Vol. 92, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 12, 1980 Pages 1042-1051

PARTIAL N-TERMINAL AMINO ACID SEQUENCE OF PRO-OPIO-MELANOCORTIN (ACTH/BETA-LPH PRECURSOR) FROM RAT PARS INTERMEDIA

F. Gossard, N.G. Seidah, P. Crine, R. Routhier and M. Chrétien
Protein and Pituitary Hormone Laboratory
Clinical Research Institute of Montreal
110 Pine Avenue West, Montreal H2W 1R7

Received October 28,1979

SUMMARY. Posterior lobes of rat pituitary (pars intermedia plus pars nervosa) were incubated with various labeled amino acids and the cell extracts analyzed by NaDodSO$_4$ polyacrylamide disc gel electrophoresis. Two forms of precursor proteins for beta-endorphin and alpha-MSH were synthesized. Both forms have been shown to contain the fragments beta-LPH 61-69 and ACTH 1-8 and are thought to have the same peptide backbone. The two forms were simultaneously submitted to automatic Edman degradation and the following partial sequence was obtained: Trp/Arg$_1$-Leu$_3$-Phe$_4$-Ser$_5$-Ser$_6$-Leu$_{11}$-Thr$_{12_13}$-Tyr$_{14}$-Ser$_{15}$-Leu$_{17_18}$-Ala$_{19}$-Ile$_{21}$-Arg$_{22_25}$-Leu$_{26_2}$ Ser$_{29}$. This sequence was compared with that reported by Nakanishi et al. (18). Their amino acids sequence was indirectly derived from DNA sequencing after isolation of mRNA from bovine pars intermedia. This comparison indicates the presence of a signal peptide of 26 amino acids in the sequence of beef ACTH/beta-LPH precursor.

INTRODUCTION

The intermediate lobe of the pituitary contains a number of peptide hormones: adrenocorticotropin (ACTH) (1,2), alpha-melanotropin (alpha-MSH, ACTH 1-13) (3,4) and corticotropin-like intermediate lobe peptide (CLIP, ACTH 18-39) (5) plus beta-lipotropin (beta-LPH)(7,8), beta-MSH (beta-LPH 41-58) (3,9) and beta-endorphin (beta-LPH 61-91) (8,10,11).

This work was supported by a Program Grant from the Medical Research Council of Canada. F.G. is a Fellow of the Medical Research Council of Canada.

ABBREVIATIONS

KRBGA: Krebs-Ringer buffer, 0.2% glucose, 0.1% bovine serum albumin.
PTH: Phenylthiohydantoin
ACTH: Adrenocorticotropin
MSH: Melanocyte-stimulating hormone
HPLC: High pressure/high performance/liquid chromatograph
LPH: Lipotropin

0006-291X/80/031042-10$01.00/0
Copyright © 1980 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Pulse-chase experiments have shown that rat pars intermedia synthesize mainly beta-endorphin and alpha-MSH, while beta-LPH appears to be an important but transient intermediate (12) in this tissue. Very little ACTH (1-39) has been found (13,14). The biosynthesis of these peptides in the pars intermedia occurs through the processing of a high molecular weight precursor (15,1). This precursor exists in two forms which are thought to differ in carbohydrate content, as suggested by differences in the incorporation of radiolabeled sugars (17) and by results obtained following incubation in the presence of tunicamycin (12). In order to account for the final active peptides in this tissue, the precursor has been named pro-opiomelanocortin (18). A similar model has been previously described for the ACTH secreting mouse pituitary tumor cell line AtT-20 by double immunoprecipitation of labeled proteins from incubated cells (19) and by the analysis of a cell free translation product from a m-RNA fraction isolated from the same cells (17).

Recently, Nakanishi et al. (20) reported the nucleotide sequence of a cloned c-DNA synthesized from m-RNA for the ACTH/beta-LPH precursor. Their results indicated that beta-LPH at the carboxylic end of the precursor is attached to ACTH with only two basic amino acids separating the two hormones. The amino terminal region was shown to contain a putative new melanotropic hormone called gamma-MSH.

The present study reports the synthesis of radiolabeled pro-opiomelanocortin by pars intermedia cells and the partial primary sequence of the N-terminal region. Our data along with those reported for the bovine precursor (20) enable us to determine the length of the signal peptide of pre-pro-opiomelanocortin.

MATERIALS AND METHODS

Preincubation of rat pars intermedia cells.
Posterior lobes (pars nervosa and pars intermedia) were carefully separated from the anterior lobe of pituitaries from deca-

pitated rats and preincubated for 1 hr at 37° in Kerbs-Ringer (KRBGA) buffer as previously described (15).

Incorporation of labeled amino acids in vitro (pulse).
At the end of the preincubation, the lobes were harvested by low-speed centrifugation and resuspended in 0.6 ml of prewarmed KRBGA buffer containing 0.5 mCi of one or several labeled amino acids (New England Nuclear: ^{3}H-alanine 16 Ci/mmole, ^{3}H-arginine 22 Ci/mmole, ^{3}H-histidine 11 Ci/mmole, ^{3}H-isoleucine 97 Ci/mmole, ^{3}H-leucine 120 Ci/mmole, ^{3}H-phenylalanine 113 Ci/mmole, ^{3}H-threonine 2 Ci/mmole, ^{3}H-tryptophan 11 Ci/mmole, ^{3}H-tyrosine 91 Ci/mmole, ^{3}H-valine 11 Ci/mmole and ^{35}S-methionine 702 Ci/mmole) (Amersham: ^{3}H-serine 19 Ci/mmole). The incubation was continued for 20 minutes under the same conditions as for the preincubation.

Peptide extraction.
After pulse labeling, the lobes were washed in 10 vol of cold phosphate buffered saline (PBS, GIBCO) containing 10 mM of the suitable unlabeled amino acid and centrifuged at low speed. The pellet was washed once more and extracted in 5 M acetic acid/bovine serum albumin 0.5 mg/ml/5mM unlabeled amino acid, by five cycles of freezing and thawing. The extract was desalted on a disposable albumin coated Sephadex G-25 medium column (9.1 ml column, Pharmacia) in 1 M acetic acid.

Microsequencing.
Peptides were subjected to automatic Edman degradations in an updated Beckman 890B sequenator using a 0.3 M Quadrol program and 150 nmole of sperm whale apomyoglobin as carrier (21). A blank run in the absence of coupling agent was done followed by a double coupling on the first cycle. The thiazolinones collected in butyl chloride were either measured for radioactivity directly in a toluene base scintillation cocktail (4 g omnifluor/liter of toluene), or, in the case of a multi amino acid labeled peptide, thiazolinones were converted to phenylthiohydantoins and separated by high performance liquid chromatography through a micro alkyl-phenyl column (Waters & Assn.) using a sodium propionate pH 4.7, methanol gradient.

RESULTS

Extracts of isolated cells of the pars intermedia or whole neurointermediate lobes incubated with tritiated phenylalanine or ^{35}S-methionine were analyzed on slab and disc gel electrophoresis with $NaDodSO_4$. No difference in radioactive patterns obtained with the two preparations was observed, indicating that the pars nervosa does not contribute significantly to the overall protein synthesis pattern in this tissue (results not shown). However, since the yield of viable cells was greatly enhanced when whole posterior lobes were incubated instead of isolated cells of the pars intermedia, the former were used for this study.

Pulse experiments.

Posterior lobes were incubated with tritiated leucine for 20 minutes (Methods). The cell extracts analyzed by sodium dodecyl sulfate polyacrylamide disc gel electrophoresis showed two proteins with apparent molecular weights of 30,000 and 32,000 (results not shown). These two proteins have been shown by Crine et al. (12) to contain ACTH and beta-endorphin, and both were immunoprecipitated by antisera against ACTH and beta-MSH. Furthermore, HPLC analysis of the tryptic fragments obtained from these two proteins labeled with various amino acids proved that they have very similar, if not identical, peptide backbones.

Microsequencing of labeled proteins.

The same pulse experiments were repeated with various amino acids, either one at a time or as a mixture. Cell extracts were desalted, directly applied to the cup of the sequenator and Edman degradation runned for 30 cycles. Fig. 1 shows the amount of radioactivity obtained with different amino acids at each cycle. When various amino acids were incorporated at the same time, the anilinothiazolinone at each cycle of the sequence was converted to PTH and passed through HPLC (Methods) and the radioactivity under labeled peaks was measured. The results (not shown) were the same as Fig. 1. All essential amino acids were well incorporated in proteins. Alanine, a non essential amino acid, was incorporated in sufficient quantity to be sequenced while glycine was not. Proline, lysine, methionine and histidine were not found in the first 30 cycles of degradation.

DISCUSSION

Previous studies have shown that short pulse incubations of pituitary pars intermedia cells with a labeled amino acid generated a high molecular weight protein. This protein was immunoprecipitated with antisera against beta-endorphin, beta-MSH and ACTH and was proposed

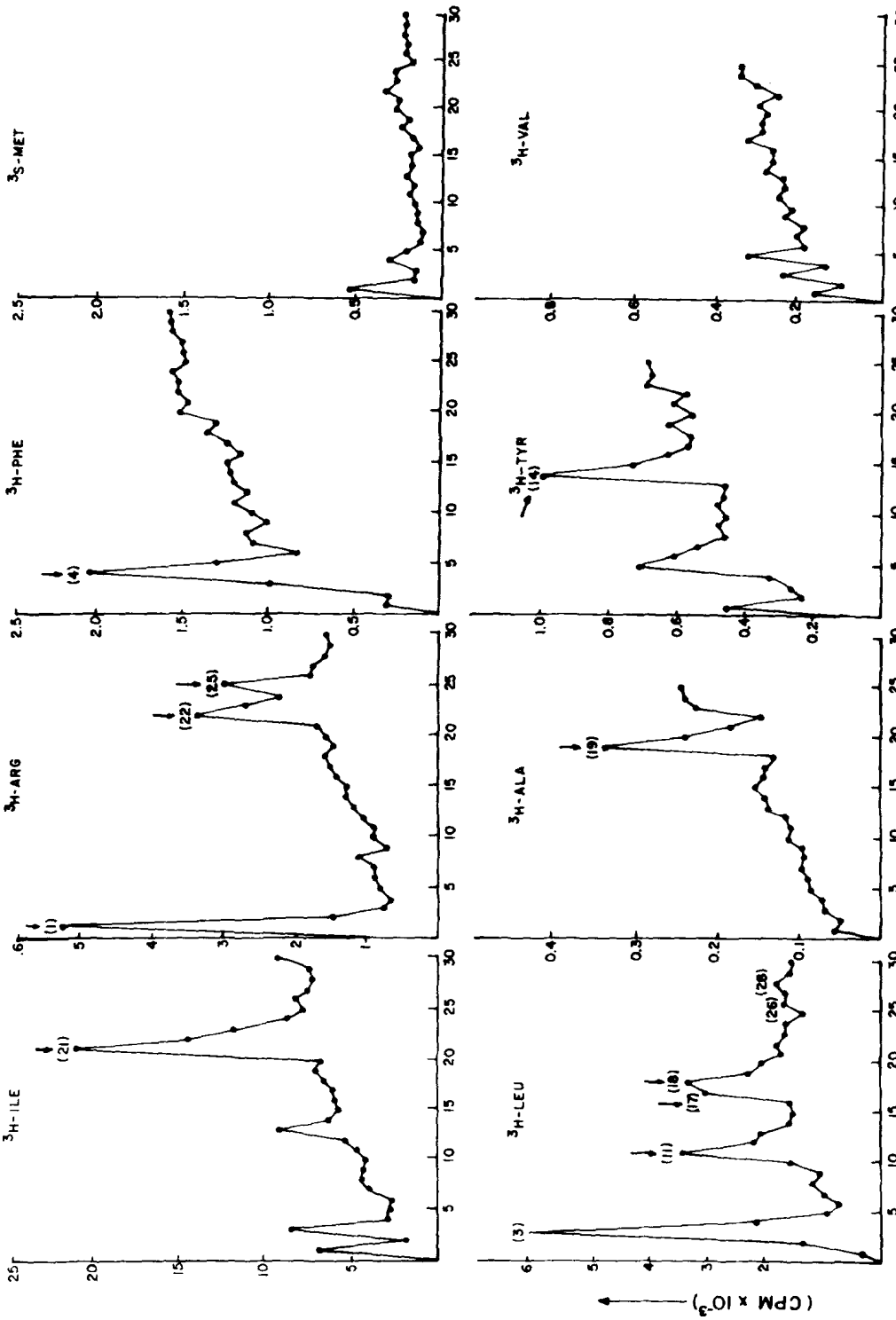

Fig. 1 Radioactivity in the butylchloride extracts recovered after each cycle of automated Edman degradations of the proteins synthesized by rat pars intermedia. The sequence position deduced from several preparations labeled with various tritiated or ^{35}S amino acids are indicated by arrows.

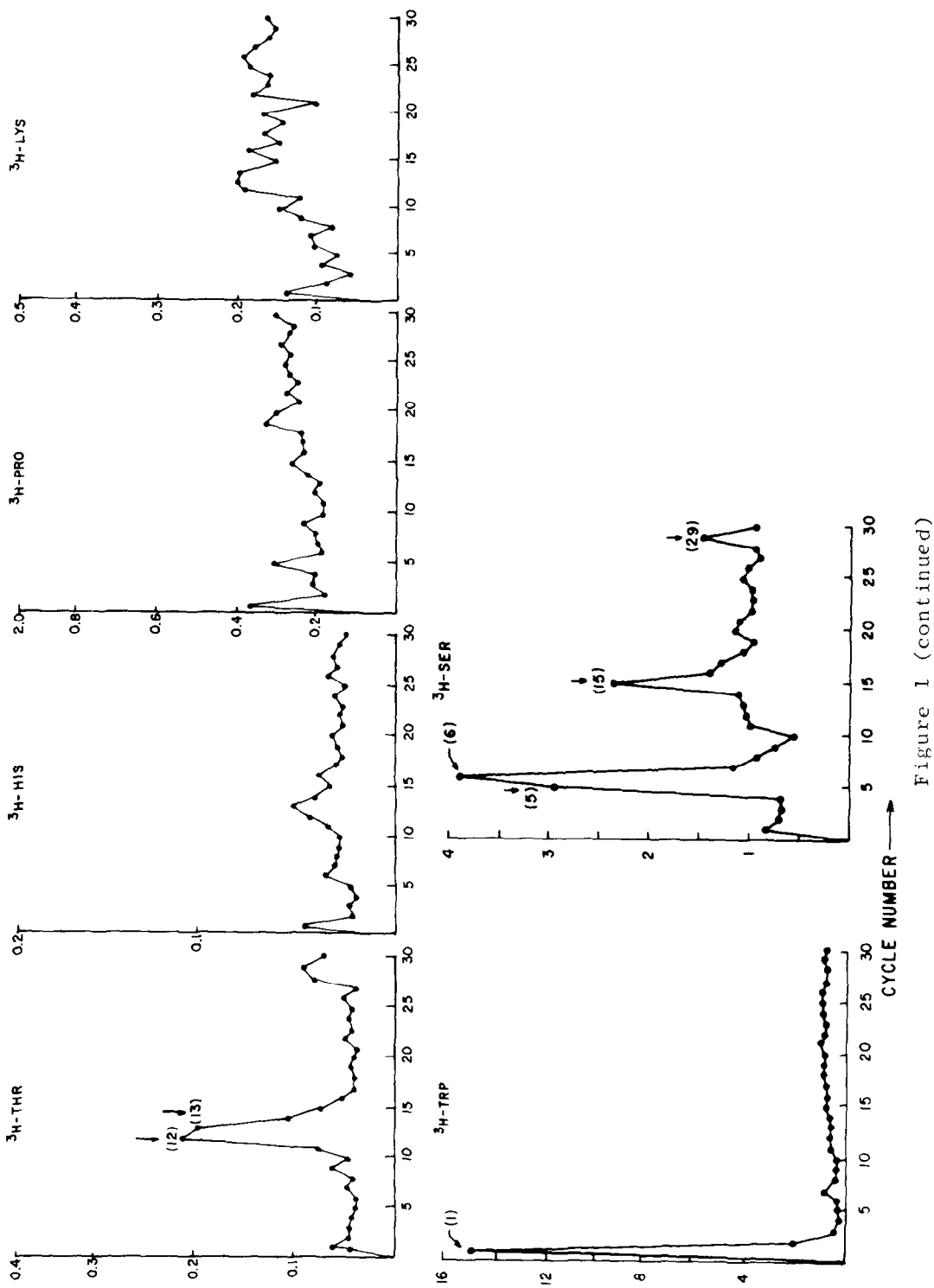

Figure 1 (continued)

to be the common precursor for these hormones in the pars intermedia (15). Recently this precursor has been resolved into two proteins of similar molecular weight (12). However both proteins exhibited the same antigenic sequences by immunoprecipitation, and the same tryptic digests with both segments ACTH 1-8 and beta-LPH 61-69 (12). These peptide maps suggested a common peptide backbone for the two precursors.

In the present study, 20 min pulse incubations of rat pituitary posterior lobes with various labeled amino acids were performed and the radioactive pro-opiomelanocortin purified and sequenced (Fig. 1). In spite of a blank run on the sequencer to minimize free amino acids sticking to the proteins, at the first cycle, tryptophan and arginine were found. Although tryptophan has been found in two other sequences of different species (Fig. 2, below), we cannot eliminate the possibility of an arginine residue. Except for the first cycle the identified positions were well characterized. Our results favor the hypothesis of one peptide backbone or at least a common amino-terminal region for both forms of the precursor.

The partial amino acid sequence obtained is illustrated in Fig. 2, along with the complete sequence of bovine precursor for ACTH/beta-LPH from Nakanishi et al. derived from DNA sequence (20) and with the partial sequence of mouse tumor AtT-20 cell precursor (22). Our sequence of the rat pars intermedia precursor shows a good homology with the one from bovine pars intermedia beginning at position 27. However, some differences are apparent, these being both conservative and non-conservative. An arginine residue instead of lysine in position 25 of the rat precursor is a conservative change easily explained by a simple point mutation. More striking differences include substitution of phenylalanine for glutamic acid at residue 3 and tyrosine for glutamic acid at residue 14 in the rat versus the bovine sequence.

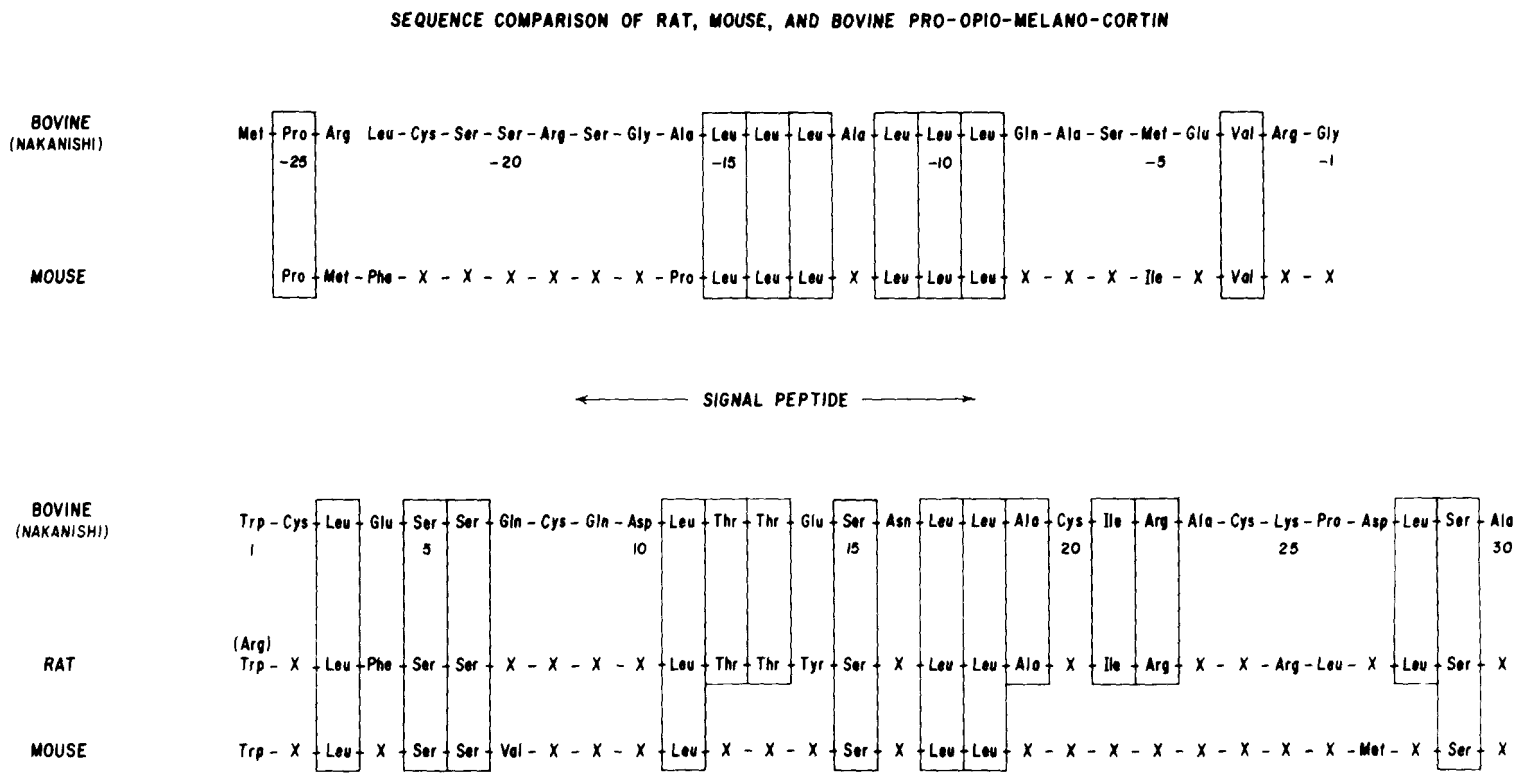

Fig. 2 Comparison of the partial sequence of pro-opiomelanocortin from rat pars intermedia cells compared with the bovine sequence obtained indirectly from the cDNA by Nakanishi et al. (20). The sequence obtained by Herbert (22) from the precursor synthesized after cell free translation of isolated AtT-20 cell mRNA is also shown.

Since the homology between these precursors begins at residue 27 of the bovine sequence derived indirectly from the c-DNA sequence, we can deduce that in the bovine precursor the first 26 amino acids constitute the signal peptide. The presence of a signal peptide has been shown for various precursors of secretory proteins and peptide hormones as reviewed by Thibodeau et al. (23). As in 75% of the 10 pre-pro-proteins computed, the signal peptide of the pro-opiomelanocortin has an hydrophobic segment from -7 to -16 of the point of cleavage, consisting almost exclusively of leucine and alanine. The cleavage point has no specific features as in other pre-proteins except for the presence of glycine at or near that site.

The signal peptide is cleaved very rapidly after or even during translation. In the case of parathyroid hormone, for example, the life of the pre-peptide is less than one minute (24). In our experiment with intact cells and comparatively long incubation times (20 min), the signal peptide of the initial translation product has probably already been cleaved from the majority of the molecules.

ACKNOWLEDGMENTS

The authors thank Mrs D. Marcil for careful typing, Pierre Demers and Normand Larivière for the drawings, G. DeSerres for technical assistance and Michael Dennis for critical reading of the manuscript.

REFERENCES

1. Shapiro, M., Nicholson, W.E., Orth, D.N., Mitchell, W.M., Island, D.P. and Liddle, G.W. (1972) Endocrinology 90:249-256.
2. Kraicer, J., Gosbee, J.L. and Bencosme, S.A. (1973) Neuro-endocrinology 11:156-176.
3. Baker, B.I. (1973) J. Endocrinol. 57:393-404.
4. Scott, A.P., Lowry, P.J., Ratcliffe, J.G., Rees, L.H. and Landon, J. (1974) J. Endocrinol. 61:355-367.
5. Scott, A.P., Ratcliffe, J.G., Rees, L.H., Landon, J., Bennett, H.P.J., Lowry, P.J. and McMartin, C. (1973) Nature (London) New Biol. 244:65-67.
6. Moon, H.D., Li, C.H. and Jennings, B.M. (1973) Anat. Res. 175:529-538.
7. LaBella, F., Queen, G., Senyshyn, J., Lis, M. and Chrétien, M. (1977) Biochem. Biophys. Res. Commun. 75:350-357.
8. Pelletier, G., Leclerc, R., Labrie, F., Côté, J., Chrétien, M. and Lis, M. (1977) Endocrinology 100:770-776.

9. Thody, A.J. (1969) Gen. Comp. Endocrinol. 13:477.
10. LaBella, F., Downey, G., Queen, G. and Pinsky, C. (1976) Can. J. Physiol. Pharmacol. 54:946-948.
11. Bloom, F., Battenberg, E.O., Rossier, J., Ling, N., Leppeluoto, J., Vargo, T.M. and Guillemin, R. (1977) Life Sci. 20:43-48.
12. Crine, M., Gossard, F., Seidah, N.G., Blanchette, L., Lis, M. and Chrétien, M. (1979) Proc. Natl. Acad. Sci. USA. 76:5085-5089.
13. Gianoulakis, C., Seidah, N.G., Routhier, R. and Chrétien, M.: (In Press) J. Biol. Chem.
14. Scott, A.P., Lowry, P.J. and Greidanus, T.B.V.W. (1976) J. Endocrinol 70:197-205.
15. Crine, P., Gianoulakis, C., Seidah, N.G., Gossard, F., Pezalla, P.D., Lis, M. and Chrétien, M. (1978) Proc. Natl. Acad. Sci. USA 75:4719-4723.
16. Eipper, B.A. and Mains, R.E. (1978) J. Supramolecular Struc. 8:247-262.
17. Roberts, J.L. and Herbert, E. (1977) Proc. Natl. Acad. Sci. USA 74:4826-4830.
18. Chrétien, M., Benjannet, S., Gossard, F., Gianoulakis, C., Crine, P., Lis, M. and Seidah, N.G. (1979) Can. J. Biochem. 57:1111-1121.
19. Mains, R.E., Eipper, B.A. and Ling, N. (1977) Proc. Natl. Acad. Sci. USA 74:3014-3018.
20. Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A.C.Y., Cohen, S.N. and Numa, S. (1979) Nature 278:423-427.
21. Seidah, N.G., Gianoulakis, C., Crine, P., Lis, M., Benjannet, S., Routhier, R. and Chrétien, M. (1978) Proc. Natl. Acad. Sci. USA 75:3153-3157.
22. Herbert, E., Budarf, M., Phillips, M., Rosa, P., Policastro, P., Oates, E., Roberts, J.L., Seidah, N.G. and Chrétien, M. (1979) submitted for publication.
23. Thibodeau, S.N., Palmiter, R.D. and Walsh, K.A. (1978) J. Biol. Chem. 253:9018-9023.
24. Habener, J.F., Potts, J.T. jr. and Rich, A. (1976) J. Biol. Chem. 251:3893-3899.