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Inhibition of preprotein processing in ascites tumor lysates by incorporation of a leucine analog

(secretory proteins/cell-free protein synthesis/preprotein cleavage/β-hydroxyleucine)

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ABSTRACT **Leucine analogs were tested in the Krebs II ascites cell-free translation system for the ability to inhibit preprotein cleavage by replacing leucine in nascent chains of bovine preprolactin, rat preprolactin, human placental prelactogen (pre-hPL), and pre-α subunit of human chorionic gonadotropin (α-hCG). In the absence of analog, ascites microsomal membranes cleaved these preproteins to their mature forms and sequestered the processed products. Also, two asparagine residues in α-hCG were glycosylated. When 4 mM β-DL-hydroxyleucine was added to the lysate instead of L-leucine, cotranslational processing and sequestration of both species of preprolactin and pre-hPL were inhibited. Sequential Edman degradation confirmed that pre-hPL was not cleaved. The inhibition of processing by β-hydroxyleucine resulted from its incorporation into protein. This was shown by reversal of the effect by addition of leucine and by inhibition of [^{3}H]leucine incorporation into protein. Of significance, the processing of pre-α-hCG was less sensitive to β-hydroxyleucine because its prepeptide contains only four scattered leucine residues, whereas the presegments of hPL and the prolactins contain six to eight clustered leucine residues. These experiments demonstrate that translocation and processing of secretory proteins require structural features determined by the primary amino acid sequence.**

The messenger RNAs of most secretory proteins encode polypeptides with extensions of 15–30 amino acids at the amino terminus of the mature protein (reviewed in ref. 1). *In vivo* and *in vitro* this extension, termed the signal peptide or prepeptide, is removed during translation by a proteolytic activity in membranes of the endoplasmic reticulum (2–5). The completed peptide chains are sequestered within the lumen of the endoplasmic reticulum and channelled into the secretory pathway (6). Prepeptides have been proposed to act as the signal that distinguishes secretory proteins from cytoplasmic proteins (3, 7). This hypothesis was inferred from binding of the mRNA–ribosome–nascent protein complex to the endoplasmic reticulum during translation and from the absence of prepeptides in cytoplasmic proteins. However, there is no direct evidence that prepeptides encode the information for secretion in eukaryotes.

The amino acid sequences of numerous prepeptides have been determined with the objective of defining the recognition regions for protein secretion (1, 8). Despite these analyses, the structural determinants for this signal function have not been identified. Prepeptides vary in length, and their amino acid sequences are heterogeneous. There is also a wide variation in the carboxy-terminal amino acid of the presegment; glycine, alanine, serine, cysteine, or threonine may be the amino acid prior to the final cleavage site. One important structural feature of prepeptides is a central hydrophobic region, which is often rich in leucine.

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An approach to probing the essential structural regions of prepeptides was suggested by the early studies of poliovirus protein precursors (9). Analogous to preprotein processing, these viral proteins were cleaved during synthesis and, thus, full-length translation products were not observed *in vivo*. Substitution of amino acid analogs for arginine, proline, and phenylalanine in the poliovirus polypeptide modified the protease cleavage sites, which then permitted isolation of the full-length polyprotein.

Because many prepeptides contain leucine-rich sequences, we examined the effect of a leucine analog on the preprotein cleavage reaction. Inhibition of cleavage could result from modification of presumed signal regions in the nascent chain, impaired translocation of the protein across the membrane, or an altered cleavage site. This leucine analog, *threo*-β-DL-hydroxyleucine, inhibited cotranslational translocation and processing of several preproteins. These studies provide direct evidence that the necessary information for the initial phases of extracellular transport of eukaryotic secretory proteins is contained within the protein structure.

MATERIALS AND METHODS

β-DL-Hydroxyleucine and *threo*-β-DL-hydroxyleucine were obtained from U.S. Biochemical and Calbiochem–Behring, respectively. [^{35}S]Methionine (500–1000 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels), [^{3}H]proline (117 Ci/mmol), and [^{3}H]leucine (120 Ci/mmol) were obtained from Amersham. Other L-amino acids were purchased from Sigma.

Antiserum to reduced, carboxymethylated α-subunit of human chorionic gonadotropin (α-hCG) was generously supplied by S. Birken and R. Canfield. Krebs II ascites cell lysates and total RNA from placenta and bovine and rat pituitaries were prepared as described (2).

Cell-free products were immunoprecipitated (10) and their amino acid sequences were determined as described (11), except that sequencer fractions were analyzed directly by liquid scintillation spectrometry. Assay mixtures for measurement of cell-free protein synthesis (4) contained 50 μM of each L-amino acid except for 5–10 μCi of labeled amino acid and the omission of leucine. Stock solutions of β-hydroxyleucine were adjusted to pH 7.6. Each 60-μl reaction mixture contained 2 or 3 μl of membranes (40 A_{260} units/ml), 12 μl of ribosome-free supernate, 0.5 μl of purified ribosomes (70 A_{260} units/ml), and 3–10 μg of total placental or pituitary RNA. Cell-free reaction mixtures were incubated for 1 hr at 30°C. Products of translation were analyzed on $NaDodSO_4$/20% polyacrylamide gels (4). Gels were soaked for 10 min in 1 M sodium salicylate, dried, and autofluorographed at −70°C (12).

Abbreviations: pre-hPL, human placental prelactogen; α-hCG, α subunit of human chorionic gonadotropin; pre-hPL*, pre-hPL synthesized in the presence of β-hydroxyleucine.

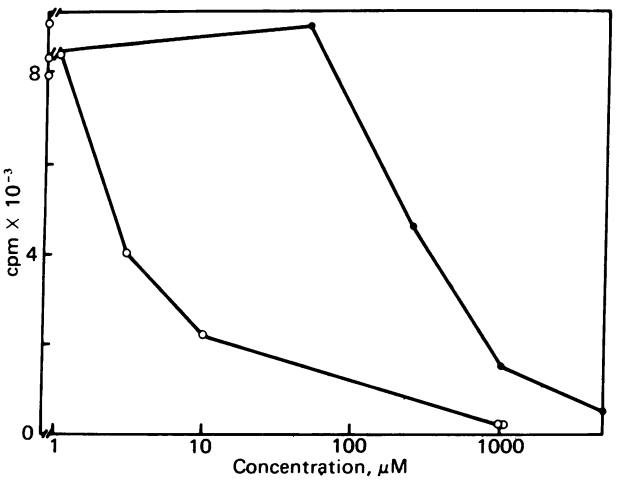

FIG. 1. Competition of L-leucine and *threo*-β-DL-hydroxyleucine with [^{3}H]leucine in the ascites cell-free system. L-Leucine (○) or *threo*-β-DL-hydroxyleucine (●) was mixed with L-[^{3}H]leucine (0.25 μM final concentration); then the other components were added. Each point represents duplicate 15-μl aliquots, the radioactivity of which was determined by the method of Mans and Novelli (13) except for omission of the heating step. The cpm reflect the incorporation of label into tRNA and protein.

RESULTS

Effect of β-Hydroxyleucine on [^{3}H]Leucine Incorporation. In ascites tumor cell-free lysates, 300 μM *threo*-β-DL-hydroxyleucine inhibited L-[^{3}H]leucine incorporation into trichloroacetic-acid-precipitable material by 50% (Fig. 1). (The incorporation of [^{3}H]leucine into protein was estimated on aliquots boiled in the presence of trichloroacetic acid; a similar inhibition curve was also observed with the analog.) Comparable inhibition with unlabeled L-leucine was seen at 3 μM. Thus, the endogenous pool of L-leucine in the lysate was about 3 μM and *threo*-β-hydroxyleucine competed 1/100th as effectively as L-leucine. Incorporation of [^{35}S]methionine into protein was inhibited only 20% by 5 mM *threo*-β-DL-hydroxyleucine, a concentration that inhibited [^{3}H]leucine incorporation more than 95%. β-DL-Hydroxyleucine (U.S. Biochemicals), which probably contains both *erythro* and *threo* stereoisomers, competed half as effectively as the purified *threo* form (Calbiochem–Behring). Automated amino acid analysis of the *threo*-β-DL-hydroxyleucine yielded a single major peak (data not shown). This analysis also revealed that less than 0.1% leucine was present in the sample of *threo*-β-DL-hydroxyleucine; thus leucine contamination could not account for the competition with [^{3}H]leucine.

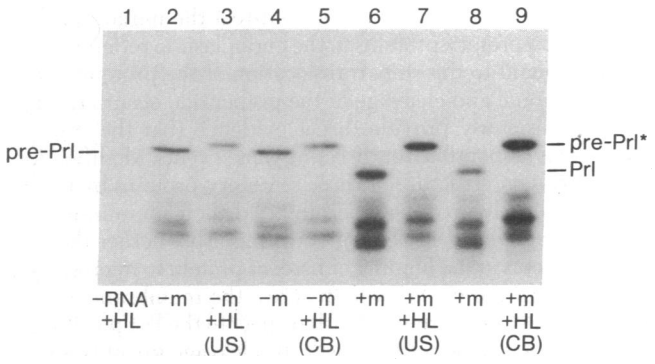

FIG. 2. Effect of 4 mM β-DL-hydroxyleucine (HL) from U.S. Biochemicals (US) or the *threo* steroisomer from Calbiochem–Behring (CB) on the processing of bovine preprolactin by ascite tumor membranes (m). Bovine pituitary RNA was translated by ascites lysates containing [^{35}S]methionine. Products were analyzed on a $NaDodSO_4$/20% polyacrylamide slab gel. Autofluorography of the dried gel was for 12 hr. Each lane represents 15 μl of reaction mixture. Products were identified as prolactin (Prl), preprolactin (pre-Prl), and preprolactin synthesized in the presence of β-hydroxyleucine (pre-Prl*).

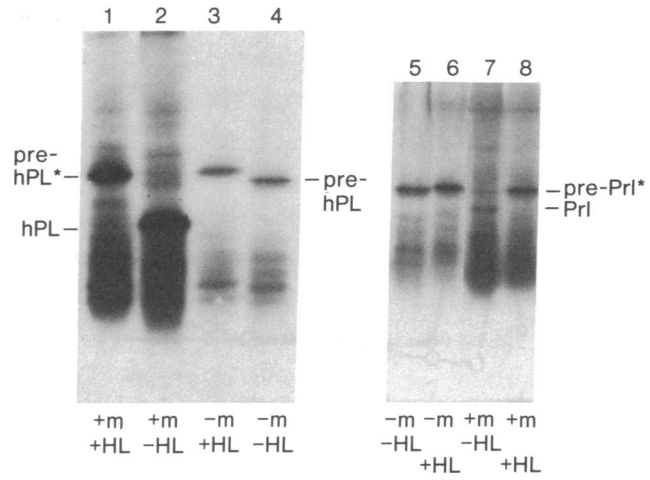

FIG. 3. Effect of 10 mM β-DL-hydroxyleucine (HL) on the processing of pre-hPL (lanes 1–4) and rat preprolactin (lanes 5–8) by ascites membranes (m). Rat pituitary (3 μg/60 μl) and placental RNA (10 μg/60 μl) were translated by ascites lysates containing [^{35}S]methionine and the products were resolved as discussed in the legend of Fig. 2.

Inhibition of Processing. Bovine and rat preprolactin and human placental prelactogen (pre-hPL) are cleaved cotranslationally to their mature forms by microsomal membranes (4, 14–16). β-Hydroxyleucine, at concentrations that completely blocked [^{3}H]leucine incorporation, was tested for its ability to inhibit preprotein processing. Bovine preprolactin synthesized in the absence of analog (Fig. 2, lanes 2 and 4) was converted to a smaller form that comigrated with an ovine prolactin standard when membranes were added at the start of translation (lanes 6 and 8). Sequence analysis of the first 10 amino acid residues of the cleaved form labeled with [^{3}H]proline revealed peaks at 2, 5, and 8 which correspond to the positions of proline in authentic bovine prolactin (14) (data not shown). However, preprolactin synthesized in the presence of β-hydroxyleucine (preprolactin*) had the same mobility whether synthesized in the absence (lanes 3 and 5) or presence (lanes 7 and 9) of membranes. Also, preprolactin* had a slightly lower electrophoretic mobility than preprolactin. This suggested that the analog was incorporated into preprolactin*. β-DL-Hydroxyleucine (U.S. Biochemicals) and the *threo* stereoisomer (Calbiochem–Behring) had identical effects, indicating that at least the *threo* form was active. Post-translational addition of β-hydroxyleucine to the lysate had no effect on the mobility of products (data not shown). Thus, the analog-induced change in mobility of preprolactin was dependent on protein biosynthesis and this change was not the result of nonspecific action on the electrophoretic migration of the protein. The change in mobility may result from decreased binding of $NaDodSO_4$ to the more polar analog-containing protein.

The membrane-dependent conversion of rat preprolactin and pre-hPL to smaller forms with the mobility of prolactin and hPL was also inhibited by β-hydroxyleucine. These preproteins* also had decreased electrophoretic mobility (Fig. 3) relative to the corresponding preproteins.

Partial amino-terminal sequence analysis was performed on the product identified as pre-hPL* to confirm that it had not been cleaved to hPL (Fig. 4). Methionine was identified at position 1 and proline at position 2 in accord with the known

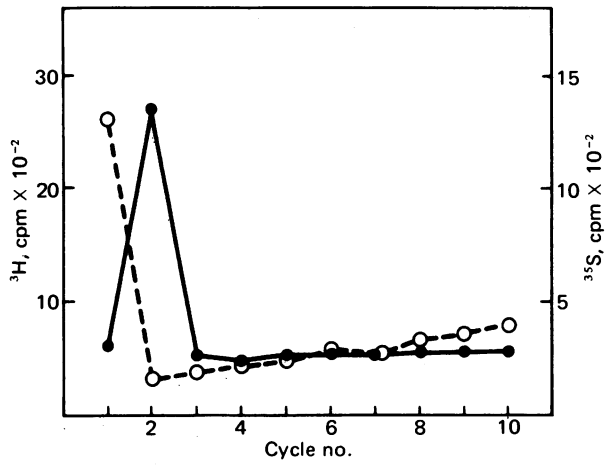

FIG. 4. Partial sequence analysis of pre-hPL*. [^{35}S]Methionine-labeled (O---O) and [^{3}H]proline-labeled (●—●) pre-hPL* synthesized in the presence of ascites of membranes was purified by electrophoresis, eluted from the gel, and mixed with 6 mg of rat serum albumin. The protein was then precipitated with 10 vol of 0.1 M HCl/acetone. Sample applied to the sequencer contained 143,000 cpm of [^{3}H]proline and 68,000 cpm of [^{35}S]methionine.

amino-terminal sequence of pre-hPL (16, 17). Proline was not recovered on the fifth cycle although it would have occupied this position if the product had been cleaved to hPL (18). Thus, pre-hPL* that accumulated in the presence of the analog contained the amino-terminal dipeptide sequence of pre-hPL and was not processed. The sequence of bovine preprolactin was not determined because it contains a blocked amino terminus (R. Jackson and G. Globel, personal communication).

Reversal of Inhibition of Processing. A crucial prediction was that, if the analog inhibited processing through its incorporation into protein, the effect should be reversed by adding leucine simultaneously with the analog. Leucine completely reversed the analog effect even though the concentration of the analog was 5-fold higher (Fig. 5*A*). Processing of bovine preprolactin to prolactin was restored (lane 7) and the change

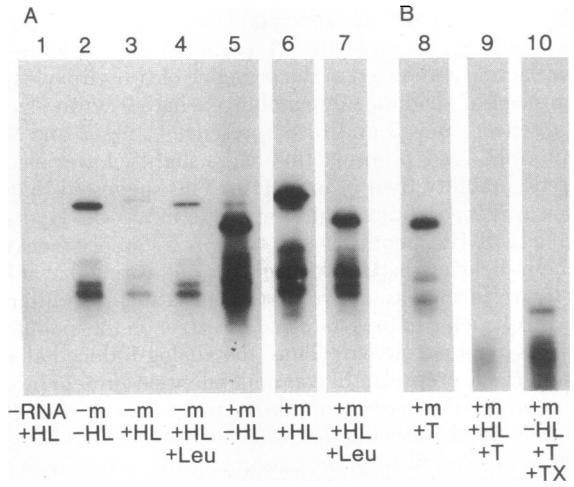

FIG. 5. (*A*) Reversal of the effects of 10 mM β-DL-hydroxyleucine (HL) on bovine preprolactin by 2 mM L-leucine (Leu) in the presence and absence of membranes (m). β-Hydroxyleucine and leucine were added together with bovine pituitary RNA to ascites lysates containing [^{35}S]methionine. Each lane represents an equal volume of reaction mixture. (*B*) Effects of β-hydroxyleucine on the sequestration of preprolactin*. After translation, cell-free products synthesized in a 60-μl reaction mixture were incubated for an additional 30 min at 24°C with 1 μg of trypsin (T). Lane 9 represents the effect of post-translational incubation with trypsin and 0.1% Triton X-100 (TX).

in electrophoretic mobility was reversed (lane 4). Thus, these data show that the action of the analog was due to its incorporation into these preproteins rather than through a direct effect on the membrane as observed with detergents (19, 20), Ca^{2+} (21), and an ovalbumin tryptic fragment (22).

In eukaryotes it is not known if cleavage of the prepeptide is obligatory for sequestration although in bacteria, secretion of an uncleaved preprotein can occur (23). Preprolactin* that accumulated in the presence of microsomes was tested for susceptibility to trypsin (Fig. 5*B*) to determine whether sequestration had occurred. None of the preprolactin* was protected from trypsin (lane 9). However, prolactin was protected from the protease (lane 8). Addition of a low concentration of detergent rendered the prolactin sensitive to trypsin (lane 10), indicating that the resistance to trypsin had been conferred by the membrane vesicles. Similar results were obtained with pre-hPL and rat preprolactin (data not shown). Thus, both sequestration and the associated cleavage of these preproteins were inhibited by the analog.

Processing of Pre-α-hCG. In the absence of membranes, first-trimester placental mRNA directed the synthesis of the pre-α-hCG. (Fig. 6, lane 3; ref. 10). In the presence of microsomal membranes, the presegment was removed and mannose-rich sugar units were attached to two asparagine residues, resulting in a product with decreased electrophoretic mobility (lane 5; refs. 10 and 24). Glycosylation and sequestration of the immunoprecipitated products occurred in the presence of β-hydroxyleucine (lanes 6 and 8) although in the same experiment processing of preprolactin (lane 11) was completely blocked by the analog (lane 12). As expected, low concentration of detergent rendered all products synthesized in the absence and presence of analog sensitive to trypsin (lanes 9 and 10). To determine if cleavage of the prepeptide had occurred, we performed sequential Edman degradation on [^{3}H]proline-labeled, immunoprecipitated sequestered product synthesized in the presence of the analog. Proline occurs in positions 2 and 8 of authentic α-hCG (25, 26), and there are no proline residues in the prepeptide (27, 28). Peaks of [^{3}H]proline were released on sequencer cycles 2 and 8 (Fig. 7), and recovery of [^{3}H]proline was similar to a previous analysis of α-hCG synthesized in the absence of the analog (24). Significant processing of pre-α-hCG shows that β-hydroxyleucine did not cause a general inhibition of preprotein processing.

DISCUSSION

Prepeptides have been proposed to direct the initial binding of nascent secretory proteins to the endoplasmic reticulum (3, 7). Subsequent to this step, translocation of the protein across the membrane and cleavage of the prepeptide occur. The results of this study provide direct evidence that the protein structure is responsible for directing these events. Modification of the primary structure of three secretory proteins by incorporation of β-hydroxyleucine inhibited their membrane-dependent segregation. We cannot distinguish whether the primary defect is in the binding of nascent protein to microsomes, the translocation of the protein across the membrane, or the cleavage of the preprotein. With respect to the last possibility, there are no leucine residues near the cleavage site of pre-hPL and bovine preprolactin, so direct modification of this site was not responsible for inhibiting cleavage.

One approach to the direct examination of whether the cleavage step is affected is to use detergent-solubilized preprotein cleavage activity (29, 30). In these preparations the cleavage reaction occurs post-translationally, and it is not dependent on the binding and sequestration steps. Preliminary data indicate that bovine preprolactin* was not processed by detergent-solubilized extracts, which did process preprolactin

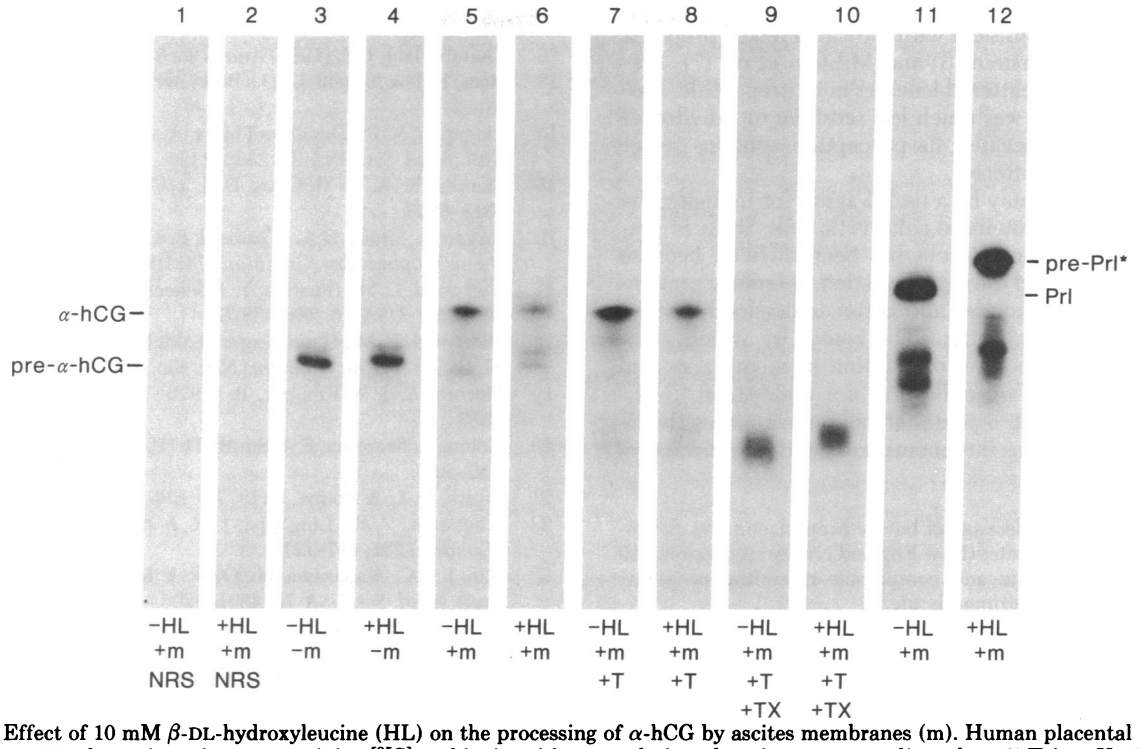

FIG. 6. Effect of 10 mM β-DL-hydroxyleucine (HL) on the processing of α-hCG by ascites membranes (m). Human placental RNA was translated in a 120-μl reaction mixture containing [^{35}S]methionine. After translation, the mixtures were adjusted to 1% Triton X-100 and 1% sodium deoxycholate and centrifuged for 15 min at 100,000 × *g* in a Beckman Airfuge. α-hCG-related products were isolated from the supernate as described (10). Lanes 1 and 2 represent products precipitated with nonimmune rabbit serum (NRS). Sequestration of products was assayed with trypsin (T) and 0.1% Triton X-100 (TX). After translation, the reaction mixtures were incubated for an additional 30 min at 24°C with 1 μg of trypsin. α-1-Antitrypsin (400 μg) was added and products were immunoprecipitated. Immunoprecipitated products (3500 cpm) were placed on lanes 3–8. Lanes 1, 2, 9, and 10 received 300, 250, 1800, and 2400 cpm, respectively, although the maximum volume was added. Total cell-free product (3600 cpm) was placed on lanes 11 and 12 to determine the effect of β-hydroxyleucine on cleavage of preprolactin (pre-Prl) to prolactin (Prl) in the same experiment.

synthesized in the absence of analog (G. Hortin, A. Strauss, and I. Boime, unpublished results). Thus, the cleavage reaction was directly inhibited. If cleavage is a prerequisite for the translocation of eukaryotic preproteins, inhibition of cleavage may cause the observed inhibition of sequestration. Study of other preproteins synthesized in the presence of analogs may clarify whether cleavage is obligatory for sequestration as well as providing further information on the structural requirements of the cleavage site.

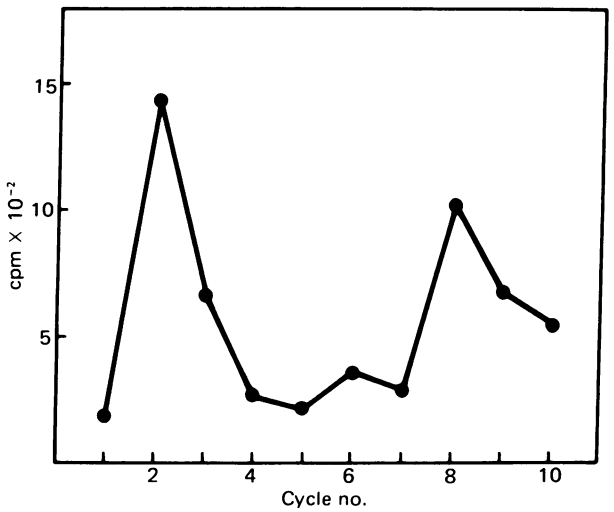

FIG. 7. Sequence analysis of [^{3}H]proline-labeled α-hCG* (15,000 cpm) synthesized in the presence of ascites membranes. After 60 min of incubation, 10 μg of trypsin was added to a scaled-up reaction mixture (1.2 ml). Incubation was continued for an additional 30 min, 4 mg of α-1-antitrypsin was added, and α-hCG was purified by immunoprecipitation (10).

Hydrophobicity may be an important characteristic of prepeptides (31), facilitating association of nascent preproteins with the endoplasmic reticulum and initiation of translocation through membranes. β-Hydroxyleucine is intermediate in hydrophobicity between leucine and threonine; each substitution of β-hydroxyleucine for leucine into protein results in a substantial decrease in hydrophobicity (approximately 800 cal/mol) (32).

Prepeptides of pre-hPL, rat preprolactin, and bovine preprolactin each contain two clusters of leucine residues, with a total of seven, six, and eight leucines, respectively (Fig. 8). Mature bovine prolactin has no segment that is nearly as leucine rich as its prepeptide (35); hPL and rat prolactin each have one such segment (18, 33). Changes in hydrophobicity and structure by the analog should be enhanced by the clustering of leucines so that, particularly for bovine preprolactin, the greatest changes in structure should be in the prepeptide. Thus, alter-

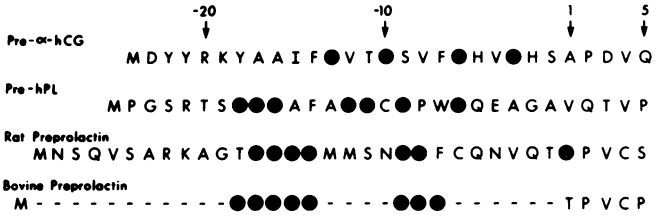

FIG. 8. Comparison of amino-terminal sequences of pre-α-hCG (25–28), pre-hPL (16, 18), rat preprolactin (33, 34), and bovine preprolactin (35, 36). Residues are numbered from the amino terminus of the mature hormones. Filled circles denote the position of leucine residues.

ation of the prepeptide probably was responsible for the inhibition of processing, consistent with the signal model proposed by Blobel and Dobberstein (3) and Milstein *et al.* (7). Pre-α-hCG has only four scattered leucines in its prepeptide. Processing of pre-α-hCG was much less sensitive to β-hydroxyleucine presumably because its prepeptide contains fewer leucines, which are scattered.

β-Hydroxyleucine may be a useful agent for inducing the accumulation of preproteins in eukaryotic cells. Study of preprotein synthesis by intact cells has been difficult because preproteins are cleaved during translation. Secretory proteins represent one of the simpler and better understood cases of protein compartmentalization. The routes by which other proteins achieve the correct orientation in membranes and segregate in specific organelles are not understood. β-Hydroxyleucine represents a potential tool for examining the insertion of these proteins into membranes and into the appropriate organelle.

Note Added in Proof. Cleavage of bovine preprolactin was also inhibited in reticulocyte lysates (New England Nuclear) containing 10 mM β-DL-hydroxyleucine and comparable quantities of ascites membranes used with the tumor lysate.

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