The major difference between the two viruses is an additional cleavage step of EMC protein A to yield proteins B and H. Protein B gives rise by a single cleavage step to structural protein α and D1. This step is analogous to the cleavage of NCVP-1 to form NCVP-3 and VP-1. EMC protein D1 is cleaved to form structural protein ε and γ in a manner similar to the formation VP-0 and VP-1 from poliovirus NCVP-3. Protein ε, a minor component of the virus, is cleaved to form σ and β. This step is analogous to the formation of VP-2 and VP-4 from VP-0 in poliovirus infected cells. Both ε and VP-0 are minor components of their respective viruses and are incorporated into virions as uncleaved precursors (11, 12,

30). A similar sequential cleavage scheme has also been demonstrated for human rhinovirus-1A (HRV-1A) and for Mengo virus infected cells (11, 51). One distinguishing feature of poliovirus and EMC, HRV-1A, and Mengo virus is the absence in the latter of a giant polyprotein analogous to NCVP-00 (4, 12, 72).

Although only poliovirus infected cells contain a detectable giant polyprotein, several lines of evidence indicate that the other picornavirus mRNAs are translated in a similar fashion. If picornavirus mRNA is translated into giant polyproteins which undergo nascent cleavages, several features of the translation products should be apparent. These translation products should arise from a single initiation site on the mRNA. If translation proceeds via a single initiation site, equimolar production of the three primary proteins (EMC precursors A, F, and C and poliovirus precursors NCVP-1, NCVP-X, and NCVP-1½) should be found <u>in vivo</u>. Analysis of the products of EMC and mengo virus RNA stimulated cell free translation products indicates that these RNAs are translated from a single initiation site (49). Oberg and Shatkin (49) labeled cell-free translation products with Met-$tRNA_m^{met}$ and with fMET-$tRNA_f^{met}$. Analysis of tryptic digests of the labeled translation products yielded a single labeled peptide when labeling with fMet-$tRNA_f^{met}$ and 30 tryptic peptides labeled with Met-$tRNA_m^{met}$ precursor (49). Analysis of the <u>in vivo</u>

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translation products from EMC, poliovirus, and HRV-1A infected cells demonstrated that the three primary proteins of these viruses were present in equimolar amounts (11, 12). However, examination of mengo virus infected cells failed to yield equimolar amounts of the three primary proteins (51). The reason for this discrepancy is unclear. The isolation of poliovirus NCVP-00 combined with the demonstration of a single initiation site of cell-free protein synthesis and the presence of equimolar amounts of the three primary proteins of these viruses provides strong evidence that picornavirus mRNA is translated from a single initiation site into giant polyproteins.

Assuming a single initiation site for picornavirus mRNA translation, the gene order of the primary and mature virion proteins can be determined by the use of pactamycin, a specific inhibitor of proteins synthesis initiation (71, 73). Radioactive labeling of proteins synthesized in the presence of pactamycin will result in selective incorporation of the label into the COOH terminal end of the molecule. This experimental approach results in extensive inhibition of labeling of residues near the NH_2-terminus and thus reflects the order of corresponding nucleotide sequences of the 5'→3' mRNA. This technique has been used by several workers as a means of genetic mapping of poliovirus genes (13, 42, 54, 71, 73). Pactamycin genetic mapping of poliovirus provides the gene order:

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NH_2—NCVP-1 - NCVP-X - NCVP-1½ - COOH for the three primary proteins (73). The corresponding proteins of EMC and HRV-1A have the same order (13, 42). The four major structural proteins of poliovirus, formed by cleavage of NCVP-1, are ordered: NH_2 - VP-4 - VP-2 - VP-3 - VP-1 - COOH (54). The corresponding EMC structural proteins are ordered NH_2 - σ - β - γ - α - COOH (13) as are the analogous proteins of HRV-1A (42).

The picornaviruses are very similar in polypeptide composition, translation, post-translational processing, and gene arrangement. The most likely explanation for the role of post-translational cleavage seems to be that proposed by Jacobson and Baltimore (31), namely that eukaryotic protein synthesizing machinery is unable to carry out internal initiation in the translation of polycistronic mRNA. Since these post-translational cleavages are highly specific they can effectively eliminate the requirement for multiple initiation sites on polycistronic messages.

Post-Translational Cleavage of Alphavirus Proteins

Although the picornaviruses were the first group of viruses rigorously examined for post-translational cleavage, alphavirus mRNA is translated in a similar manner. The alphaviruses are "plus" strand viruses with a 42S genomic RNA and a 26S mRNA (65). These viruses contain

three structural proteins, the core protein (CP) and two envelope proteins (EP1 and EP2) with molecular weights of 30,000, 45,000, and 53,000, respectively (52). The two alphaviruses most frequently studied have been Sindbis virus and Semliki Forest virus (SFV).

Using a temperature sensitive mutant of Sindbis virus, Scheele and Pfefferkorn (60) were able to demonstrate the accumulation of a 130,000 dalton putative precursor at the expense of the viral structural proteins in pulse labeled cells. Tryptic peptide analysis was used to confirm a precursor product relationship between the 130,000 dalton protein and Sindbis virus CP, EP1, and EP2 (61, 62). This precursor has been isolated more recently without the aid of a temperature sensitive mutant in Sindbis virus infected HeLa cells (66). Both direct and indirect evidence exists for a single initiation site on Sindbis virus 26S mRNA (14, 62). Pulse labeling in the presence of pactamycin selectively inhibits incorporation of radioactive amino acids into the CP, thus inferring a single site of protein synthesis initiation (62). Recent work by Cancedda et al. (14), employing incorporation of labeled methionine from fMET-tRNA$_f^{met}$ into cell free translation products, demonstrates a single labeled peptide upon tryptic peptide analysis of 26S mRNA stimulated translation products (14). These same workers, however, could detect two initiation sites by the same technique when Sindbis

virus 42S genomic RNA was used to stimulate cell free protein synthesis.

The translation of SFV mRNA is very similar to that seen with Sindbis virus mRNA. SFV infected cells have proven to be more efficient for the study of post-translational cleavage, hence a more complete picture is available. The structural proteins CP, EP1, EP2, and a small glycoprotein EP3 (10,000 daltons) are synthesized as part of a 130,000 dalton precursor (36, 39, 40, 43). This precursor undergoes several cleavage steps to generate both the CP and EP1. A 62,000 dalton cleavage product of the 130,000 dalton polypeptide is relatively stable and ultimately gives rise to EP2 and EP3 (43, 67).

Pactamycin gene ordering experiments provide the gene order: NH_2-CP-EP3-EP2-EP1-COOH for the structural proteins of SFV (40). This order is analogous to that of Sindbis virus with the exception of the additional component EP3 of SFV. Analysis of tryptic digests of cell free translation products labeled with ^{35}S fMET-$tRNA_f^{met}$ indicates a single initiation site for translation of SFV 26S mRNA (16).

While no giant polyprotein analogous to poliovirus NCVP-00 has been isolated in alphavirus infected cells, the generation of viral structural from precursors of 130,000 daltons is similar to that in poliovirus infected cells. The suggestive evidence from pactamycin gene ordering

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experiments and selective labeling of cell free translation products with fMET-tRNA$_f^{met}$ hint that alphavirus mRNA may indeed be translated into an as yet undetectable giant polyprotein.

Post-Translational Cleavage of DNA Virus Proteins

Post-translational cleavage of DNA virus proteins appears to be linked to virus assembly rather than as a means of translating a polycistronic mRNA. Vaccinia virus infected cells contain precursor proteins P4a and P4b of structural proteins 4a and 4b, respectively (32, 33, 44). Pulse chase labeling (32) and tryptic peptide analysis (44) were used to establish a precursor-product relationship. Rifampicin inhibited the formation of 4a from P4a (32, 33). Continued rifampicin treatment resulted in the incorporation of P4a into assembled virions as a core component (33). Kinetic evidence and the exclusive particulate nature of 4a have led Moss et al. to conclude that cleavage of P4a occurs immediately prior to or concomitantly with virus assembly (33).

The post-translational cleavage of adenovirus-2 proteins also appears to be linked to virus assembly. The conversion of five low molecular weight precursors to mature virion proteins is associated with the conversion of empty capsids to mature virus particles (1, 29, 83).

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The available data from DNA virus precursor proteins suggests that post-translational cleavage is associated with virus morphogenesis in a manner reminiscent of T4 protein maturation (20). This is in contrast to the primary role of post-translational cleavage in the picornaviruses and the alphaviruses. The brief review of endogenous eukaryotic proteolytic mechanisms presented initially gains perspective when taken in concern with "plus" strand RNA virus mRNA translation. The pre-existing proteolytic machinery of eukaryotic cells has not only eliminated the requirement of these viruses to code for all of the enzymes necessary for protein maturation, but has allowed the translation of viral polycistronic mRNA without evolutionary selection, by eukaryotes, for the ability to translate mRNA with internal initiation sites. This molecular arrangement thus allows the translation of a cumbersome viral mRNA molecule by a more evolutionarily advanced host cell.

Post-Translational Cleavage of RNA Tumor Virus Proteins

During the past three years there have been several reports of the existence of high molecular weight precursors of oncornavirus structural proteins. Before reviewing this evidence, a general summary of oncornavirus structure and replication will be presented.

Oncornavirus Structure

These enveloped viruses contain a 60-70 S RNA genome, which under denaturation conditions sediments as 28—35S subunits (75). A unique feature of the oncornaviruses is the presence of an RNA directed DNA polymerase, which makes possible the synthesis of a full length DNA copy of the virus genome (75).

The avian and mammalian oncornaviruses have a rather complex protein structure which is, however, similar among both groups of viruses. The avian oncornaviruses contain five nonglycosylated structural proteins termed: p10, p12, p15, p19, and p27 (9). Oncornavirus proteins are designated p or gp for protein or glycoprotein with molecular weight values times 10^{-3} (e.g., p30 = p30,000) (3). The mammalian viruses contain four nonglycosylated proteins: p10, p12, p15, and p30 (9). Recently a fifth nonglycosylated protein, termed p15E, has been identified for the murine viruses (28). These viruses generally contain a major glycoprotein, gp85 in the avian viruses and gp69/71 in the mammalian viruses as well as a minor glycoprotein, gp37 and gp45 in the avian and mammalian viruses, respectively (9).

Recently two groups of workers (6, 8, 17, 84) have determined the genetic complexity of avian oncornavirus genomic RNA as well as physical and genetic maps of the genome. Evidence from chemical analysis (8) and genetic

recombination (6) demonstrates that the 60-70S genome is polyploid with a genetic complexity of 3.5 x 10^6 daltons. RNAase T1 oligonucleotide mapping reveals a unique arrangement of the oligonucleotides demonstrating that all 35S subunits are similar in sequence (8). T1 oligonucleotide mapping of recombinant avian leukosis and sarcoma viruses with specific markers for the viral glycoprotein, the sarcoma phenotype, and the RNA directed DNA polymerase produced the gene order: 5'- nonglycosylated structural proteins - RNA directed DNA polymerase - envelope glycoproteins - sarcoma gene product - 3' (84).

Oncornavirus Replication

The existence of a virion RNA directed DNA polymerase suggests that these viruses synthesize a DNA copy of their genetic information. Viral specific DNA sequences have been detected in infected cells in a stable integrated association with host cell DNA (5, 78). The obvious link in the conversion of ssRNA to ds integrated DNA is a ds nonintegrated DNA intermediate. This unintegrated DNA, termed proviral DNA, has recently been isolated as a closed circular form and is a precursor of the integrated form of the viral specific DNA (23, 26).

The evidence to date indicates that shortly after infection viral DNA is made in the cytoplasm of infected cells. This DNA is a linear or open circular form similar in length to genomic RNA subunits. Viral DNA migrates to

the nucleus and assumes a closed circular form just prior to its integration into host cell DNA (26). The integrated DNA can serve as template for the production of "plus" strand RNA which could be translated into viral proteins and assembled into mature viral proteins (75).

Translation of Oncornavirus mRNA

Evidence from murine leukemia virus infected cells indicates that oncornavirus mRNA may consist of three distinct molecules which sediment as 35S, 21S, and 16S entities (22, 25, 79). These molecules are all poly-A containing, polyribosome associated RNAs and presumably should function as mRNAs. The mechanism for generating these size classes of mRNA is unknown, however, recent work (Nicholaus Mueller-Lantzsch personal communication) (63) suggest that a post-translational cleavage of oncornavirus mRNA may occur. If the 35S mRNA molecule is functional, and were translated 5'→3' from a single initiation site, one would expect a product of 250-300,000 daltons.

In examining oncornavirus translation products one is severely hampered by the immortalizing nature of this viral infection. Viral protein synthesis accounts for approximately 2% of the total host macromolecular synthesis. This biological relationship necessitates a specific immune probe for viral translation products. This probe has invariably been antibody generated against

viral structural proteins. The inadequacy of this probe lies in its inability to monitor non-virion associated proteins coded for by the virus.

During the past three years, evidence has accumulated that oncornaviruses direct the synthesis of their structural proteins as precursors which are cleaved to form mature viral proteins. Vogt and Eisenman first reported the isolation of an oncornavirus precursor polypeptide (80). These workers used a combination of pulse-chase labeling and immune precipitation with antisera to avian myeloblastosis virus (AMV) to isolate a 76,000 dalton precursor (Pr76) of AMV structural proteins. Tryptic peptide analysis of Pr76 and AMV confirmed this precursor product relationship (80). More recent work employing tryptic peptide analysis of Pr76 and cleavage intermediates with tryptic peptide maps of purified AMV structural proteins is evidence that Pr76 is a precursor of AMV p10, p14, p17, and p28 (81). Two groups of workers have been able to synthesize Pr76 in cell-free translation systems stimulated with AMV genomic RNA (56, 57, 82).

Work with mammalian oncornaviruses has been limited, almost exclusively, to Rauscher leukemia virus infected cells. One group of workers have been able to isolate 200,000, 90,000, 80,000 and 65,000 dalton precursors of the structural proteins of this murine leukemia virus (2, 45). These workers have been able to translate RLV genomic RNA

in a cell-free system into 180,000 dalton products (46). Recently the genomic RNA of Moloney murine leukemia virus (MLV) has been translated in a cell-free system (37). Polypeptides of 60,000, 70,000, and 160,000 daltons were detected in response to added MLV RNA (37). This work (2, 37, 45, 46) employing both in vivo and in vitro methods is the only support for a giant polyprotein as the initial translation product of oncornavirus mRNA. A second group of workers using similar techniques both in vivo and in vitro have not been able to detect translation products larger than 82,000 daltons (24, 58,77). These results are in direct conflict with the isolation of a 200,000 dalton oncornavirus polyprotein (2, 45). The reasons for these conflicting results are not clear. Three recent reports have appeared which support 60-70,000 dalton precursors of RLV nonglycosylated structural proteins. Stephenson et al. (69) have isolated a 60-70,000 dalton protein in temperature sensitive mutants of RLV infected cells, which appears to be an uncleaved precursor of RLV p30, p15, and p12. Mouse L929 cells also contain a 70,000 dalton uncleaved precursor of murine p30 (50). Shapiro et al. (64) were able to identify a 65,000 dalton precursor of RLV p30 and p15 in RLV infected rat kidney cells as well as a 90,000 dalton precursor of the main glycoprotein (gp69/71). These infected cells also contained a 260-300,000 dalton immune precipitable polypeptide, which could

not be confirmed by tryptic peptide analysis to be a precursor polyprotein.

When the work described in manuscript I and II was initiated, a single report of oncornavirus precursor polypeptides existed. We have found (manuscript I and II) that FeLV infected cells contain 60,000 and 70,000 dalton precursors of FeLV p30, p15, p11, and p10. Our findings are consistent with those of AMV infected (56, 57, 80, 81, 82) cells and with the work of several other workers (24, 50, 58, 64, 69, 77). Oncornavirus mRNA translation remains an unresolved phenomenon. Further work will be necessary to reconcile the appearance of large polyproteins in some oncornavirus infected cells and the absence of these polyproteins in many other oncornavirus systems.

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MANUSCRIPT I

Analysis of Intracellular Feline Leukemia Virus Proteins. V. Identification of a 60,000 Dalton Precursor of FeLV p30

By

Gregory F. Okasinski and Leland F. Velicer

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ANALYSIS OF INTRACELLULAR FELINE LEUKEMIA VIRUS PROTEINS. I. IDENTIFICATION OF A 60,000 DALTON PRECURSOR OF FeLV p30

Gregory F. Okasinski and Leland F. Velicer

Department of Microbiology and Public Health

Michigan State University

East Lansing, MI 48824

Running Title: FeLV p30 precursor polypeptide

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ABSTRACT

The synthesis and release of feline leukemia virus (FeLV) 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 FeLV p30 was isolated from infected cells by immune precipitation with antiserum to p30 and subsequent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 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 which co-migrated with FeLV p30 in SDS-PAGE. 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 0.5-24 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.

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INTRODUCTION

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 5-7 major structural proteins with molecular weights ranging from 10,000-85,000 daltons (5). While the polypeptide composition of the oncornaviruses has been rigorously studied, information concerning the synthesis and processing of these polypeptides has only recently appeared (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, 36, 6). All three types of nononcogenic RNA virus mRNA are translated into polypeptides which 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).

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of approximately 3 x 10^6 daltons (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 of polyribosomes of viral specific RNA with a mol. wt. 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 with limited success (8, 30, 35). Recently, however, polypeptides (mol. wts. of 140,000-185,000 and 50,000-75,000) have been synthesized using Rauscher leukemia virus (RLV) genomic RNA in a cell free protein synthesizing system (24), and 75,000-80,000 dalton polypeptides 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

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200,000, 80,000 and 65,000 dalton precursor polypeptides in the latter. While precursor polypeptides have been isolated, evidence for a 300,000 dalton precursor polypeptide is lacking.

The work reported here was undertaken (1) to determine if a precursor polypeptide of FeLV p30 existed, and (2) to monitor the incorporation of intracellular p30 into extracellular virus. Data is presented which demonstrates a 60,000 dalton precursor polypeptide (Pp60) of FeLV p30 and suggests that most of the newly synthesized intracellular p30 is incorporated into extracellular FeLV.

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 was obtained from cells incubated for 3 h 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 1μCi of isotope/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 of ^{14}C amino acid mixture (1μCi/10^{6} cells) at a cell

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density of 50 x 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 1000 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 overlayed onto a discontinuous gradient, consisting of 5 ml of 40% sucrose (wt/wt) in TNE buffer (0.01M Tris, 0.1M NaCl, 0.001M 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 a SW 27 rotor (Beckman). The banded virus was collected, diluted with an equal volume of TNE buffer, and pelletized by centrifugation at 25,000 rpm for 1.5 h in a SW 27 rotor. The viral pellet was resuspended in sample buffer or lysis buffer (see below and Figure 1) for SDS-PAGE and detergent disruption respectively.

Preparation of subcellular fractions. Cells were collected by centrifugation, washed in Hank's balanced salt solution (HBSS), resuspended in lysis buffer (0.5% NP-40, 0.15 M NaCl, 0.01 M Tris, pH 7.4), vortexed for 20 sec, and then incubated for 5 min at 4°C. The disrupted cells were then centrifuged at 2400 rpm for 5 min in an International PR-6 centrifuge. The supernate was removed and centrifuged at 100,000 x g for 1 h in a SW 50.1 rotor (Beckman). The 100,000 g supernate (cytoplasmic extract) was carefully removed and the pellet resuspended in lysis buffer containing 0.2% deoxycholate. The cytoplasmic extract (CE) was also made 0.2% deoxycholate in one experiment (Figure 6). Both the resuspended pellet and CE were rapidly freeze-thawed 8 times. The solubilized pellet and the CE were then centrifuged at 100,000 x g for 1 h in a SW 50.1 rotor. The supernate from the solubilized pellet was termed the particulate fraction (PF) or NP-40 insoluble fraction, while 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 Dr. E. Sanders (Michigan State University).

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 x g for 0.5 h in a SW 50.1 rotor. Clarified antisera 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 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 TCA 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 HCl, 5mM EDTA, 1% SDS, and 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 using 3a 70B scintillation cocktail (17).

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 eluted with 0.4 ml of 0.1% SDS at 37°C for 24 h. Small aliquots of each fraction were assayed for radioactivity to locate labeled polypeptides. ^{3}H amino acid labeled FeLV was prepared, electrophoresed, and p30 eluted in a similar manner.

The eluted polypeptides plus 1 mg of BSA as carrier, were precipitated with 15% TCA and 1 volume of ethanol. The precipitated protein was centrifuged and the pellet washed 4 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 formic acid + 0.5 ml 30% hydrogen peroxide kept at 25°C for 1.5 h) for 1 h at 4°C. Fifteen ml of distilled H_2O was added followed by lyophilization. The lyophilized proteins were resuspended in 15 ml of distilled H_2O and lyophilized again.

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

resuspended in 3 ml of distilled H_2O 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 (29), using a high pressure column of type P chromobeads (Technicon) maintained at 52.5°C. The tryptic peptides were resuspended in 1.5 ml of pH 3.1 buffer (16 ml pyridine, 278 ml acetic acid/liter) and then centrifuged at 1000 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 pH 3.1 buffer and 300 ml of pH 5.0 buffer (161 ml pyridine, 143 ml acetic acid/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 (RPI Corp., Elk Grove Village, Ill.).

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 (Figure 1a) obtained was similar to that seen with ^{3}H amino acid labeled FeLV (17, and Figure 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 (Manuscript in Preparation). SDS-PAGE of the immune precipitate (Figure 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

Fig. 1.--SDS-PAGE of immune precipitated p30 from NP-40 disrupted FeLV. ^{35}S labeled FeLV was prepared from 100 x 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 then rapidly freeze-thawed 15 times. The disrupted virus was incubated with 200 µl of anti-p30 and an immune precipitate collected, resuspended in sample buffer, and electrophoresed, as described in materials and methods.

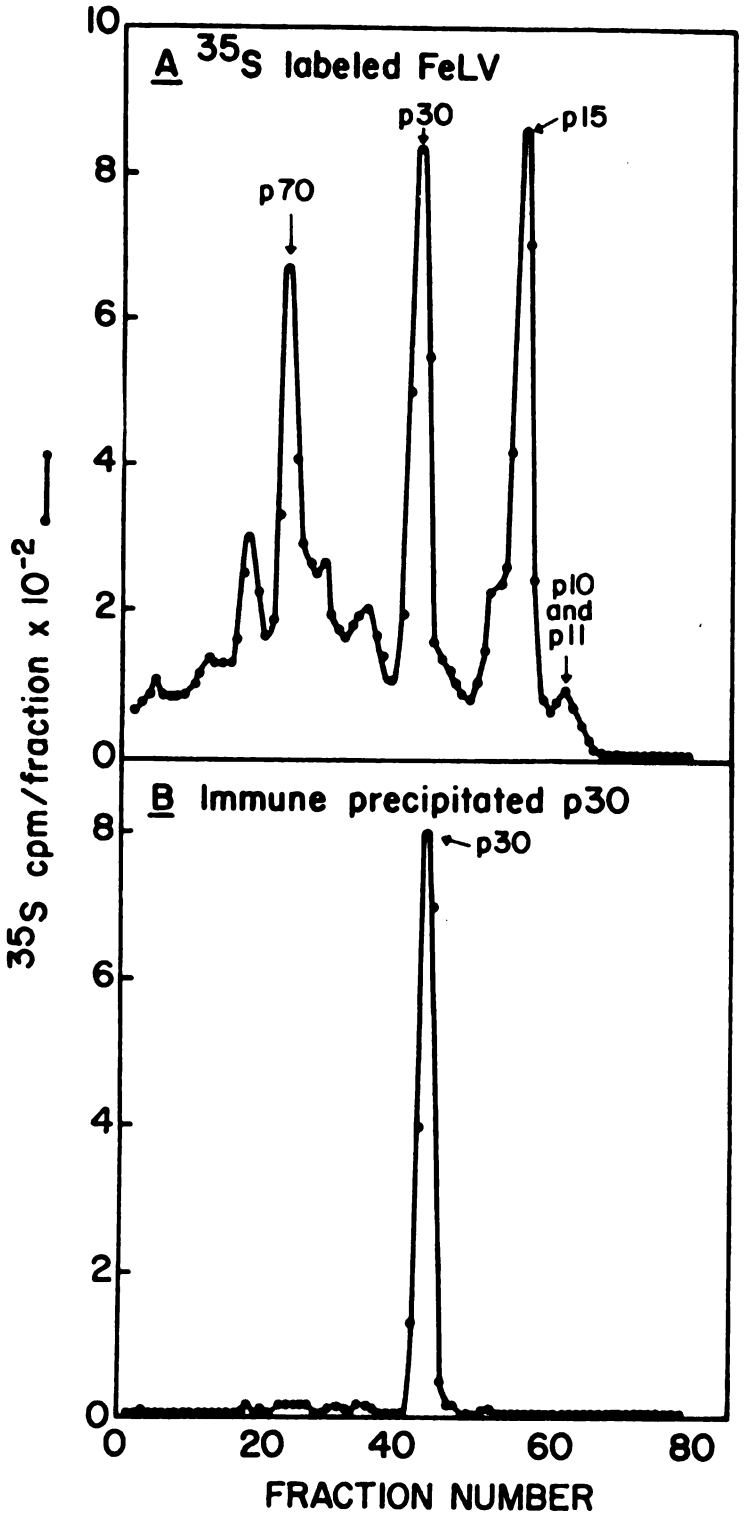
A 35S labeled FeLV
p70
p30
p15
p10 and p11
B Immune precipitated p30
p30
35S cpm/fraction x 10-2
FRACTION NUMBER
0
2
4
6
8
10
0
20
40
60
80

Figure 1

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 cytoplasmic extract and particulate fraction 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 (Figure 2a). This antiserum had previously been shown to be monospecific with respect to FeLV proteins in both immune precipitation (Figure 1b) and immunodiffusion (17). Antiserum to BSA was used in similar immunodiffusion experiments with no precipitin lines evident (data now shown).

Estimation of the level of intracellular p30 synthesis. To estimate the percent of host cell protein synthesis directed toward synthesis of p30 a PF and CE from long term labeled cells (200×10^6) were each divided into equal aliquots (cpm/aliquot) and incubated with increasing amounts of anti-p30. The immune precipitable cpm in each aliquot of the CE and PF are expressed as the percent of total cpm (cpm in the CE aliquot and cpm in the PF aliquot). The CE contained 90% of the total cpm in this experiment (data not shown). Maximal immune precipitation

Fig. 2.--Immunodiffusion of the cytoplasmic extract (CE), particulate fraction (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 x 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 Figure 1.

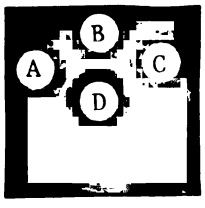

Figure 2

occurred with 50 µl of antiserum (Figure 3), which was equivalent to 400 µl of anti-p30 to maximally immune precipitate intracellular p30 from the CE or PF of 100 x 10^6 cells. The data in Figure 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 cpm present in this fraction. Nonspecific precipitation was determined from a parallel experiment employing 5 µg of BSA per aliquot and increasing anti-BSA. Total nonspecific precipitation was less than 5% of the anti-p30 immune precipitable cpm. Tht total cpm (cpm in CE + cpm in PF) in this experiment represented greater than 95% of the total TCA precipitable cpm 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 precipitated 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 which co-migrated with FeLV p30 (Figure 4a and 4c). In addition two very small peaks (a and b) are consistently seen in both

Fig. 3.--Maximal immune precipitation of intracellular p30. $250_3 \times 10^6$ cells were labeled for 3 h with 250 µCi of ^3H amino acid mixture in 125 ml of growth medium, and a CE and PF prepared as described in materials and methods. Each subcellular fractions was divided into six equal aliquots (cpm/aliquot) and 5 µg of unlabeled NP-40 disrupted virus (prepared as in Figure 1) added. The aliquots were incubated with either 5, 10, 25, 50, 100, or 200 ul of anti-p30 and immune precipitates collected as described in materials and methods. The immune precipitates were resuspended in 1% SDS and the radioactivity was assayed. The cpm immune precipitated from each aliquot are expressed as the percent of total cpm (cpm of a CE aliquot + cpm of PF aliquot).

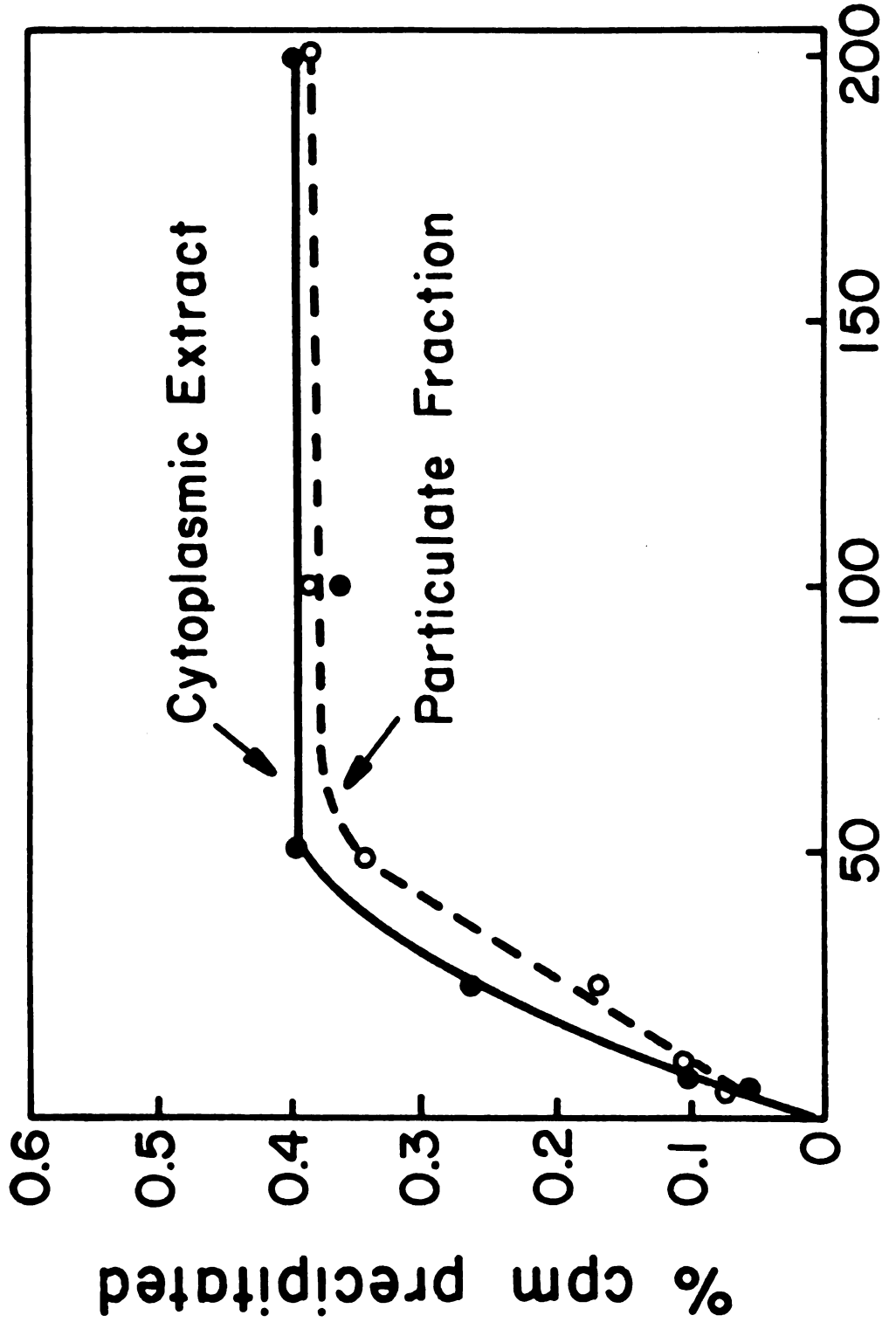
% cpm precipitated
0.6
0.5
0.4
0.3
0.2
0.1
0
50
100
150
200
anti-p30 (μl)
Cytoplasmic Extract
Particulate Fraction

Figure 3

Fig. 4.--SDS-PAGE of immune precipitates from the cytoplasmic extract and the particulate fraction of long term labeled cells. A CE and PF were prepared from 100×10^6 cells labeled for 3 h with 100 µCi of ^{35}S methionine in 50 ml of growth medium as described in materials and methods. The CE and the PF were each divided into two equal aliquots (cpm/aliquot) and incubated with either 200 ul of anti-p30 or with 5 ug of BSA and 200 ul of anti-BSA. The immune precipitates were collected and co-electrophoresed with ^{3}H amino acid labeled FeLV as described in materials and methods. (A) CE and anti-p30. (B) PF and anti-p30. (C) CE with 5 ug BSA and anti-BSA. (D) PF with 5 ug BSA and anti-BSA.

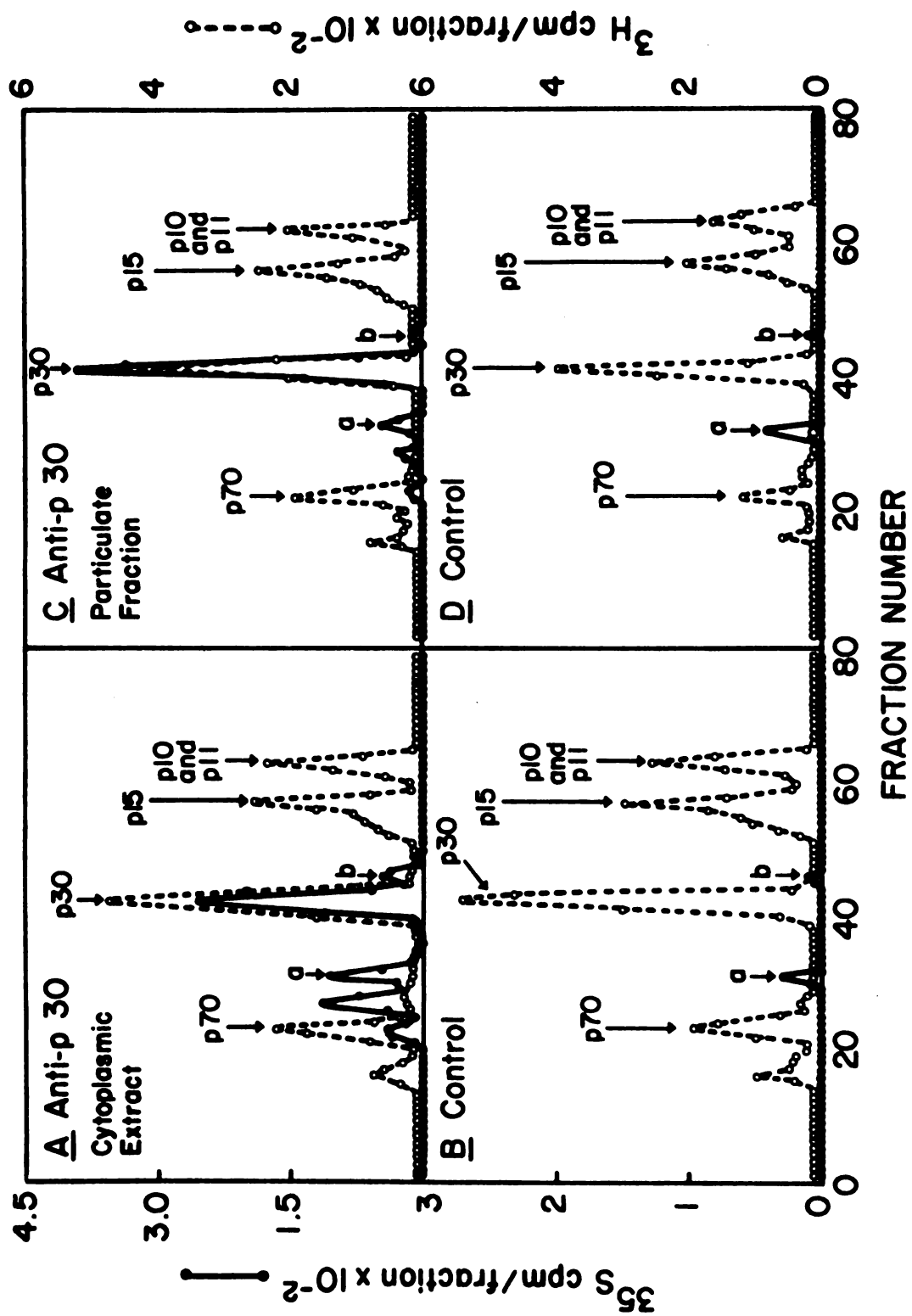
A Anti-p 30
Cytoplasmic Extract
B Control
C Anti-p 30
Particulate Fraction
D Control
p70
p30
p15
p10 and p11
a
b
35S cpm/fraction x 10-2
3H cpm/fraction x 10-2
FRACTION NUMBER

Figure 4

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experimental and control (Figures 4b and 4d) profiles. Two polypeptides which 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 (RSV) 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 if 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 (PMSF, Sigma) 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 (Figure 5a) contained a single polypeptide with molecular weight of 60,000 daltons (Pp60), while the immune precipitate from the CE (Figure 6a) contained a single polypeptide that co-migrated with FeLV p30. Control experiments employing BSA and anti-BSA

Fig. 5.--SDS-PAGE of immune precipitates from particulate fractions of pulse-chase labeled cells. 700×10^6 cells were pulse labeled for 2.5 min with 1 mCi of ^{35}S methionine and then divided into seven aliquots, one of which was lysed immediately while 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 100×10^6 cells). A PF fraction was prepared from both pulse labeled and pulse-chase labeled cells, using lysis buffer containing 300 ug/ml of phenyl methyl sulfonyl fluoride, as described in materials and methods. NP-40 disrupted FeLV (5 ug) was added to each PF followed by 500 ul of anti-p30. Immune precipitates were collected and co-electrophoresed with ^3H amino acid labeled FeLV as described in materials and methods. Arrows indicate positions of non-glycosylated ^3H amino acid labeled FeLV polypeptides. (A) PF from cells pulse labeled for 2.5 min. (B-F) PFs from cells chased for 0.5-6 h.

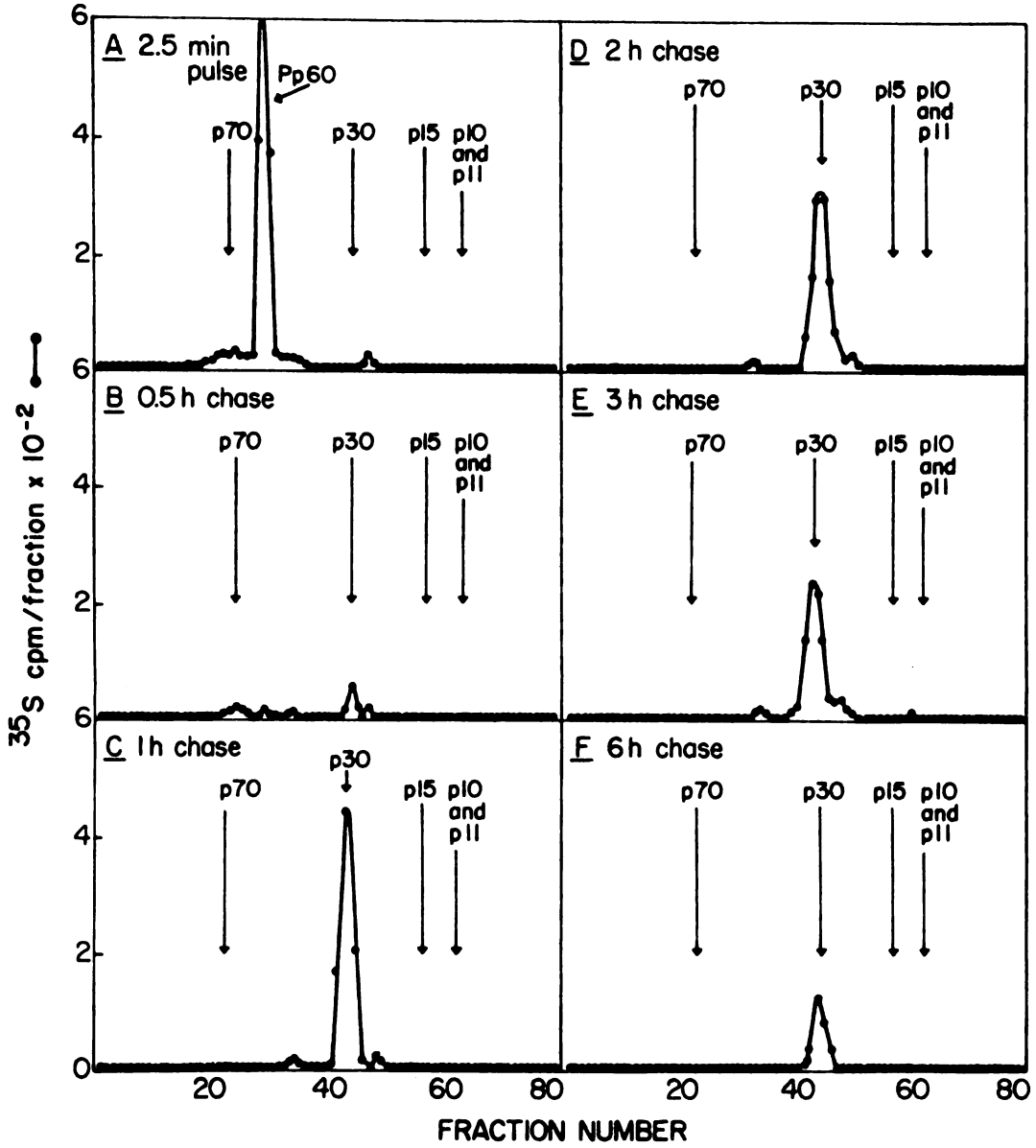

A 2.5 min pulse
Pp60
p70
p30
p15
p10 and p11
B 0.5 h chase
C 1 h chase
D 2 h chase
E 3 h chase
F 6 h chase
^{35}S cpm/fraction x 10^{-2}
FRACTION NUMBER
0
2
4
6
20
40
60
80

Figure 5

Fig. 6.--SDS-PAGE of immune precipitates from cytoplasmic extracts of pulse chase labeled cells. Cytoplasmic extracts were prepared from the same 700 x 10^6 cells labeled as described in Figure 5, using lysis buffer containing 300 ug/ml of PMSF as described in materials and methods. Deoxycholate was added to a final concentration of 0.2%. NP-40 disrupted FeLV (5 ug) was added to each CE followed by 500 ul of anti-p30. Immune precipitates were collected and co-electrophoresed with ^3H amino acid labeled FeLV as described in methods and materials. Arrows indicate positions of nonglycosylated ^3H amino acid labeled FeLV polypeptides. (A) CE from cells pulse labeled for 2.5 min. (B-F) CEs from cells chased for 0.5-6 h.

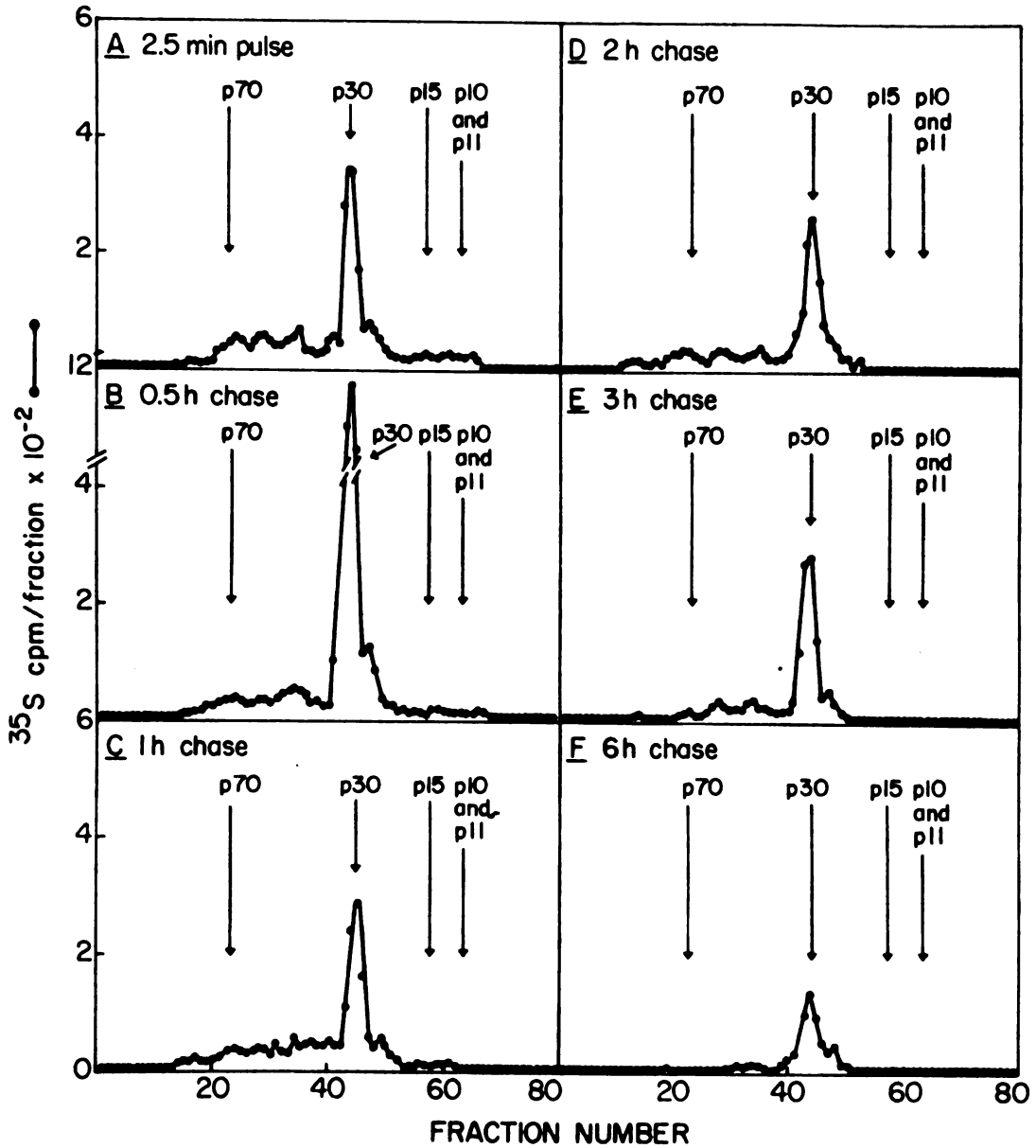

A 2.5 min pulse
B 0.5h chase
C 1h chase
D 2h chase
E 3h chase
F 6h chase
p70
p30
p15
p10 and p11
35S cpm/fraction x 10-2
FRACTION NUMBER

Figure 6

yielded a polypeptide profile (data not shown) identical to that seen with long term labeled subcellular fractions (Figures 4b and 4d). 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 co-migrated with FeLV p30 (Figure 5b), while the CE contained a 30,000 dalton polypeptide in quantities much greater than seen at the end of the pulse (Figure 6b).

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

Immune precipitates of cells chased for 1, 2, and 3 h contained p30 in both the PF (Figure 5c-e) and the CE (Figure 6c-e). The level of immune precipitable p30 in the PF decreased during this two hour chase period while 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 (Figure 5f and 6f) was relatively equal and both were reduced as compared to the 3 h chase interval. No labeled p30 could be recovered at the 24 h chase time (data not shown).

Fig. 7.--Analysis of intracellular and extracellular p30 levels during a 0.5-24 h chase period following a 2.5 min pulse. The levels of p30 are expressed as the percent 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 Figure 5 and 6). Total extracellular p30 is the entire amount of virion associated FeLV p30 released during a 0.5-24 h chase following a 2.5 min pulse (determined from SDS-PAGE profiles of labeled FeLV released during this experiment). Symbols: ●-● Percent of total labeled immune precipitable intracellular p30 found in both the PF and the CE; o-o Percent of total labeled extracellular FeLV p30.

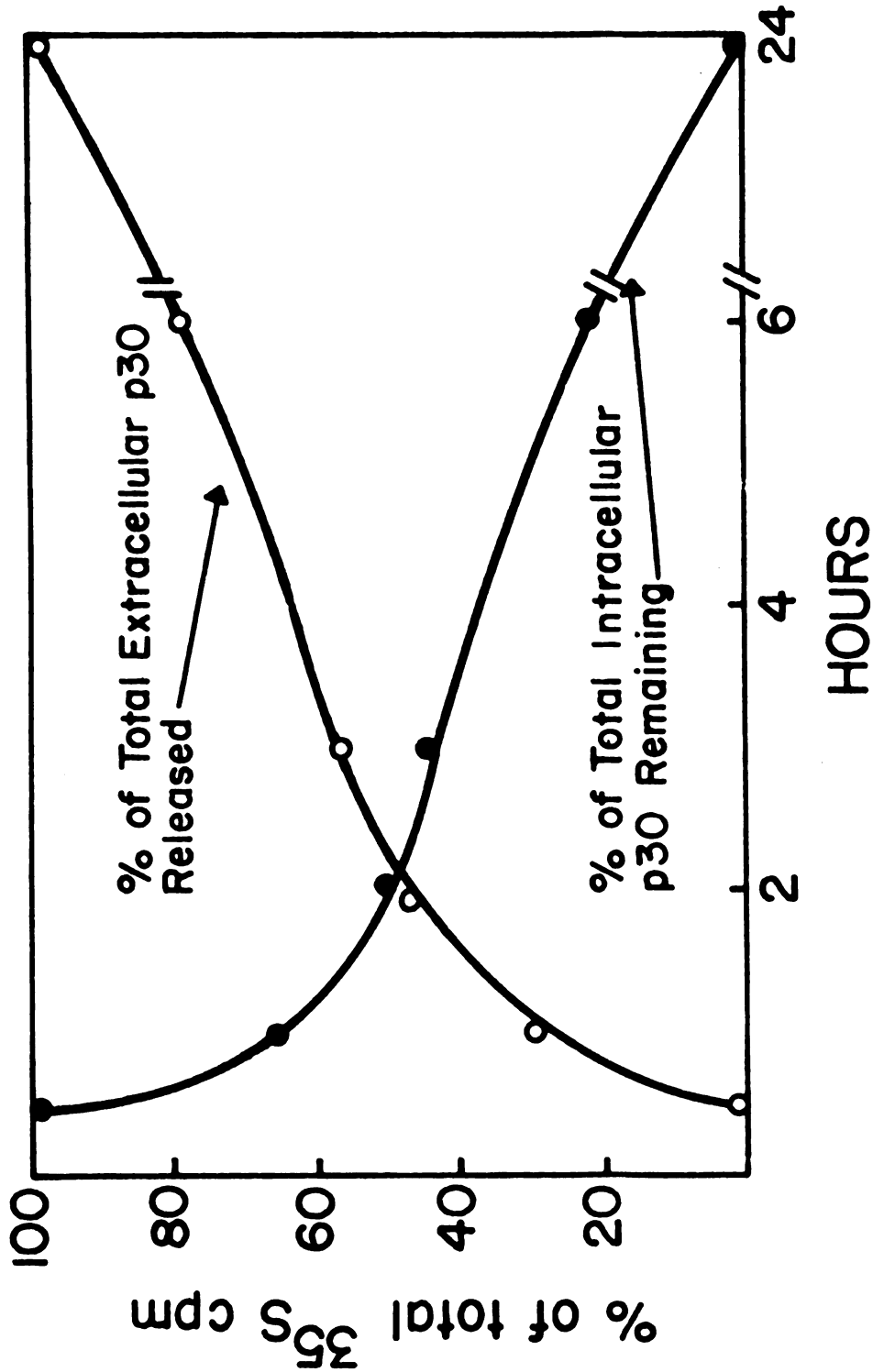

% of total ^{35}S cpm
100
80
60
40
20
2
4
6
24
HOURS
% of Total Extracellular p30 Released
% of Total Intracellular p30 Remaining

Figure 7

Deoxycholate was added to the cytoplasmic extracts (NP-40 soluble fraction) in this experiment (Figure 6) to demonstrate that Pp60 is found only in the PF under identical detergent conditions. Identical results to those presented in Figure 6 were obtained in absence of deoxycholate (data not shown).

To determine if 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 x 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 subcellular 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 0.5-24 h chase period following a 2.5 min pulse (Figure 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 (Figures 5 and 6). Unlabeled carrier virus (100 ug) was added to each chase aliquot (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 the resulting electropherograms (electropherograms not shown). The 24 h chase aliquot provided the total extracellular FeLV p30 value, while all remaining values were represented as a percent of the total extracellular FeLV p30 (Figure 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 total.

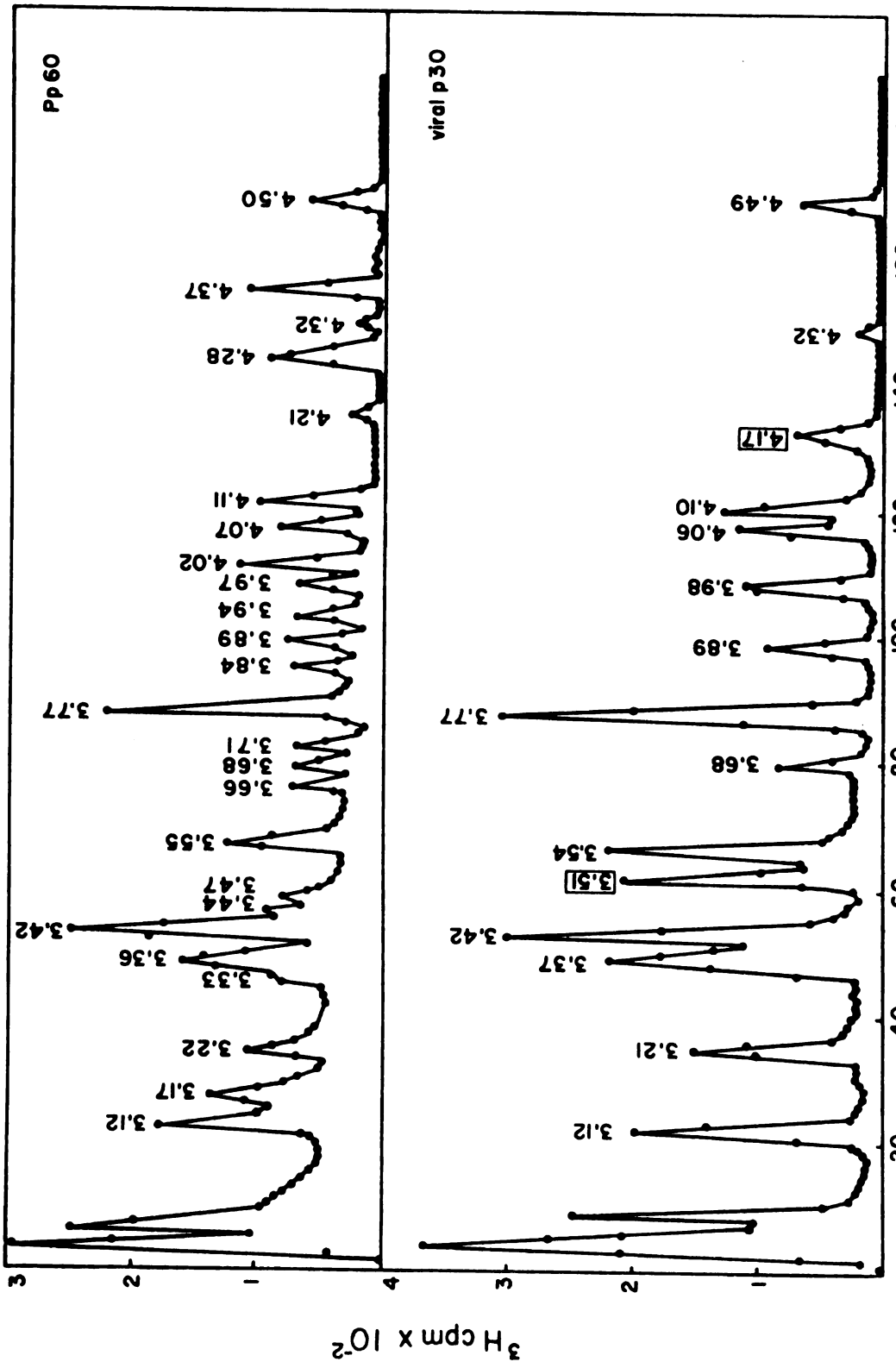
Intracellular p30 levels during the 0.5-24 h chase period were obtained from the data shown in Figure 5b-f and 6b-f. The 0.5 h chase time provided the total (100%) intracellular level of p30 in this analysis. The cpm co-migrating with viral p30 from both the CE and PF were used to determine the level of intracellular p30 at each chase time. The level of p30 at each chase time is represented as the percent of total intracellular p30.

Analysis of the data presented in Figure 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 if 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 (Figure 8) contained 25 ^{3}H labeled peptides present in Pp60 while ^{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.

Fig. 8.--Tryptic peptide analysis of Pp60 and FeLV p30 eluted from SDS polyacrylamide gels. Labeled Pp60 was immunoprecipitated from the PF of 500 x 10^6 cells pulse labeled for 2.5 min with 500 µCi of ^{3}H 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 prepared as described in materials and methods. Percent recovery for the entire procedure was 70-80%. 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 a absent in Pp60.


Pp60
viral p30
3.12
3.17
3.22
3.33
3.36
3.42
3.44
3.47
3.55
3.66
3.68
3.71
3.77
3.84
3.89
3.94
3.97
4.02
4.07
4.11
4.21
4.28
4.32
4.37
4.50
3.12
3.21
3.37
3.42
3.51
3.54
3.68
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3.89
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FRACTION NUMBER
^{3}H cpm x 10^{-2}

Figure 8

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DISCUSSION

Evidence from tryptic peptide analysis and pulse-chase labeling demonstrates that Pp60 is a rapidly cleaved precursor of FeLV p30. 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 indicated 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 (Figure 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 (MSV [MLV]) infected rat cells (28). 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 (Figures 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 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 1-3 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 (Figures 5 and 6),

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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 oncornavirus 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 following pulse labeling (Figure 6) may suggest further post-translational 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 Figure 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

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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 determined in this analysis.

Tryptic peptide analysis of Pp60 and p30 demonstrates that Pp60 contains p30 (Figure 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 precursors 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. While a final judgement 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 of the feline leukemia virus pseudotype of Maloney sarcoma virus [MSV(FeLV)] may be an uncleaved precursor of MSV(FeLV)p30. While 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 not only investigate 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 3×10^6 dalton viral genome subunits (12, 16, 31) and translated from a single initiation site, one would expect a precursor polypeptide with a mol. wt. of approximately 300,000 daltons. The failure to detect such a giant precursor polypeptide in our experiments may be due to several factors: (1) the p30 antigenic determinant of such a large polypeptide may be inaccessible to antibody; (2) Pp60 may be a cleavage product of a nascent polypeptide; (3) 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; (4) the hypothesized 300,000 dalton precursor may not exist.

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MANUSCRIPT II

ANALYSIS OF INTRACELLULAR FELINE LEUKEMIA VIRUS PROTEINS. II. THE GENERATION OF FeLV STRUCTURAL PROTEINS FROM PRECURSOR POLYPEPTIDES

By

Gregory F. Okasinski and Leland F. Velicer

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ANALYSIS OF INTRACELLULAR FELINE LEUKEMIA VIRUS PROTEINS. II. THE GENERATION OF FeLV STRUCTURAL PROTEINS FROM PRECURSOR POLYPEPTIDES

Gregory F. Okasinski and Leland F. Velicer

Department of Microbiology and Public Health

Michigan State University

East Lansing, MI 48824

Running Title: Precursor polypeptides of FeLV Proteins

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ABSTRACT

The synthesis and processing of feline leukemia virus (FeLV) polypeptides was studied in a chronically infected feline thymus tumor cell line, F422, which produces the Rickard strain of FeLV. Immune precipitation with antiserum to FeLV p30 and subsequent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to isolate intracellular FeLV p30 and possible precursor polypeptides. SDS-PAGE of immune precipitates from cells pulse labeled for 2.5 min with ^{35}S methionine revealed the presence of a 60,000 dalton precursor polypeptide (Pp60) as well as a 30,000 dalton polypeptide. When cells were grown in the presence of the proline analogue, L-azetidine-2-carboxylic acid, a 70,000 dalton precursor polypeptide (Pp70) was found in addition to Pp60 after a 2.5 min pulse. The cleavage of Pp60 could be partially inhibited by the general protease inhibitor, phenyl methyl sulfonyl fluoride (PMSF). This partial inhibition was found to occur only if PMSF was present during pulse labeling.

Intracellular Pp70 and Pp60, and FeLV virion p70, p30, p15, p11, and p10 were subjected to tryptic peptide

analysis. The results of this tryptic peptide analysis demonstrated that intracellular Pp70 and virion p70 were identical and that both contained the tryptic peptides of FeLV p30, p15, p11, and p10. Pp60 contained the tryptic peptides of FeLV p30, p15, and p10, but lacked the tryptic peptides of p11. The results of pactamycin gene ordering experiments indicated that the small structural proteins of FeLV are ordered p11-p15-p10-p30. The data indicate that the small structural proteins of FeLV are synthesized as part of a 70,000 precursor. A cleavage scheme for the generation of FeLV p70, p30, p15, p11, p10 from precursor polypeptides is proposed.

INTRODUCTION

The generation of stable virion proteins from large precursor polypeptides is a well-documented phenomenon observed in many animal virus infected cells. Post-translational cleavage of precursor polypeptides was initially described for poliovirus infected cells (8, 23). This phenomenon has been shown to be widespread; occurring in coxsackievirus (11), Sindbis virus (21, 22), Semliki Forest virus (4, 12, 15), Mengo virus (20), and encephalomyocarditis virus infected cells (3, 14). The synthesis of large precursor polypeptides has been proposed as a mechanism by which polycistronic mRNA is translated in eukaryotic cells (2).

During the past three years several reports of precursor polypeptides in oncornavirus infected cells have appeared. Avian myeloblastosis virus infected primary chick fibroblasts contain a 76,000 dalton precursor polypeptide (27). Tryptic peptide analysis of this precursor polypeptide reveals the presence of four virion proteins within the 76,000 dalton molecule (27, 28). Immune precipitation of Rauscher leukemia virus (RLV) proteins from infected cells was used to demonstrate 82,000 and 65,000

dalton presumptive precursor polypeptides in JLS-V9 cells (26) and 200,000, 90,000, 80,000 and 65,000 dalton polypeptides in JLS-V16 cells (1, 17). Jamjoom et al. (9) demonstrated that RLV contains a non-glycosylated 70,000 dalton polypeptide which is identical to an intracellular polypeptide, immune precipitable by antiserum to RLV. The virion and intracellular 70,000 dalton polypeptide contain RLV p30 tryptic peptides, and the authors suggest a possible precursor-product relationship.

Recently, we identified a 60,000 dalton precursor polypeptide (Pp60) of feline leukemia virus (FeLV) p30 (18). We employed both immune precipitation of FeLV polypeptides from pulse labeled cells and tryptic peptide analysis of Pp60 and FeLV p30 to demonstrate a precursor-product relationship. In this report we present evidence: (1) for a 70,000 dalton precursor polypeptide (Pp70) of FeLV p30, p15, p11, and p10, (2) that the cleavage of Pp70 results in the generation of Pp60 and p11, (3) that the cleavage of Pp60 can be partially inhibited by the general protease inhibitor, phenyl methyl sulfonyl fluoride (PMSF), (4) that intracellular Pp70 and FeLV virion p70 contain identical tryptic peptide profiles, and (5) that the gene order of the low molecular weight FeLV structural proteins is p11-p15-p10-p30.

Our evidence indicates that the small structural protein of FeLV differ from those of avian viruses in both

arrangement within and cleavage from precursor polypeptides. FeLV appears to be similar to RLV in that both contain uncleaved precursors of structural protein (9 and this publication). The inhibition of precursor cleavage by a general protease inhibitor reported here seems to be unique for FeLV.

MATERIALS AND METHODS

Source of cells and virus. The chronically 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. F-422 cells were pulse labeled with ^{35}S methionine (1 μCi/10^6 cells) or ^{3}H amino acid mixture (2 μCi/10^6 cells). This pulse was terminated and cells chased in the presence of 10 times the normal concentration of methionine as previously described (18).

Purification of FeLV. ^{14}C or ^{3}H amino acid labeled FeLV and unlabeled FeLV was purified from F-422 culture fluids by discontinuous sucrose gradient centrifugation as previously described (18).

Preparation of subcellular fractions. Particulate fractions (PF) and cytoplasmic extracts (CE) were prepared from NP-40 disrupted cells as previously described (18). Briefly, cells were disrupted with lysis buffer (0.5% NP-40, 0.15 M NaCl, 0.01 M Tris, pH 7.4) and centrifuged

at 2400 rpm for 5 min in an International PR-6 centrifuge. The supernatant was removed and centrifuged at 100,000 x g for 1 h. The 100,000 x g supernatant (cytoplasmic extract) was removed and the pellet solubilized with lysis buffer containing 0.2% deoxycholate (particulate fraction). Both the CE and PF were sonicated for 2-3 min in a 150-W Branson Ultrasonic Cleaner (Branson Instruments Co., Stamford, Conn.) and centrifuged again at 100,000 x g for 1 h.

Immune precipitation. Rabbit antiserum to FeLV p30 was prepared and clarified as previously described (7, 18). Rabbit antiserum to bovine serum albumin (BSA) was obtained from Dr. E. Sanders (Michigan State University). Clarified antisera 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 sedimentation through 1 ml of 10% sucrose (wt/wt) in lysis buffer at 2,000 rpm for 20 min in an International PR-6 centrifuge. This was repeated one additional time and the final precipitate solubilized for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (18).

Double detergent disruption of FeLV. Labeled or unlabeled purified FeLV was disrupted by resuspension of viral pellets in lysis buffer containing 0.5% deoxycholate followed by 5 min of sonication in a 150-W Branson

Ultrasonic Cleaner. The disrupted virus was centrifuged at 100,000 x g for 1 h in a SW 50.1 rotor (Beckman) and the supernatant used as a source of labeled or unlabeled soluble FeLV polypeptides.

SDS-PAGE. Electrophoresis in the presence of SDS was done as previously described (18) using a 9% polyacrylamide gel described by Fairbanks et al. (5). The gels were fractionated and assayed for radioactivity using 3a70b scintillation cocktail (RPI Corp., Elk Grove Village, Ill.) as previously described (7).

Tryptic peptide analysis. Tryptic peptides of immune precipitated polypeptides and labeled FeLV polypeptides, eluted from SDS polyacrylamide gels, were prepared as previously described (18). One mg of BSA was added to FeLV proteins separated by gel filtration in the presence of 6 M GuHCl and the proteins precipitated by dilution of GuHCl to 0.6 M followed by addition of TCA to a final concentration of 25%. Tryptic peptides were then prepared as previously described (18). Cation exchange chromatography of tryptic peptides was done using a high pressure column of type P chromobeads (Technicon) as previously described (18). Tryptic peptides were lyophilized and stored at -76 C until chromatography.

Pulse labeling in the presence of pactamycin.

Pactamycin was obtained from Dr. George B. Whitfield of the Upjohn Company. The drug was stored as a 5×10^{-5} M solution in 1 mM acetic acid at -20 C.

250×10^6 cells were incubated for 30 sec with pactamycin at a final concentration of 5×10^{-7} M and pulse labeled with ^3H amino acid mixture (2 μCi/10^6 cells) or ^{14}C amino acid mixture (0.2 μCi/10^6 cells) in a final volume of 5 ml.

RESULTS

The effect of phenyl methyl sulfonyl fluoride (PMSF) on the cleavage of Pp60. To determine the effect of PMSF on cell viability and protein synthesis during the short period of exposure to be employed in these experiments, cells were incubated with PMSF for 2 min and then pulse labeled for 2.5 min in the presence of PMSF. Both hot TCA precipitable radioactivity and trypan blue dye exclusion were monitored. The results of this experiment (Figure 1) indicated that PMSF reduced TCA precipitable radioactivity by 40% (at 50 μg/ml) during the short period of exposure used in these experiments and reduced cell viability by 10-15%.

Pulse-chase labeling experiments were done in the presence of PMSF to determine if PMSF, a general protease inhibitor, could inhibit the cleavage of Pp60. In the first experiment (Figure 2) cells were pulse labeled for 2.5 min and then chased for 30 min in the presence or absence of PMSF. SDS-PAGE of immune precipitates from subcellular fractions of pulse labeled cells demonstrated the presence of Pp60 in the particulate fraction (PF) and p30 in the cytoplasmic extract (CE) (Figure 2A and E) as

Fig. 1.--The effect of phenyl methyl sulfonyl fluoride (PMSF) on cell viability and protein synthesis. Six aliquots of cells (50 x 10^6 cells per aliquot) were incubated with 0, 5, 10, 15, 20 or 50 µ/ml of PMSF for 2 min and then pulse labeled for 2.5 min with 10 µCi of ^{35}S methionine/aliquot in the presence of PMSF. The pulse labeling was terminated and the cells chased for 3.0 h in the presence of PMSF as described in materials and methods. Cell viability was determined by trypan blue dye exclusion immediately after the pulse label (4.5 min exposure to PMSF) and at 1.5 h and 3.0 h. The effect of PMSF on protein synthesis was determined by assaying hot TCA precipitable radioactivity from NP-40 disrupted cells.

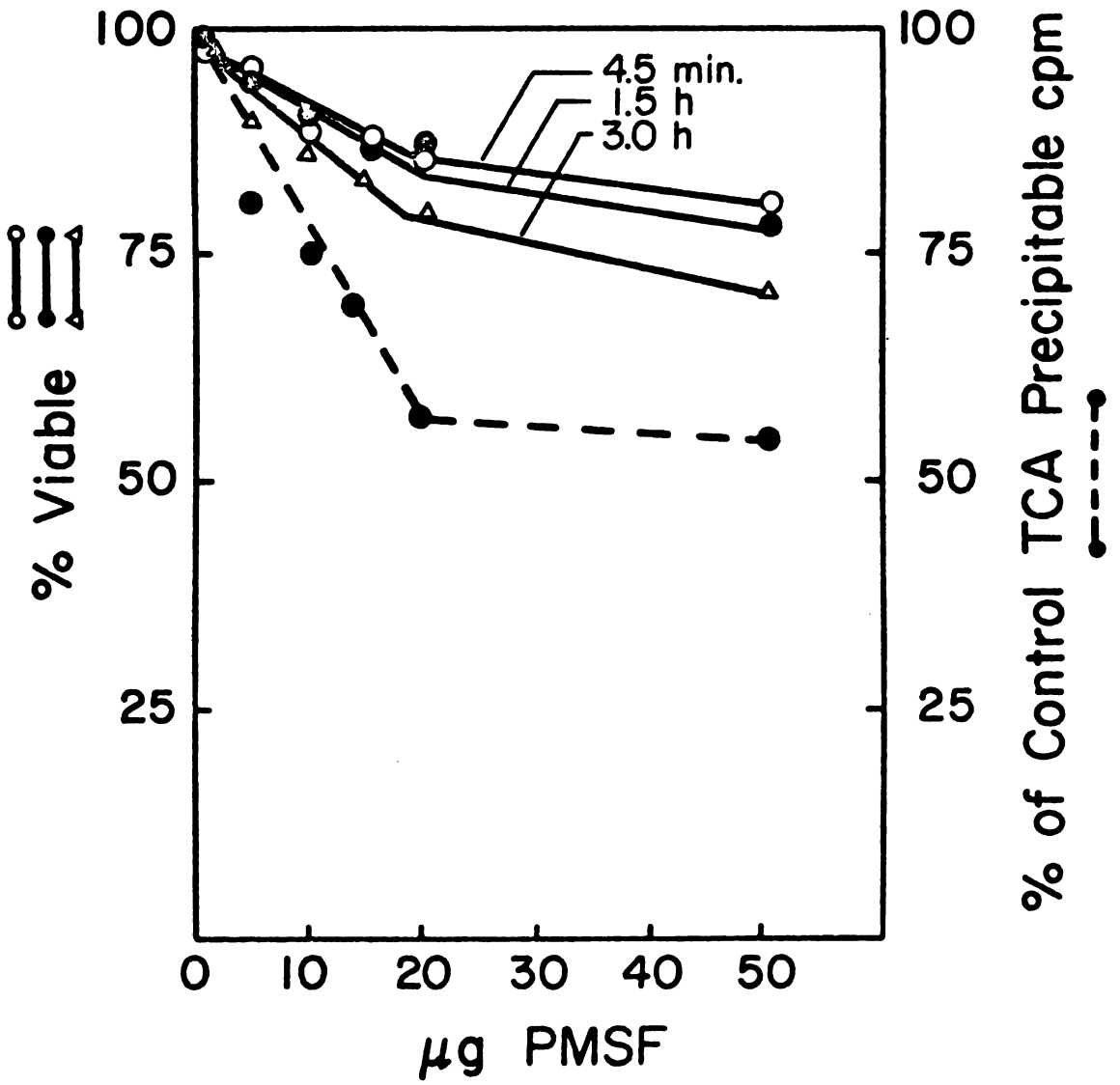
100
75
50
25
% Viable
4.5 min.
1.5 h
3.0 h
0
10
20
30
40
50
μg PMSF
% of Control TCA Precipitable cpm

Figure 1

Fig. 2.--SDS-PAGE of immune precipitates from particulate fractions and cytoplasmic extracts of cells incubated with and without (PMSF) following pulse labeling. 200 x 10^6 cells were pulse labeled with 200 µCi of ^{35}S methionine in 4 ml of growth medium. The pulse labeled cells were divided into 4 aliquots, two of which were chased for 0.5 h either with or without PMSF (50 µg/ml) and the remaining aliquots used as a source of pulse labeled polypeptides. Particulate fractions and cytoplasmic extracts were prepared from both pulse labeled and pulse-chase labeled cells. Polypeptides were immune precipitated from each fraction with anti-p30 and co-electrophoresed with ^{3}H amino acid labeled FeLV as described in materials and methods. Solid arrows indicate position of non-glycosylated FeLV polypeptides. (A) and (E) PF and CE, respectively, from pulse labeled cells. (B) and (F) a PF and CE, respectively, from cells chased in the absence of PMSF. (C) and (G) a PF and CE, respectively, from cells chased in the presence of 50 µg/ml PMSF. (D) and (H) SDS-PAGE of immune precipitates from a PF and CE, respectively, of pulse labeled cells incubated with 5 µg of BSA and 200 µl of anti-BSA.

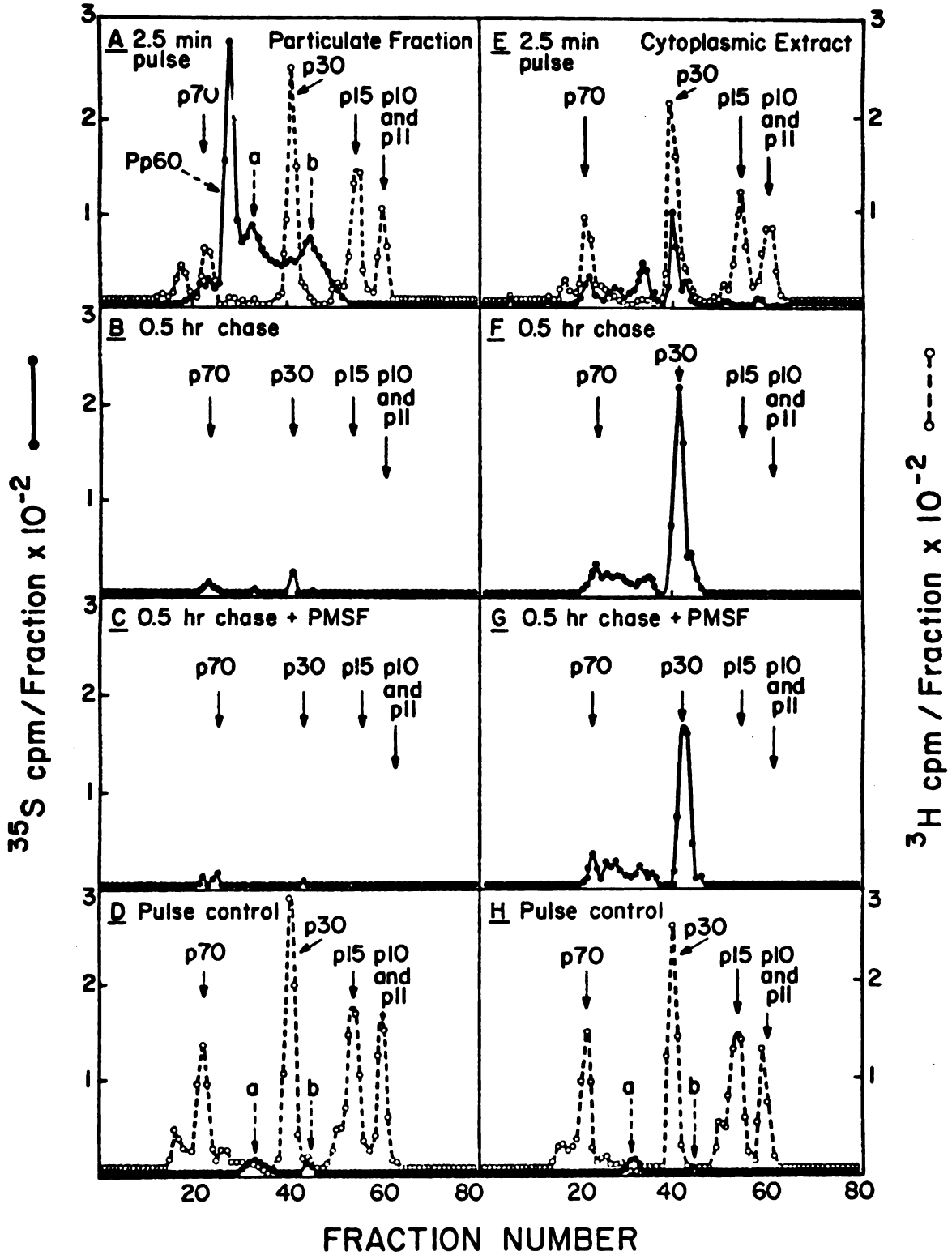
A 2.5 min pulse
Particulate Fraction
p30
p70
p15 p10 and p11
Pp60
a
b
E 2.5 min pulse
Cytoplasmic Extract
B 0.5 hr chase
F 0.5 hr chase
C 0.5 hr chase + PMSF
G 0.5 hr chase + PMSF
D Pulse control
H Pulse control
35S cpm/Fraction x 10-2
3H cpm / Fraction x 10-2
FRACTION NUMBER
20
40
60
80

Figure 2

previously reported (18). When cells were chased for 0.5 h in the presence or absence of PMSF, Pp60 was absent from both subcellular fractions and p30 was found mainly in the CE (Figure 2B, F, C, and G) indicating no inhibition of Pp60 cleavage.

The effect of PMSF of Pp60 cleavage was also examined with the protease inhibitor present during pulse labeling. In this experiment (Figure 3) cells were incubated with PMSF for 2 min and then pulse labeled for 2.5 min in the presence of PMSF. SDS-PAGE of immune precipitates from subcellular fractions of cells pulse labeled in the presence of PMSF demonstrated the presence of Pp60 in both the PF and CE and little if any p30 (Figure 3A and D). When cells pulse labeled in the presence of PMSF were chased in the presence or absence of PMSF for 0.5 h, Pp60 was present in both subcellular fractions as was a small amount of p30, indicating partial inhibition of Pp60 cleavage.

Nonspecific immune precipitation was monitored by incubating labeled subcellular fractions with 5 µg of BSA and 200 µl of anti-BSA. Figure 2D and H depict typical results obtained from subcellular fractions of pulse labeled cells. We routinely detect only two polypeptides (labeled a and b) in all control experiments. Identical results were obtained from subcellular fractions of cells incubated with PMSF (data not shown).

Fig. 3.--SDS-PAGE of immune precipitates from particulate fractions and cytoplasmic extracts of cells pulse labeled with ^{35}S methionine in the presence and absence of PMSF. 300 x 10^6 cells were resuspended in 6 ml of growth media deficient in methionine but containing 50 mg/ml of PMSF for 2 min. The cells were then pulse labeled for 2.5 min, divided into 3 aliquots and 2 aliquots chased in the presence or absence of PMSF. Particulate fractions and cytoplasmic extracts were prepared and then immune precipitated with anti-p30 and electrophoresed as described in materials and methods. (A) and (D) a PF and CE, respectively, from cells pulse labeled in the presence of PMSF. (B) and (G) a PF and CE, respectively, from cells pulse labeled and chased in the presence of PMSF (50 μg/ml). (C) and (F) a PF and CE, respectively, from cells pulsed in the presence of PMSF and chased in the absence of PMSF.

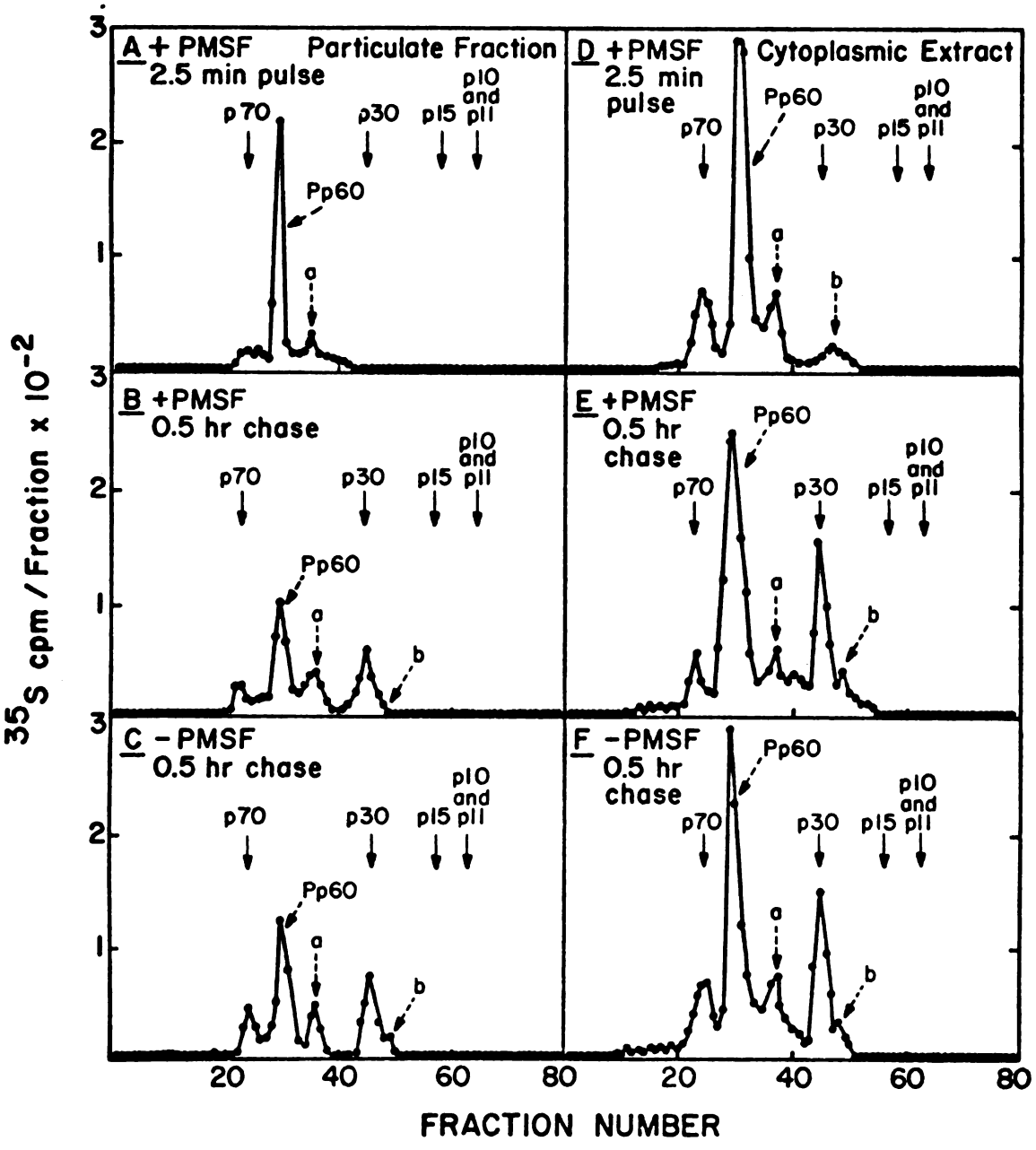
A + PMSF
2.5 min pulse
Particulate Fraction
D + PMSF
2.5 min pulse
Cytoplasmic Extract
B + PMSF
0.5 hr chase
E + PMSF
0.5 hr chase
C - PMSF
0.5 hr chase
F - PMSF
0.5 hr chase
p70
p30
p15
p10 and p11
Pp60
a
b
35S cpm / Fraction x 10-2
FRACTION NUMBER
20
40
60
80
1
2
3

Figure 3

The effect of L-azetidine-2-carboxylic acid on the synthesis of FeLV precursor polypeptides. We have previously demonstrated a precursor-product relationship between Pp60 and p30 (18). Several amino acid analogues have been tested for their effect on the appearance and processing of FeLV precursor proteins. Of the analogues tested (fluorophenylalanine, canavanine, and L-azetidine-2-carboxylic acid) only L-azetidine-2-carboxylic acid has yielded any detectable effect. SDS-PAGE of immune precipitates from subcellular fractions of cells pulse labeled in the presence of L-azetidine-2-carboxylic acid demonstrated the presence of large amounts of a 70,000 dalton polypeptide (Pp70) as well as reduced levels of Pp60 and p30 (Figure 4). Comparison of these results (Figure 4) with those seen in Figure 2A and E, indicated that the 70,000 dalton polypeptide seen in relatively low amounts in the absence of analogues was now the principal immune precipitable polypeptide when pulse labeling was done in the presence of L-azetidine-2-carboxylic acid. This suggests that the proline analogue may be inserted into the initial translation product and subsequently inhibit the cleavage step resulting in the formation of Pp60. Control experiments employing immune precipitation of BSA with anti-BSA yielded results similar to Figure 2D and H (data not shown).

Fig. 4.--SDS-PAGE of immune precipitates from the cytoplasmic extract and particulate fraction of cells pulse labeled with ^{35}S methionine in the presence of L-azetidine-2-carboxylic acid. Prior to pulse labeling, 100 x 10^6 cells were incubated in growth medium deficient in methionine and proline but containing L-azetidine-2-carboxylic acid (0.21 mg/ml). The cells were pulse labeled for 2.5 min with 100 µCi of ^{35}S methionine in the presence of L-azetidine-2-carboxylic acid (0.21 mg/ml) and then a cytoplasmic extract and a particulate fraction was prepared. The fractions were incubated with 300 µl of anti-p30 and 5 µg of disrupted FeLV. The immune precipitates were collected and a co-electrophoresed with ^{3}H amino acid labeled FeLV as described in materials and methods. Solid arrows indicate position of labeled FeLV polypeptides.

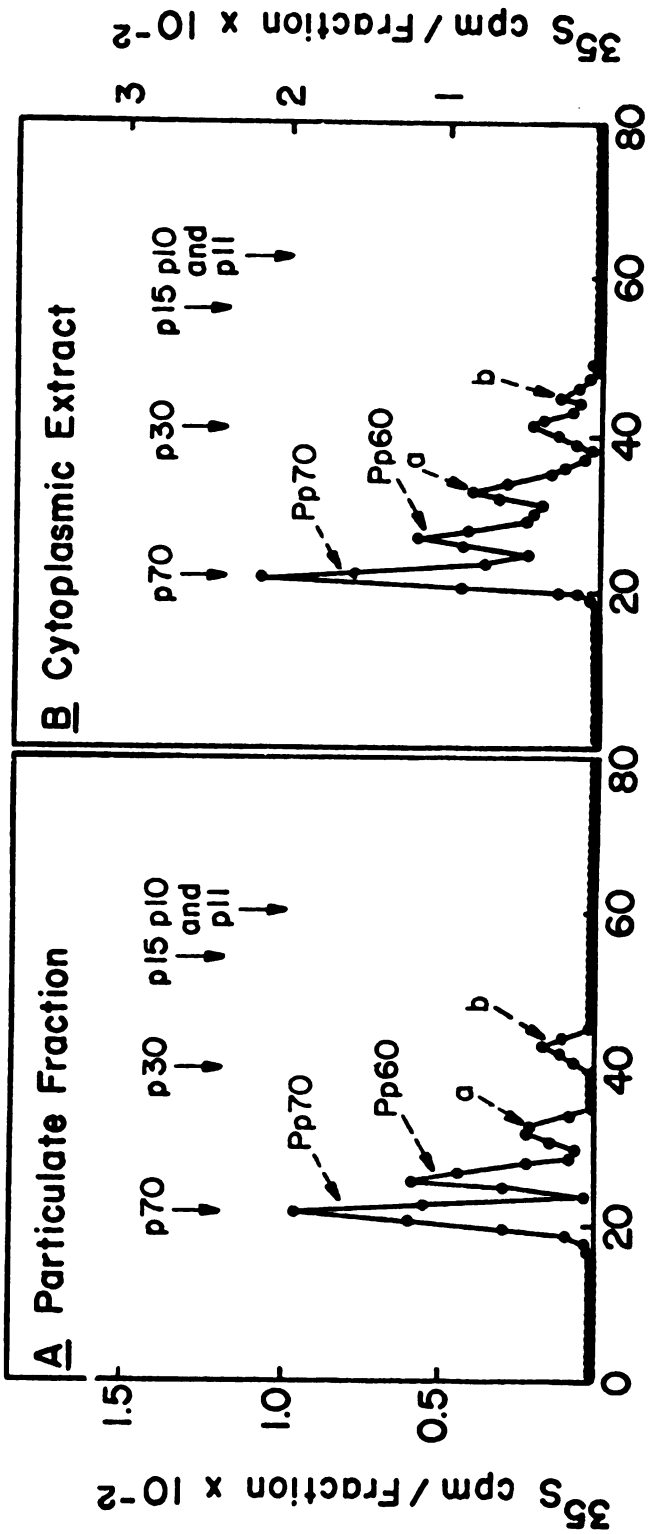
A Particulate Fraction
B Cytoplasmic Extract
p70
p30
p15 p10 and p11
Pp70
Pp60
a
b
35S cpm / Fraction x 10-2
FRACTION NUMBER

Figure 4

Immunoprecipitation of p30 and p70 from disrupted FeLV. The presence of an intracellular 70,000 dalton polypeptide that is both immune precipitable by anti-p30 and electrophoretically identical to virion p70 led us to examine virion p70 for p30 antigenic determinants. In a previous report (18) we had demonstrated that the anti-p30 serum could only immunoprecipitate viral p30 from 0.5% NP-40 disrupted virus. Subsequent experiments have shown that 0.5% NP-40 alone does not solubilize viral p70 to any considerable degree (data not shown).

^{14}C amino acid labeled FeLV was disrupted by treatment with 0.5% NP-40 and sodium deoxycholate and then immune precipitated with anti-p30. The detergent disruption procedure employed in these experiments solubilizes the major FeLV polypeptides (Figure 5A) although only 60-70% of the FeLV p70 is solubilized (data not shown). SDS-PAGE of the resulting immune precipitate demonstrated two labeled FeLV polypeptides. Both FeLV p30 and p70 were immune precipitated (Figure 5B). FeLV p70 was only partially immune precipitated (30% of the total solubilized p70 cpm) in this experiment. The data indicated that FeLV p70 contained some p30 antigenic determinants. 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 (data not shown).

Fig. 5.--SDS-PAGE of immune precipitated p30 and p70 from detergent disrupted FeLV. ^{14}C labeled FeLV was prepared from 25 x 10^6 cells incubated with 25 µCi of ^{14}C amino acid mixture for 24 h in 12.5 ml of growth medium. The virus was purified as described in materials and methods. The viral pellet was resuspended in 0.6 ml of lysis buffer containing 0.5% NP-40 and 0.5% deoxycholate by 3 min of sonic oscillation in a 150-W Branson Ultrasonic Cleaner (Branson Instruments Co., Stamford, Conn.). The disrupted virus was then centrifuged for 1 h at 100,000 x g. (A) FeLV polypeptides (20,000 cpm) were precipitated with nine volumes of acetone and 50 µg of BSA as carrier and then electrophoresed. (B) FeLV polypeptides (20,000 cpm) were immune precipitated with 100 µl of anti-p30 and electrophoresed as described in materials and methods.

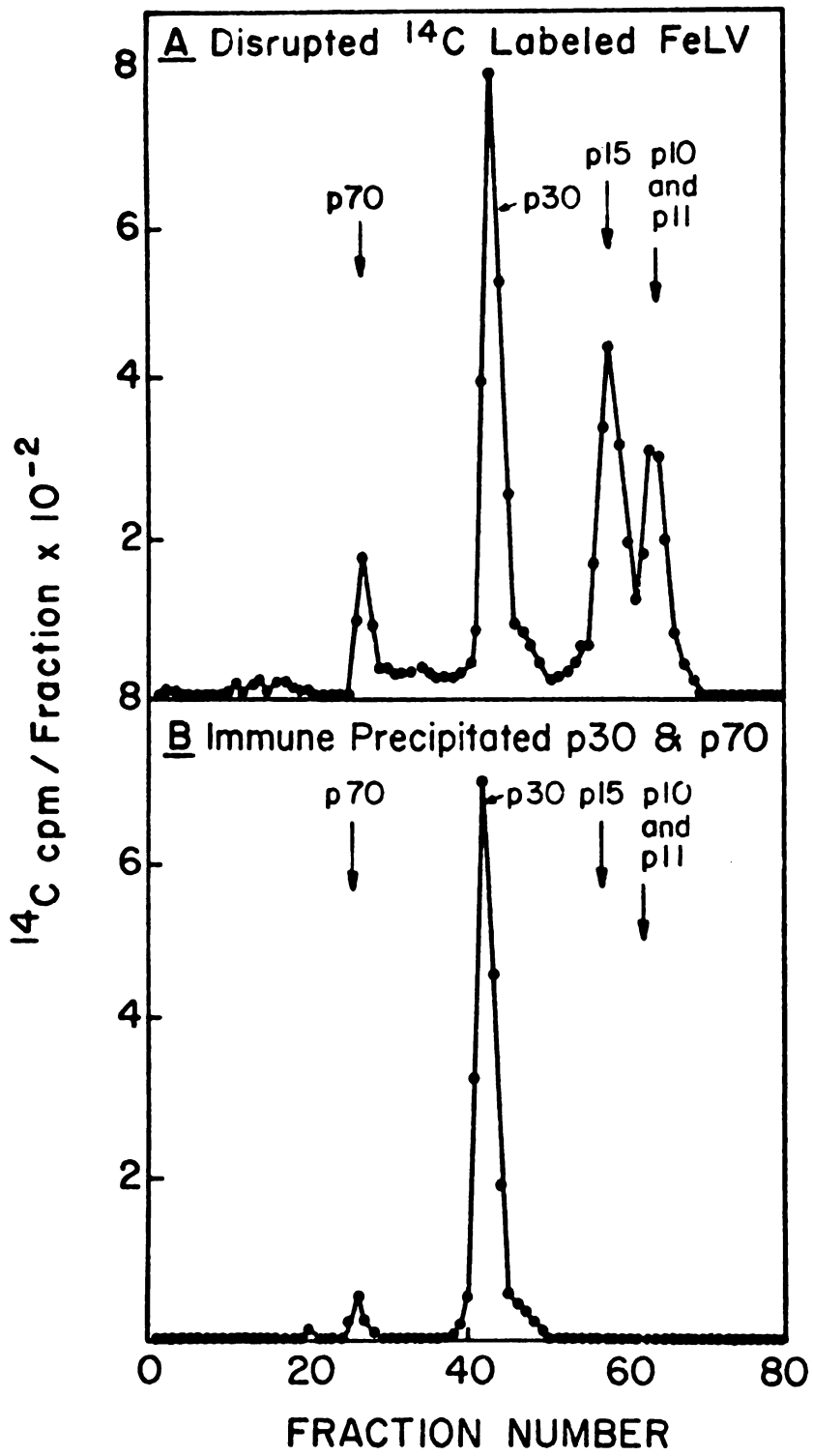
A Disrupted ^{14}C Labeled FeLV
p70
p30
p15
p10 and p11
B Immune Precipitated p30 & p70
p70
p30
p15
p10 and p11
^{14}C cpm / Fraction x 10^{-2}
8
6
4
2
8
6
4
2
0
20
40
60
80
FRACTION NUMBER

Figure 5

Tryptic peptide analysis of intracellular Pp70, Pp60, and FeLV virion p70, p30, p15, p11, and p10. Intracellular ^{3}H amino acid labeled Pp70 and Pp60 tryptic peptides were subjected to high pressure cation exchange chromatography on a column of type P chromobeads (Figure 6). The tryptic peptides are identified by the elution pH of the peak fraction (Figure 6). Pp70 contained 29 detectable peptides while Pp60 was resolved into 25 peptides. Twenty three of the 25 peptides present in Pp60 were also found in Pp70. Two Pp60 tryptic peptides, with elution pH values 3.85 and 3.94, were not detectable in the tryptic peptide profile of Pp70. Pp70 contained in addition to the 23 Pp60 tryptic peptides, 6 peptides with elution pH values of 3.28, 3.62, 3.73, 3.81, 4.12 and 4.14. Because Pp70 and FeLV virion p70 were both immune precipitable with anti-p30 (Figure 4 and 5) and since Pp70 contained the tryptic peptides of Pp60 (Figure 5), tryptic peptide analysis of the structural proteins of FeLV was done to determine (1) if intracellular Pp70 and FeLV virion p70 were similar, and (2) if the additional tryptic peptides seen in Pp70 but not in Pp60 could be assigned to any of the FeLV structural proteins.

Tryptic peptide analysis of FeLV virion p70 yielded 29 peptides (Figure 7) which were indistinguishable from the 29 peptides of intracellular Pp70. The results demonstrated that intracellular Pp70 and virion p70 (Figure 7

Fig. 6.--Tryptic peptide analysis of Pp70 and Pp60 eluted from SDS polyacrylamide gels. Pp70 was prepared by SDS-PAGE of immune precipitates from the particulate fraction and cytoplasmic extract of 500 x 10^6 cells pulse labeled for 2.5 min with 500 µCi of ^3H amino acid mixture in the presence of L-azetidine-2-carboxylic acid. Pp60 was prepared in a similar manner from the particulate fraction of 500 x 10^6 cells pulse labeled (without L-azetidine-2-carboxylic acid) with 500 µCi of ^3H amino acid mixture. Both polypeptides were eluted from gels and tryptic peptides prepared as described in materials and methods. Tryptic peptides were eluted from a column of Type P chromobeads with a linear gradient of pyridine acetate (pH 3.1-4.9) at a flow rate of 30 ml/h. 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). Percent recovery ranged from 70-80% of the radioactivity eluted from polyacrylamide gels. [____] Elution pH values of Pp60 tryptic peptides absent in Pp70.

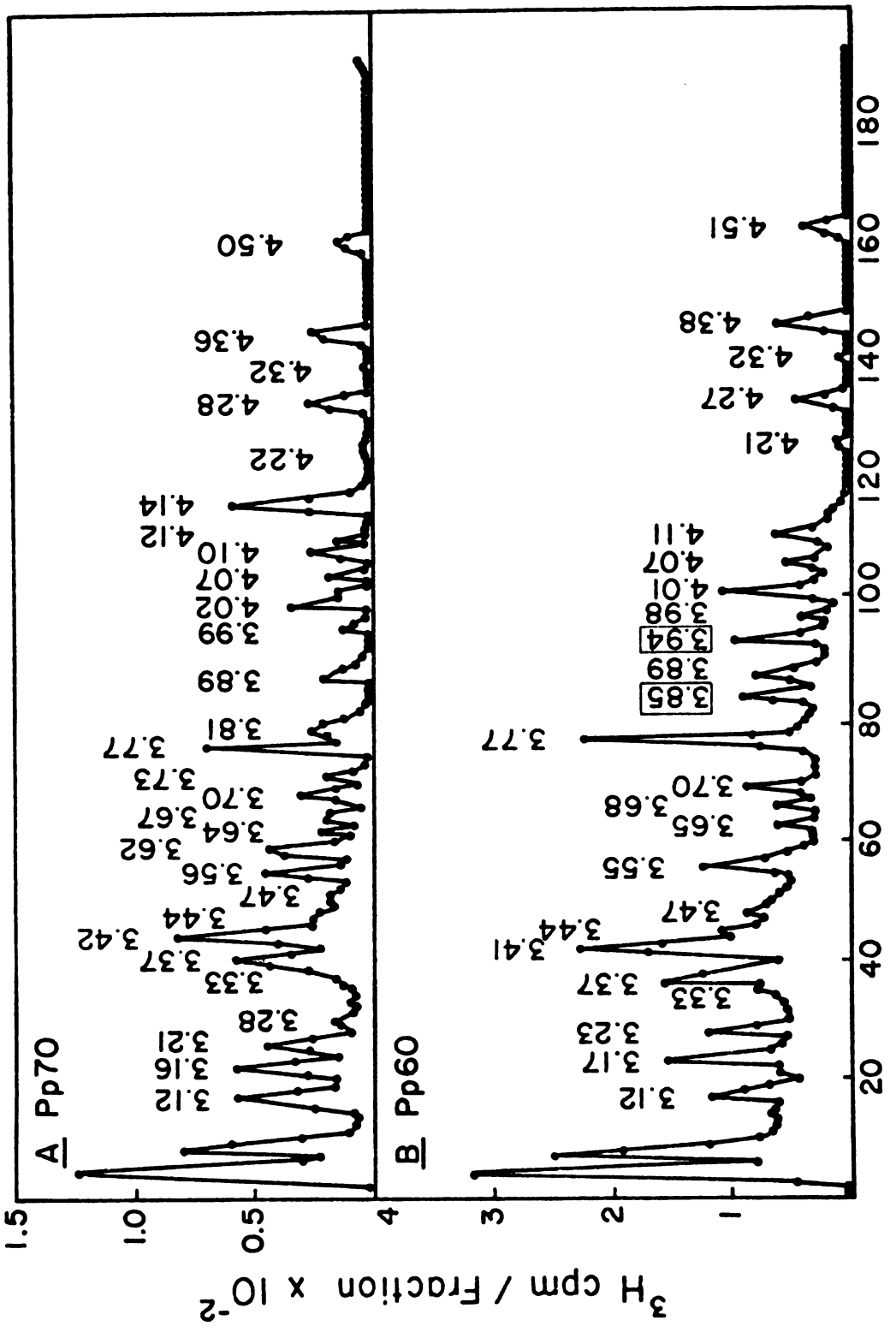
A Pp70
B Pp60
3H cpm / Fraction x 10-2
FRACTION NUMBER
3.12
3.16
3.21
3.28
3.33
3.37
3.42
3.44
3.47
3.56
3.62
3.64
3.67
3.70
3.73
3.77
3.81
3.89
3.99
4.02
4.07
4.10
4.12
4.14
4.22
4.28
4.32
4.36
4.50
3.12
3.17
3.23
3.33
3.37
3.41
3.44
3.47
3.55
3.65
3.68
3.70
3.77
3.85
3.89
3.94
3.98
4.01
4.07
4.11
4.21
4.27
4.32
4.38
4.51

Figure 6

Fig. 7.--Tryptic peptide analysis of FeLV virion p70, p30, p15, p11, and p10. $FeLV_3$p70, p30, and p15 were prepared by SDS-PAGE of ^3H amino acid labeled FeLV. FeLV p11 and p10 were purified by gel filtration of labeled FeLV in GuHCl. Tryptic peptides of the 5 virion proteins were prepared as described in materials and methods. Tryptic peptides were eluted and pH values determined as described in Figure 6. Percent recovery ranged from 60-80% of the radioactivity eluted from polyacrylamide gels or gel filtration columns. [____] Elution pH values of tryptic peptides absent in virion p70 and Pp70 of Figure 6 ( ) p70 tryptic peptides present in both p70 and virion proteins p30, p15, p11, and p10.

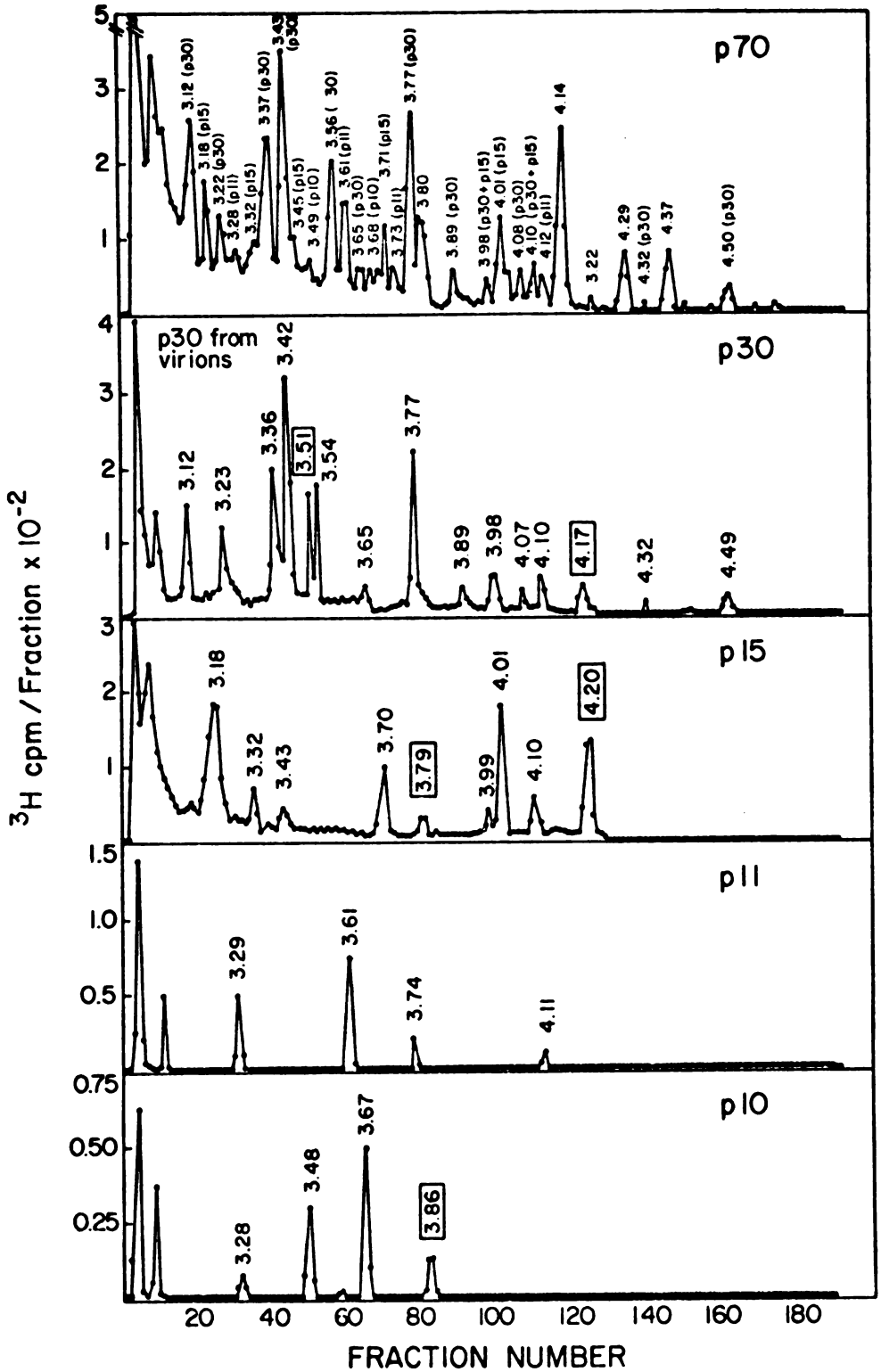
p70
3.12 (p30)
3.18 (p15)
3.22 (p30)
3.28 (p11)
3.32 (p15)
3.37 (p30)
3.45 (p15)
3.49 (p10)
3.56 (p30)
3.61 (p11)
3.65 (p30)
3.68 (p10)
3.71 (p15)
3.73 (p11)
3.77 (p30)
3.80
3.89 (p30)
3.98 (p30+p15)
4.01 (p15)
4.08 (p30)
4.10 (p30+p15)
4.12 (p11)
4.14
3.22
4.29
4.32 (p30)
4.37
4.50 (p30)
p30 from virions
p30
3.12
3.23
3.36
3.42
3.51
3.54
3.65
3.77
3.89
3.98
4.07
4.10
4.17
4.32
4.49
p15
3.18
3.32
3.43
3.70
3.79
3.99
4.01
4.10
4.20
p11
3.29
3.61
3.74
4.11
p10
3.28
3.48
3.67
3.86
^{3}H cpm / Fraction x 10^{-2}
FRACTION NUMBER

Figure 7

and 6) were identical by tryptic peptide analysis. Thirteen of 15 p30 tryptic peptides were present in virion p70 and intracellular Pp60 and Pp70 (Figure 6 and 7). Virion p15 contained 9 tryptic peptides of which seven were present in virion p70 and intracellular Pp60 and Pp70 (Figure 7 and 6). The assignment of the p15 tryptic peptide with an elution pH of 3.79 remains uncertain due to the low level of radioactivity found in the p15 tryptic peptide and the high level of radioactivity present in the p70 tryptic peptides with elution pH values of 3.77 and 3.80. The pH values are accurate to ± 0.01 pH units and thus could allow assignment of this p15 peptide to either the p70 peptides 3.77 or 3.80. We chose to consider this peptide as not being present within p70 because of the relatively low level of radioactivity. The tryptic peptides of p11 had elution pH values of 3.29, 3.61, 3.74, and 4.11 (Figure 7). All four of these p11 tryptic peptides were present in both virion p70 and intracellular Pp70 but were absent in the Pp60 tryptic peptide profile (Figure 6 and 7). These data demonstrate that both virion p70 and intracellular Pp70 contain p11, while Pp60 lacks p11. Tryptic peptide analysis of virion p10 revealed 4 tryptic peptides with elution pH values of 3.28, 3.48, 3.67, and 3.86. Two of the p10 tryptic peptides (pH values 3.48 and 3.67) were present in p70, Pp70, and Pp60, indicating the presence of p10 in all three molecules.

The p10 tryptic peptide with an elution pH value of 3.86 was not present in p70 or Pp70 but was present in Pp60, while the p10 peptide designated 3.28 is also seen p70, and Pp70 but is absent in Pp60. We judge the presence of the p10 peptides 3.86 and 3.28 and the Pp60 peptide 3.85 (Figure 6) to be expected anomalies of tryptic digestion of precursor and product proteins.

The tryptic peptide analysis of intracellular and virion polypeptides was done in parallel rather than by a double label procedure, because of the relatively small amounts of p10 and p11 available from FeLV and the expense of generating large quantities of precursor polypeptides of FeLV. To help insure uniformity in this parallel analysis, all of the tryptic peptides of the proteins examined (Figure 6 and 7) were prepared in parallel and stored as lyophilized tryptic peptides until chromatography (no longer than 3 weeks). Tryptic peptides were eluted with buffers prepared in large quantities and the column of type P chromobeads was washed with sodium hydroxide, pyridine, and starting buffer before each run. FeLV p10 and p11 were purified by gel filtration in GuHCl (7). To determine the effect of GuHCl treated proteins on the tryptic peptide profiles, tryptic peptides were prepared and analyzed from FeLV p30 and p15 prepared both from SDS-PAGE and gel filtrations in GuHCl. Polypeptides prepared by both procedures yielded qualitatively identical results

when their tryptic peptides were analyzed by high pressure cation exchange chromatography (data not shown). The reproducibility of the pH values assigned to the tryptic peptides was examined from several tryptic peptide profiles of FeLV p30 and was found to vary by ± 0.01 pH units (data not shown).

Pactamycin gene ordering. The order of FeLV p30, p15, p11, and p10 within Pp70 was determined by preferential labeling of the carboxy terminal end of newly synthesized polypeptides. Our experimental approach was similar to that employed by other workers to order the proteins of poliovirus (24, 25), encephalomyocarditis virus (3), and recently AMV (28). In our experiments pactamycin was used to selectively inhibit initiation of polypeptide synthesis, while permitting chain elongation. Cells were incubated for 30 sec with 5×10^{-7} M pactamycin, a concentration requiring 10-15 min to completely inhibit incorporation of labeled amino acids, then pulsed labeled in the presence of pactamycin with ^{3}H or ^{14}C amino acid mixtures for 5 min. A parallel culture was pulse labeled in the absence of pactamycin with the opposite (i.e., either ^{3}H or ^{14}C amino acid mixture) labeled amino acid mixture. After pulse labeling, both cultures were chased for 20 h and released virus was purified from the growth medium. Labeled FeLV proteins were separated by gel filtration in the presence of 6 M GuHCl to allow resolution

of FeLV p11 and p10 which are not resolved by SDS-PAGE (7). The relative labeling ratios were determined from the percent of label in each protein synthesized in the presence of pactamycin over the percent of label in each protein synthesized in the absence of pactamycin.

Table 1 depicts the results of one such experiment. Examination of this data indicates that pactamycin treatment lead to a preferential loss of label in p11 and p15 relative to p10 and p30. The relative labeling ratios obtained in this experiment indicate the following order for FeLV proteins: N-p11-p15-p10-p30-C.

Table 1.--The effect of pactamycin[a] on the relative labeling of FeLV virion proteins.

Viral protein	Relative labeling ratio[b]
p11	0.44
p15	0.65
p10	1.06
p30	1.21

[a]Cells were pulse labeled in the presence or absence of pactamycin then chases for 20 h. Purified virus was disrupted and proteins chromatographed by gel filtration in the presence of 6 M GuHCl.

[b]Ratios are the percent of label in each protein synthesized in the presence of pactamycin over the percent of label in each protein synthesized in the absence of pactamycin.

DISCUSSION

In this communication we have presented evidence that the major non-glycosylated structural proteins of FeLV are synthesized as part of a large precursor polypeptide. The largest precursor detected in these experiments was a 70,000 dalton polypeptide, present both intracellularly (Figure 4) and as a component of FeLV (Figures 5, 6 and 7). Tryptic peptide analysis demonstrated that Pp70 and virion p70 were identical and that both contained the tryptic peptides of FeLV p30, p15, p11, and p10 (Figures 6 and 7). The presence of p11 tryptic peptides in intracellular Pp70 and the absence of these tryptic peptides in Pp60 indicates that the conversion of Pp70 → Pp60 involves release of p11 from Pp70. We have also presented evidence suggesting that the conversion of Pp70 → Pp60 can be inhibited by L-azetidine-2-carboxylic acid (Figure 4) while the conversion of Pp60 → FeLV structural proteins was partially inhibited by a general protease inhibitor (Figures 2 and 3). Pulse labeling experiments in the presence of pactamycin indicated that the order of small FeLV structural proteins is p11-p15-p10-p30 (Table 1).

The presence of a virion associated protein (p70) with a tryptic peptide profile identical to intracellular Pp70 indicates incorporation of an uncleaved precursor into mature virions. A similar finding has recently been demonstrated for RLV and RLV infected cells (9) as well as evidence for a possible uncleaved precursor as a component of the feline leukemia virus pseudotype of Moloney sarcoma virus (MSV) (FeLV) (19). Subviral particles prepared from FeLV contain p70 within the core of FeLV rather than as an envelope component (Behnke and Velicer manuscript in preparation).

While immune precipitates (using anti-p30) from pulse labeled cells routinely contain low levels of a labeled 70,000 dalton moiety (Figures 2 and 3) (18), this polypeptide becomes the principal immune precipitable polypeptide when cells are grown in the presence of L-azetidine-2-carboxylic acid. We attribute the routine presence of Pp70 at low levels in immune precipitates during normal pulse labeling conditions (Figure 2A and B) and with long term labeling conditions (18) to reflect an intracellular level of uncleaved Pp70 which eventually becomes incorporated into FeLV as virion p70. However, a portion of this Pp70 may also be destined for further cleavage. The proline analogue, L-azetidine-2-carboxylic acid could affect cleavage of Pp70 in two ways. The analogue could be incorporated directly into the cleavage

enzyme and alter the activity of the enzyme, or the analogue could be incorporated into the substrate (Pp70). In our experiment the cells were pre-incubated with L-azetidine-2-carboxylic acid for 45' prior to pulse labeling (Figure 5). Similar results are obtained when the cells are pre-incubated for 0, 5, and 10 min with the analogue (data not shown). If the proline analogue were altering the cleavage enzyme directly, the enzyme would have to have an extremely short half-life. The most likely alternative is that L-azetidine-2-carboxylic acid is incorporated directly into Pp70 and thus prevents the conversion of Pp70 → Pp60.

The effect of the general protease inhibitor upon the cleavage of Pp60 does not provide an unequivocal interpretation. We cannot attribute the rather dramatic changes in the levels and distribution of Pp60 (Figure 3) to general effect of PMSF on cell viability because the decrease in cell viability is rather small. While PMSF does reduce the level of protein synthesis, this reduction appears to be quantitative rather than selective for viral protein synthesis. Furthermore, the data in Figure 3 indicate that a change in the total length of exposure from 4.5 min (Figure 3, Panel A and D plus C and F) to 34.5 min (Figure 3, Panel B and E) did not cause any significant changes in the electrophoretic profiles. The data presented in Figures 2 and 3 indicate that partial

inhibition of Pp60 by PMSF only occurs if PMSF is present during and shortly after the synthesis of Pp60. It is difficult to attribute the inhibition of Pp60 cleavage to a simple inactivation of a protease due to the short time frame of activity seen in these experiments. The data in Figure 2 argue that Pp60 becomes committed to cleavage within the first few minutes following synthesis while the data in Figure 3 indicate that PMSF inhibition only requires a short exposure of PMSF during pulse labeling. Because PMSF inhibition requires the presence of the protease inhibitor only during pulse labeling we interpret its activity to an aberration of the normal juxtaposition of a substrate Pp60 and enzyme, possibly induced by interaction of PMSF with either Pp60 or a protease. In support of our interpretation is the change in distribution of Pp60 within the particulate fraction (PF) and cytoplasmic extract (CE). Under normal conditions Pp60 is limited to the PF, while in the presence of PMSF Pp60 is found in both the PF and CE immediately after pulse labeling. Lindell has recently reported (13) that addition of PMSF to isolated nuclei inhibits the release of RNA polymerase, which also indicates that PMSF may have additional activities aside from the protease inactivation activity. Further experimentation involving isolation of intracellular membranes and precursor polypeptides will be required to more clearly elucidate the activity of PMSF on Pp60

cleavage. Previous work with both AMV infected cells (27, 28) and RLV infected cells (26) has not provided evidence for any inhibition of precursor cleavage by general protease inhibitors. Our results with FeLV infected cells may reflect differences in experimental design (i.e., concentrations of protease inhibitor, time of addition of inhibitor, type of inhibitor) or in differing susceptibilities of avian, murine, and feline oncornavirus precursor polypeptides.

Tryptic peptide analysis of intracellular precursor polypeptides and virion structural proteins (Figures 6 and 7) demonstrates that the major structural non-glycosylated polypeptides of FeLV are synthesized as part of a 70,000 dalton precursor. The presence of p30, p15, p11, and p10 tryptic peptides within the Pp70 molecule and the absence of the p11 tryptic peptides in Pp60 indicates that the conversion of Pp70 → Pp60 results in the formation of p11. These data would suggest that Pp70 contains the p11 moiety at either its NH_2-terminal or COOH-terminal end and that removal of p11 results in the formation of Pp60.

The results of pactamycin gene ordering experiments (Table 1) confirm the suggested position of p11 within Pp70. These results combined with the tryptic peptide analysis and immune precipitation experiments lead us to propose the cleavage scheme presented in Figure 8 for the generation of FeLV non-glycosylated structural proteins.

Fig. 8.--Proposed scheme for the generation of the non-glycosylated structural proteins of FeLV. The order of FeLV proteins within Pp70 was that obtained from pactamycin gene ordering experiments. The existence of Pp25 is uncertain.

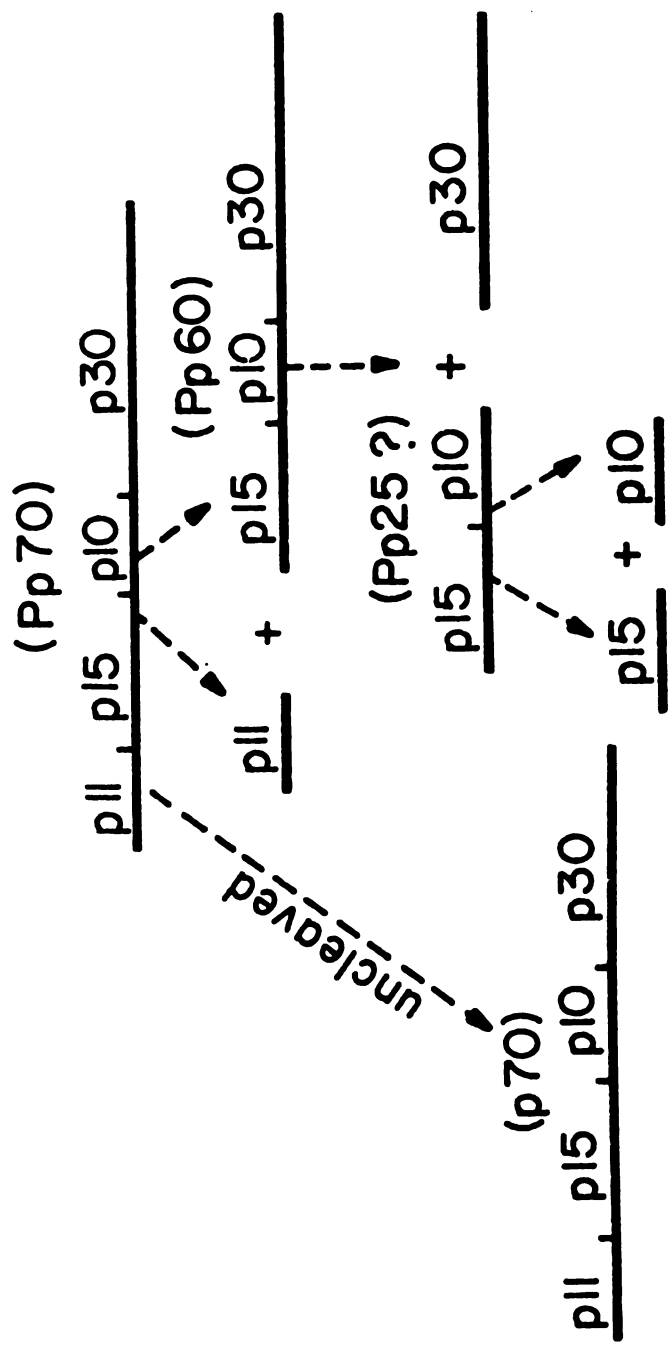
(Pp70)
p11
p15
p10
p30
uncleaved
(p70)
p11
p15
p10
p30
(Pp60)
p11
+
p15
p10
p30
(Pp25?)
p15
p10
+
p30
p15
+
p10

Figure 8

The first step in this scheme involves the generation of p11 and Pp60 from Pp70. Because p11 is the NH_2-terminal moiety, this cleavage may occur on a nascent polypeptide and thus could explain the detection of only low levels of Pp70 under normal labeling conditions (Figure 2a and b). The absence of any anti-p30 immune precipitable intracellular polypeptides other than Pp60 and p30 (Figure 2) argues that p30 is generated by direct cleavage of Pp60. The order of FeLV structural proteins also supports this interpretation in that p30 is carboxy-terminal on the Pp60 moiety. The generation of p30 from Pp60 should result in the formation of a precursor polypeptide of about 25,000 daltons in molecular weight (Pp25) containing p15 and p10. We have been unable to detect such a precursor with anti-p15 serum (G. Okasinski unpublished results) which leads us to believe that p15 and p10 may be rapidly generated directly from a would-be Pp25. The final feature of this proposed generation of structural proteins is the incorporation of Pp70 into assembled virus as p70.

Comparison of the arrangement and proposed processing of FeLV structural proteins with that recently reported for AMV reveals some distinct differences. The order of AMV structural proteins within a 76,000 dalton precursor (Pr76) is N-p17-p28-p14-C with the position of p10 being uncertain (28). The major group specific antigen (p28) is located internally within AMV Pr76 while the

corresponding FeLV protein is COOH-terminal on FeLV Pp70. Vogt et al. (28) have proposed that the first cleavage of Pr76 results in precursors of 66,000 daltons and 12,000 daltons in molecular weight (Pr66 and Pr12) the latter arising from cleavage at the COOH-terminal end and ultimately giving rise to AMV p14. Thus AMV precursor processing may differ from that proposed for FeLV in that the first cleavage is at the COOH-terminal end in the former and at the NH_2-terminal end in the latter. Avian Pr76, thus, requires complete or almost complete synthesis prior to the first cleavage while feline Pp70 could be cleaved as a nascent polypeptide. These differences could explain the relative ease of avian Pr76 isolation and the absence of abundant feline Pp70 under normal labeling conditions. Pr66 is believed to be further processed to a 60,000 dalton precursor (Pr60). At this point the two proposed schemes become very similar. It is believed that AMV p28, p10, and p17 are generated directly from AMV Pr60. This process may be analogous to the generation of FeLV p30, p15, and p10 from FeLV Pp60. Although Vogt et al. (28) have detected a 32,000 dalton precursor (Pr32), it is not believed to be an essential step in the generation of AMV proteins. Recent work with RLV precursor proteins has also failed to detect precursors of RLV p30 smaller than 65,000 daltons (1, 17) and may suggest a similar phenomenon in RLV precursor processing. Thus FeLV appears to differ from AMV

in both the arrangement of structural proteins in precursor polypeptides and in the initial cleavage from precursors but may be similar in the processing of 60,000 dalton precursor polypeptides. The incorporation of a precursor polypeptide into assembled virions also appears to be a unique phenomenon of FeLV and RLV (9) while absent from AMV.

The identification of precursor polypeptides in FeLV infected cells is consistent with similar reports of precursor polypeptides in AMV infected cells (31, 32) and in RLV infected cells (21, 30, 12). Precursor polypeptides similar to those identified in AMV and RLV infected cells have also been synthesized in cell-free translation systems (9, 13, 20, 33). In addition to the presence of precursor polypeptides in cells productively infected with oncornaviruses, these precursors have in every instance been indicated to have a membrane association (9, 21, 22, 30, 31, 32). While the synthesis of oncornavirus proteins in productively infected cells results in formation of high molecular weight membrane associated precursor polypeptides, the size of detectable precursors and their subsequent processing appears to vary. When amino acid analogues have been utilized to inhibit precursor processing, the results have varied from no effect in AMV infected cells (27) with fluorophenylalanine and canavanine to inhibition of formation of a 65,000 dalton precursor in RLV infected cells

with canavanine and no effect with fluorophenylalanine and L-azetidine-2-carboxylic acid (26). In our hands L-azetidine-2-carboxylic acid results in accumulation of Pp70 (Figure 5) while canavanine and fluorophenylalanine yield no detectable alterations in electrophoretic profiles of immune precipitable precursor polypeptides (G. Okasinski unpublished results). Our finding of partial inhibition of Pp60 cleavage by a general protease inhibitor (Figures 3 and 4) is to our knowledge, first such published report and serves to point out what may be fundamental differences in various oncornavirus precursor polypeptides and/or in host cell influences on precursor processing.

The synthesis and processing of RLV precursor polypeptides as discerned by two different groups of workers using different host cells may best exemplify the effects of host cells on precursor processing (17, 26). One group of workers, using JLS-V9 cells infected with RLV, could identify presumptive precursor polypeptides with molecular weights of 82,000 and 65,000 daltons. These precursor polypeptides are believed to be precursors of RLV p30, p15, and p12b (26). Results of immune precipitation of intracellular protein with anti-RLV indicated that very little if any RLV p30 was present intracellularly and suggested that production of p30 from precursor polypeptides is immediately followed by assembly of RLV p30 into extracellular virus. When JLS-V16 cells were examined for the

presence of RLV precursor polypeptides, four large polypeptides were identified with molecular weight of 200,000 90,000, 80,000, and 65,000 respectively (1, 17). JLS-V16 cells, however, do contain readily identifiable intracellular RLV p30, suggesting relatively larger pools of intracellular RLV p30. These differences in the number of identifiable precursor polypeptides, their size, and their processing rates (as judged by intracellular RLV p30 levels) all argue that due caution must be exercised in developing a general model of oncornavirus protein synthesis and processing.

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