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mRNA-dependent synthesis of authentic precursor to human placental lactogen: Conversion to its mature hormone form in ascites cell-free extracts

(*in vitro* protein cleavage/protein hormone/secretory proteins)

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ABSTRACT **Messenger RNA derived from term placenta directs the synthesis of human placental lactogen (hPL, molecular weight 22,200) in an ascites 30,000 × *g* post-mitochondrial supernate (S-30). When the S-30 is fractionated into ribosome and cell-sap (S-100) fractions, and these are recombined for incubation, term placental mRNA directs the synthesis of a protein with a molecular weight of 25,000. This protein contains authentic hPL tryptic peptides. This suggested that during the separation of ribosomes and S-100 a component responsible for cleavage was lost. A 1.0 M sucrose cushion was used for the preparation of ribosomes and S-100 and membranous material accumulated at the sucrose interphase. When this membrane fraction was added back to the ribosome-S-100 system only hPL was formed. Cleavage was greatest when membranes were added within the first few minutes of incubation. In a run-off system composed of term polysomes, ascites S-100, and the inhibitor of initiation, pactamycin, the 25,000 molecular weight material, referred to as pre-hPL, was also synthesized.**

These data strongly suggest that (*i*) pre-hPL is an authentic precursor to hPL, (*ii*) cleavage of the precursor primarily occurs on nascent, ribosome-bound peptide chains, and (*iii*) pre-hPL is the primary gene product.

A large number of proteins are synthesized in various precursor forms. Recently it has been shown that mRNAs that code for secretory proteins direct the synthesis in some cell-free systems of proteins larger than the circulating forms. Typically these contain an additional sequence of about 30 amino acids not found in their corresponding finished products (1–7). In the cell-free system derived from ascites tumor cells, both the larger and the finished forms have been observed. While these larger proteins have been presumed to be precursors, they have not been detected *in vivo*. This has usually been attributed to rapid cleavage to the final product in the cell. Cleavage of these proteins to their corresponding finished products would lend support to the physiological nature of these heavier proteins.

Messenger RNA isolated from term placenta directs the synthesis, in the 30,000 × *g* supernate (S-30) derived from wheat germ, of a protein that is larger (molecular weight 25,000) than the hormone human placental lactogen (hPL, molecular weight 22,200), but which nevertheless contains authentic hPL tryptic peptides (6). However, when the same mRNA was added to an S-30 fraction from ascites tumor cells hPL itself was synthesized (6). Here we show that when the ascites S-30 is fractionated into ribosomal and cell-sap fractions, term placental mRNA directs the synthesis of the same larger protein observed in the wheat germ system. The major factor responsible for the synthesis of finished product in the ascites S-30 is a particle-bound cleavage activity. Addition of this particulate fraction resulted in a shift of synthesis of the heavier protein to hPL. Furthermore, polysomes derived from term placenta, and incubated under run-off conditions, synthesized a 25,000 molecular weight protein that also contained the authentic hPL tryptic peptides.

Abbreviations: hPL, human placental lactogen; S-30, S-100, 30,000 and 100,000 × *g* supernates, respectively.

MATERIALS AND METHODS

[^{35}S]Methionine was obtained from Amersham/Searle and sucrose was purchased from Schwarz/Mann. Human placental lactogen (95% pure) was purchased from Nutritional Biochemicals.

Ribosomes, ribosome-free supernate (S-100) and the preincubated 30,000 × *g* post-mitochondrial supernate (S-30) were prepared from Krebs ascites tumor cells (8). To obtain ribosomes and S-100, the preincubated S-30 was placed on a layer of 1.0 M sucrose containing buffer A [30 mM Tris·HCl (pH 7.5), 120 mM KCl, 5 mM magnesium acetate, 7 mM 2-mercaptoethanol] and centrifuged for 5 hr at 50,000 rpm in a Spinco 60 Ti rotor. Wheat germ S-30, term placental mRNA, and polysomes were prepared as previously described (6, 9).

RNA-dependent protein synthesis was assayed in a 0.18 ml reaction mixture. Each reaction mixture contained 30 mM Tris·HCl (pH 7.5), 80 mM KCl, 2.6 mM magnesium acetate, 2.5 mM dithiothreitol, 0.3 mM GTP, 1.5 mM ATP, 1.0 mM creatine phosphate, 0.12 mg/ml of creatine kinase, 40 μM (each) of nonradioactive amino acids (minus methionine), 1.4 μM (15 μCi) [^{35}S]methionine, and term placental RNA. The amounts of extracts are noted in the experiments. Incubation was at 30° for 90 min and subsequent treatment was as previously described (6).

The products synthesized *in vitro* were examined on 20% polyacrylamide slab gels and by tryptic fingerprinting (6, 10).

RESULTS

Translation of placental RNA in ascites S-30 resulted in the synthesis of hPL primarily in the finished form (molecular weight 22,200) (Fig. 1A). However, when the ascites S-30 was fractionated into ribosome and cell-sap fractions (S-100) and these were recombined for the incubation, term placental mRNA directed the synthesis of a protein with a molecular weight of about 25,000 (referred to as heavy hPL). Its migration was the same as the hPL-containing protein synthesized in the wheat germ S-30 (ref. 6 and Fig. 1B).

hPL contains six tryptic peptides, each of which contains one methionine residue (11). The labeled protein synthesized in the ascites S-100–ribosome system contained methi-

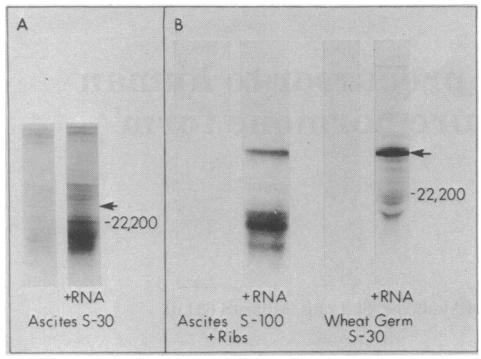

FIG. 1. Autoradiograph of a sodium dodecyl sulfate–polyacrylamide gel electropherogram containing proteins synthesized in cell-free extracts derived from ascites tumor cells and wheat germ. In part A the equivalent of 300 μg of S-30 (preincubated) protein was used. The RNA-dependent lane contains about 100,000 cpm of radioactivity and the lane denoting no RNA addition contains about 30,000 cpm. Part B shows the proteins synthesized in response to 30 μg of RNA in a mixture of ascites S-100 and ribosomes (100,000 cpm) and wheat germ S-30 (150,000 cpm). The equivalent of 20,000 cpm of labeled protein synthesized in the absence of RNA was applied to the control lanes. The ascites system contained 450 μg of S-100 and 20 μg of preincubated ribosomes. The equivalent amount of reaction mixture was added to each lane. The position of heavy hPL (arrows) and hPL (22,200) are indicated in both panels.

onine-labeled tryptic peptides (Fig. 2, panel B) that were coincident with authentic hPL peptides (Fig. 2, panel A). These labeled peptides were not observed when the ascites system was incubated in the absence of RNA. It is significant that the heavy hPL synthesized in the wheat germ displayed these same labeled tryptic peptides (6).

The marked difference in distribution between the synthesis of heavy hPL in the S-100–ribosome and the synthesis of hPL in the S-30 (Fig. 1) systems derived from ascites tumor cells suggested that a cleavage activity had been removed during separation of ribosomes and S-100. Removal of a particulate component was one strong possibility, since a 1.0 M sucrose cushion was used for separating these fractions. To test this point the material accumulating at the sucrose interphase was collected, diluted with buffer A, and centrifuged at 200,000 × *g* for 90 min. The pellet was resuspended and saturating amounts of this fraction (referred to as membranes) were added to incubation mixtures containing ribosomes, S-100, and mRNA at the start of incubation (Fig. 3B). The presence of this interfacial fraction results in the disappearance of most if not all of the heavy hPL protein and a corresponding appearance of hPL. When the membrane fraction was incubated in the absence of ribosomes, a small quantity of hPL was also synthesized (Fig. 3C), but the amount is negligible in comparison to the amount formed in the complete system (Fig. 3B). No heavy hPL was detected. The residual synthesis of hPL probably results from some contaminating ribosomes in the membrane fraction.

The above interpretations were substantiated by tryptic fingerprinting. The regions in preparative polyacrylamide gels corresponding to heavy hPL and hPL were eluted and subjected to fingerprint analysis. In the presence of ribosomes, S-100, membranes, and RNA (Fig. 3B) the eluted material from the region corresponding to heavy hPL contained no detectable methionine-labeled tryptic peptides related to hPL, whereas the region corresponding to finished hPL contained the six methionine-labeled tryptic peptides.

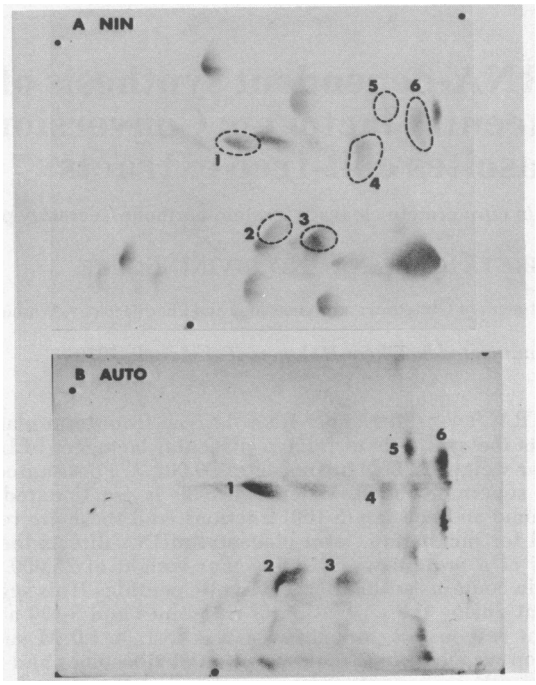

FIG. 2. Two dimensional tryptic fingerprint analyses of a mixture of carrier hPL and the labeled 25,000 molecular weight protein synthesized in the ascites S-100–ribosome system. Panel A has been stained with ninhydrin; panel B is the corresponding autoradiograph. The ninhydrin-positive peptides of hPL which show the same mobility as the labeled peptides are denoted by the broken rings. About 400,000 cpm was applied to the map. Peptide 5 stains weakly with ninhydrin. The labeled peptide immediately to the right of no. 1 does not coincide with the ninhydrin spot near no. 1.

When heavy hPL is generated by incubating 30–90 min in the absence of membranes; subsequent addition of membranes did not result in detectable cleavage. This suggests that the presumed cleavage activity takes place very rapidly on ribosome-bound nascent chains. It takes at least 10 min to synthesize heavy hPL. Therefore, if cleavage is an early

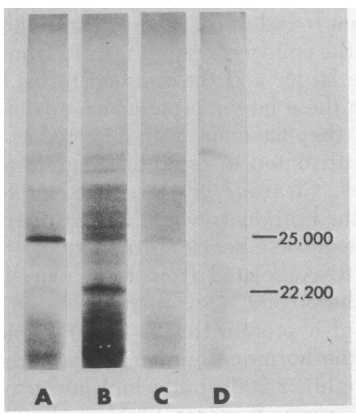

FIG. 3. Sodium dodecyl sulfate gel electrophoresis of proteins synthesized in the reconstituted ascites S-100–ribosome cell-free system in the presence and absence of membranes. Equivalent amounts of protein were added to each lane and the amounts of radioactivity applied were the following: A (S-100 + ribosomes), 60,000 cpm; B (S-100 + ribosomes + membranes), 130,000 cpm; C (S-100 + membranes), 70,000 cpm; and D (S-100 + membranes − RNA), 38,000 cpm. The migrations of hPL (22,200) and heavy hPL (25,000) are indicated. The complete reaction mixture contained 25 μg of term placental mRNA, the protein equivalent of 450 μg of S-100, 100 μg of membranes, and the RNA equivalent of 20 μg of purified ribosomes.

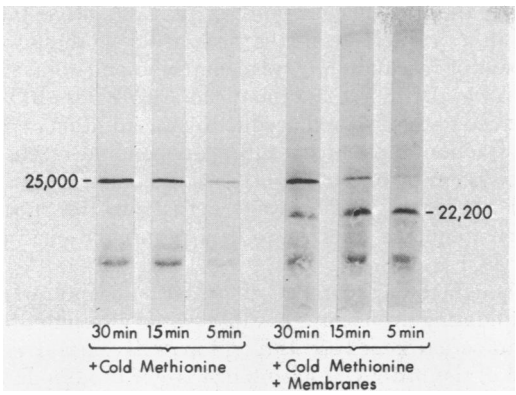

FIG. 4. Sodium dodecyl sulfate gel electrophoresis of proteins synthesized in response to placental RNA. Following incubation at the indicated times additions were made of 0.2 mM unlabeled ("cold") methionine and membranes or unlabeled methionine alone. After addition, incubation was continued for a total of 90 min. Equivalent amounts of protein were applied to each lane. The amounts of radioactivity added from incubations with both membranes and unlabeled methionine were: 5 min, 55,000 cpm; 15 min, 100,000 cpm; and 30 min, 120,000 cpm. The cpm added from incubations with unlabeled methionine alone were: 5 min, 32,000; 15 min, 55,000; and 30 min, 80,000. The quantity of cell-free components is the same as described for Fig. 3.

event during synthesis, we might see cleavage if the membrane fraction were added to the complete system during the first few minutes of incubation. Accordingly, reaction mixtures containing mRNA, S-100, and ribosomes were incubated for 5, 15, and 30 min, and then membranes and excess unlabeled methionine were added together. (The unlabeled methionine was added to prevent the appearance of labeled hPL synthesized in the presence of the membrane fraction.) The incubation was continued for a total of 90 min and samples were then taken for gel analysis (Fig. 4). It is clear that the addition of membranes after 5 min results in the synthesis primarily of hPL (confirmed by tryptic fingerprinting); very little heavy hPL was synthesized. In contrast, when unlabeled methionine alone was added after 5 min of initial incubation and the incubation was continued to 90

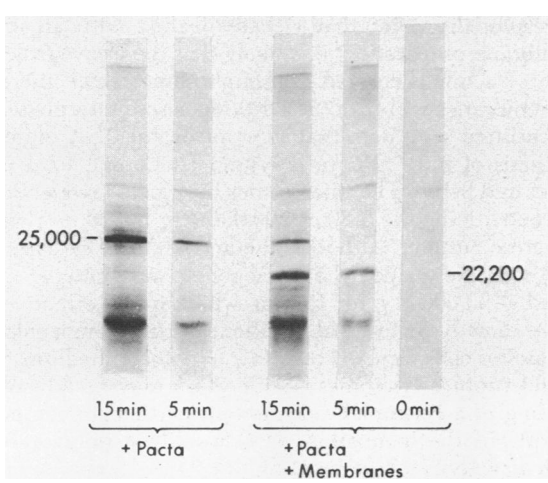

FIG. 5. Sodium dodecyl sulfate gel electrophoresis of proteins synthesized in response to placental RNA. Incubations were the same as in Fig. 4, except 1.0 μM pactamycin was added instead of unlabeled methionine. The following amounts of radioactivity were applied from reactions with membranes plus pactamycin: 0 min, 20,000 cpm; 5 min, 45,000 cpm; and 15 min, 75,000 cpm. The radioactivities from reactions treated only with pactamycin were: 5 min, 30,000 cpm and 15 min, 50,000 cpm.

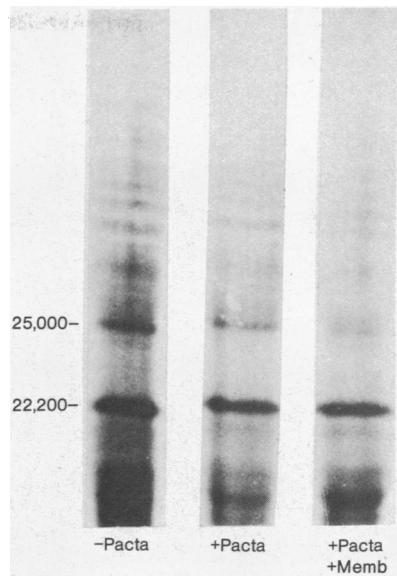

FIG. 6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of proteins synthesized in a run-off system composed of term placental polysomes and ascites S-100. Where indicated, 1.0 μM pactamycin and membranes were added.

min, only heavy hPL was synthesized. After longer periods of initial incubation there was a progressive increase in the amount of heavy hPL synthesized despite the addition of the membrane fraction. Therefore, it is apparent that cleavage of heavy hPL occurs before it is released from the ribosome or just after release. All of the finished hPL which appears following the addition of membranes at 30 min (and part of that at 15 min) probably reflects reinitiation by ascites ribosomes prior to membrane addition; this provides a supply of nascent, ribosome-bound substrate for cleavage by the membranes.

This experiment was repeated using 1 μM pactamycin, instead of a chase of unlabeled methionine, to block membrane-dependent protein synthesis (Fig. 5). Pactamycin at this concentration blocks placental-mRNA-dependent initiation in the ascites system. Again only finished product was synthesized in substantial amounts when membranes and pactamycin were added after 5 min, whereas significant amounts of the heavy form were observed when they were added after 15 min. Thus the data indicate that a precursor–product relationship exists between heavy hPL and its mature hormone form.

Further evidence that heavy hPL may be a physiological intermediate comes from work with detergent-treated placental polysomes. If, under run-off conditions (where ribosomes complete the synthesis of nascent chains), reinitiation is prevented, any synthesis of heavy hPL would indicate that the protein had been preinitiated in the tissue. To examine this point the run-off system was incubated in the presence of pactamycin. In a cell-free system containing pactamycin, term placental polysomes, and ascites cell-sap, a protein having a molecular weight of 25,000 and hPL were synthesized (Fig. 6). There was a reduction in synthesis of the heavier protein with pactamycin, indicating that some reinitiation could otherwise occur. The migration of the heavier protein was the same as that of the protein synthesized in the wheat germ S-30 and homologous ascites systems programmed with exogenously added placental mRNA. This protein contained the same [^{35}S]methionine-labeled tryptic peptides corresponding to hPL (Fig. 7). The synthesis of heavy hPL

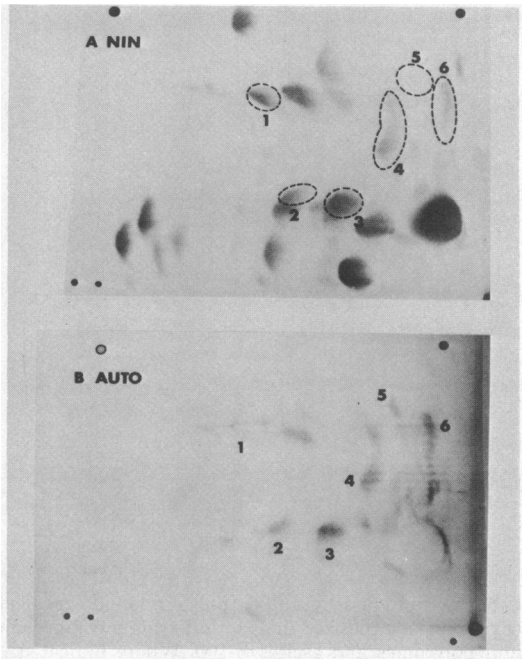

FIG. 7. Two-dimensional tryptic fingerprint analysis of a mixture of carrier hPL and labeled heavy hPL synthesized in a run-off system containing 1.0 μM pactamycin. (A) ninhydrin stain; (B) autoradiograph.

probably reflects the fact that detergent treatment releases polysomes from membranes and these membranes would be eliminated by accumulating at the 1.0 M sucrose interphase during the subsequent step in preparation of the polysomes (9). The finished product which also appears presumably indicates the fraction of nascent protein which had already been cleaved *in vivo*.

Further evidence that the endogenously synthesized heavy hPL did not arise as a result of reinitiation is shown by the distribution of tryptic peptides on the maps. Of the six methionine tryptic peptides, peptide I is closest to the amino terminus of hPL (9). The methionine in this peptide represents the 14th amino acid residue from the amino terminal end. This tryptic peptide was the most radioactive when derived from heavy hPL synthesized in the homologous ascites system programmed with exogenously added mRNA (Fig. 2). However, under non-reinitiating conditions, this peptide, as would be expected, was the least radioactive (Fig. 7).

Additional evidence that this protein is similar to that synthesized in the mRNA-dependent system is shown in Fig. 6. Adding the ascites membrane fraction at the beginning of incubation to the run-off system in the presence of pactamycin markedly diminished the level of the hPL precursor. Therefore, the hPL precursor synthesized under run-off conditions is also susceptible to cleavage by ascites membranes.

DISCUSSION

The data presented support the hypothesis that the heavy hPL (or pre-hPL) synthesized in the wheat germ cell-free system programmed with term placental mRNA represents an authentic precursor. First, the synthesis of this protein was not restricted to the wheat germ system, since the protein was also synthesized in the S-100-ribosome system derived from ascites tumor cells. Second, more direct evidence that heavy hPL is an authentic precursor was the demonstration that the protein was synthesized from placental polysomes in a "run-off" cell-free system. Its synthesis was not the result of reinitiation by placental ribosomes and thus the protein was preinitiated in the tissue. Finally, pre-hPL could be cleaved to the finished product by the addition of a membrane fraction to either the mRNA-dependent reconstituted ascites system or to the run-off system containing term placental ribosomes. The cleavage activity in this membrane fraction might be referred to as a preprotein cleavage enzyme (PCE).

Although the appearance of pre-hPL did not arise from false initiating events, it is possible that as a result of improper termination some read-through may have occurred at the 3′ end of the mRNA, resulting in a larger protein. However, as the data show, pre-hPL is probably cleaved before completion of synthesis, which indicates a structural difference at the amino terminus. This is analogous to the finding of Kemper *et al.* (5) that the structural difference between the pre-proparathyroid hormone synthesized in the wheat germ and the mature hormone form is at the amino terminus; the carboxyterminal cyanogen bromide peptides were the same. Heavy forms of immunoglobulin light chain also contain differences at the amino termini of the proteins (ref. 4, D. Swan, Marion Nau, D. McKean, and P. Leder, personal communication). Therefore, it seems safe to conclude that pre-hPL is a physiologic intermediate and represents a precursor form of its corresponding finished product.

It may be surmised that similar proteins synthesized in cell-free systems programmed with immunoglobulin, parathyroid, and growth hormone mRNAs are authentic intermediates. These data are also consistent with the idea that these precursors represent the primary gene products.

The lack of detectable cleavage activity in wheat germ extracts accounts for the synthesis of these precursor proteins. In contrast, an mRNA coding for the nonsecretory protein globin directs the synthesis of only the mature form in wheat germ extracts (12). The fact that tissue-specific secretory proteins are cleaved to finished products by a membrane fraction from a different cell type (ascites tumor) suggests that a variety of precursors contain the same or very similar recognition sites for the cleavage activity.

Despite the point that we are dealing with an impure membrane preparation, it is likely that the cleavage activity in this fraction is derived from microsomes, since microsomal membranes will band at a 1.0 M sucrose interphase. This is consistent with data that show placental RNA directs the synthesis of only hPL in a system containing total microsomes and S-100. The microsomes in this case were obtained by centrifuging the S-30 at 200,000 × *g* for 90 min without a sucrose cushion. Although the activity may be lysosomal, this is unlikely since the homogenate was initially centrifuged at 30,000 × *g* for 15 min, which would effectively remove most lysosomes. In addition, during homogenization, the ascites cells were exposed to a hypotonic medium, which would rupture lysosomes. While there may be adventitious binding of a lysosomal enzyme to a particulate component, the pH of the incubations (7.4) would be unfavorable for such an activity.

It appears that the transient existence of these precursors in the tissue results from a rapid cleavage of the protein while it is synthesized on the ribosome, as was suggested earlier (2, 5). This is supported by data showing that the released precursor cannot be cleaved by subsequent additions of ascites membranes; cleavage was only observed when the membrane fraction was added prior to the synthesis of com-

pleted chains. Milstein *et al.* (2) also showed that the immunoglobulin light chain precursor synthesized in a reticulocyte cell-free system could not be cleaved by subsequent addition of ascites microsomes. Perhaps the protein has to be attached to peptidyl tRNA in order to be recognized, and after release the protein is not cleaved.

It has been suggested that, during the synthesis of secretory proteins, the nascent chain is vectorially released into the cisternal space of the endoplasmic reticulum (13–16). Thus it is possible that, as a nascent chain, pre-hPL has to penetrate the membrane in order to be cleaved. This penetration would be aided by the membrane-anchored ribosome. However, once the protein is released such penetration might be difficult.

There is a precedent for such a cleavage mechanism in the synthesis of picornaviral proteins. It appears that the processing of poliovirus-specific proteins requires several maturation-cleavage steps. At least one of these involves cleavages of ribosome-bound nascent virus-specific proteins (17, 18). Early cleavage of one of the translational products of encephalomyocarditis virus RNA has been shown in infected ascites extracts (19).

Note Added in Proof. Similar conclusions were reached with the myeloma cell-free system by G. Blobel and B. Dobberstein (1975) *J. Cell Biol.* **67**, 835–851.

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