CELL-FREE TRANSLATION PRODUCT CONTAINING CORTICOTROPIN AND β-ENDORPHIN ENCODED BY MESSENGER RNA FROM ANTERIOR LOBE AND INTERMEDIATE LOBE OF BOVINE PITUITARY

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1. Introduction

Corticotropin (ACTH), which is produced in the pituitary, is a single polypeptide with approx. mol. wt 4500. Our previous work with the use of a cell-free protein-synthesizing system has demonstrated that the initial, direct product of translation of ACTH mRNA from the anterior lobe of bovine pituitary has approx. mol. wt 35 000 [1]. This result has aroused interest in the biological role of the extra-portion in the large molecular form of ACTH. Previous investigations have suggested that ACTH and β-LPH are produced and stored in the same pituitary cell and released together in the same physiological or pathological situations [2–5]. Furthermore, studies on peptide structures have indicated that several biologically active peptides can be elaborated from the same precursor; γ-LPH, β-MSH, endorphins and methionine-enkephalin are derived from β-LPH [6,7], while α-MSH and CLIP are formed from ACTH [8]. Thus, it appeared plausible to assume that ACTH and β-LPH are formed by post-translational cleavage of a common precursor encoded by the same mRNA.

Another interesting feature of ACTH is the fact that the occurrence of α-MSH and CLIP depends on the anatomical localization of their precursor; ACTH, when synthesized in the intermediate lobe, is converted to α-MSH and CLIP, while ACTH, when synthesized in the anterior lobe, is released as intact ACTH [9]. Although this difference is believed to be due to the presence of specific peptidases, the cell-free translation product of ACTH mRNA from the intermediate lobe remained to be examined in comparison with that of ACTH mRNA from the anterior lobe.

In the present investigation, evidence has been provided by the use of a cell-free protein-synthesizing system to indicate that the translation product of ACTH mRNA in fact contains the β-endorphin sequence and that this product is encoded by mRNA not only from the anterior lobe but also from the intermediate lobe of bovine pituitary.

Abbreviations: ACTH, corticotropin; ACTH-(1–24), synthetic peptide with residues 1–24 of the amino acid sequence of corticotropin; β-LPH, β-lipotropin; γ-LPH, γ-lipotropin; α-MSH, α-melanotropin; β-MSH, β-melanotropin; CLIP, corticotropin-like intermediate lobe peptide; IgG, immunoglobulin G

2. Materials and methods

2.1. *Isolation of RNA*

Bovine pituitaries were obtained from a local abbattoir. The anterior lobe was separated from the neurointermediate lobe at the cleft dividing the two lobes. RNA was isolated from the membrane fraction of 20–25 g anterior lobes or 6–8 g neurointermediate lobes, collected from 20 pituitaries, as described [1]. The RNA isolated from anterior lobes was subjected to oligo(dT)-cellulose chromatography to separate poly(A)-containing RNA as described [1].

2.2. *Cell-free protein synthesis*

The cell-free protein-synthesizing system used was derived from rabbit reticulocytes. Reticulocyte lysates

were prepared as described previously [10]. The reaction mixture (total vol. 50 μl) contained 20 mM Tris–HCl, pH 7.5, 2 mM magnesium acetate, 0.1 M KCl, 1.8 mM dithiothreitol, 20 μM hemin, 1 mM ATP, 0.2 mM GTP, 8 mM creatine phosphate, 40 μg/ml creatine phosphokinase (EC 2.7.3.2), 0.1 mM each of 19 unlabelled amino acids, 0.2 mCi/ml L-[^{35}S]-methionine (635 Ci/mmol) or L-[4,5-^{3}H]leucine (57 Ci/mmol), 0.4 ml/ml reticulocyte lysate and either 40–60 μg/ml poly(A)-containing RNA from anterior lobes or 250–300 μg/ml RNA from neurointermediate lobes. In some experiments, the reaction mixture was scaled up 10- to 20-fold. Incubation was carried out at 25°C for 90 min.

2.3. *Immunological procedures*

Rabbit antisera were prepared by repeated immunization with ACTH-(1–24) covalently bound to rabbit serum albumin [11]. Anti-ACTH was purified by subjecting the crude IgG fraction, obtained by ammonium sulfate precipitation at 40% saturation, to affinity chromatography on ACTH-(1–24)–Sepharose 4B [12]. This antibody was shown by radioimmunoassay [13] to be specific for ACTH, being not reactive with β-MSH; the antibody failed to bind ^{125}I-labelled β-MSH, and an excess of β-MSH did not compete with ^{125}I-labelled ACTH for binding to the antibody. Rabbit antiserum to β-endorphin (RB100-11/76) [14] was a generous gift of Dr R. Guillemin, and the IgG fraction from this antiserum was isolated as described [1]. Indirect immunoprecipitation of the cell-free translation product was performed as described [1].

2.4. *Electrophoretic analysis of immunoprecipitates and cyanogen bromide cleavage products*

The immunoprecipitates were analyzed by electrophoresis on discontinuous sodium dodecylsulfate–polyacrylamide slab-gel containing 10% acrylamide according to the procedure [15] with minor modifications. Autoradiography was performed by exposing the dried gel to Kodak RP-Royal K-Omat film for 3–10 days. Elution of radioactive material from the gel was carried out as follows:
After electrophoresis, the gel was sliced into 2 mm segments, and the individual segments were homogenized in a Teflon–glass homogenizer with 1.5 ml 50 mM Tris–HCl, pH 7.5, containing 0.1 mg/ml human serum albumin (Miles Lab., Kankakee, USA) and 0.06% sodium dodecylsulfate. After standing at 37°C for 12 h and then at 4°C for 12 h, the homogenates were centrifuged to remove the gel. The fractions containing radioactivity were combined and dialyzed for two days against 50 mM Tris–HCl, pH 7.5, containing 0.15 M NaCl to be subjected to a second immunoprecipitation.

For cyanogen bromide cleavage, the immunoprecipitates (approx. 0.2 mg protein) were dissolved in 0.3 ml 70% formic acid containing 20 mg cyanogen bromide and were incubated at 25°C for 20 h. The mixture was lyophilized, dissolved in 1 ml distilled water and then lyophilized again. The residue was washed once with 10% trichloroacetic acid and then three times with acetone–ether (1:1, by vol.). This material was analyzed by electrophoresis on sodium dodecylsulfate–urea–polyacrylamide slab gel containing 10% acrylamide as described [16]. Fluorography was conducted according to the procedure [17].

2.5. *Reagents and determinations*

Porcine β-endorphin was a generous gift of Dr N. Ling. Human β-MSH was kindly provided by Dr H. Yajima. ACTH-(1–24) was obtained from Daiichi Seiyaku Co. (Tokyo, Japan). RNA concentrations were determined by assuming $A^{1\%}_{254}$ 250. The protein contents of the immunoprecipitates were determined by the method of Lowry et al. [18] with bovine serum albumin as the standard. Other reagents and determinations were as described [1].

3. Results

In the experiments presented in this paper, RNA was isolated from the anterior and the neurointermediate lobes of bovine pituitaries. In view of the negligible content of ACTH mRNA in the neural lobe (unpublished results), we used the neurointermediate lobe instead of the intermediate lobe, because the separation of the two lobes occasionally caused damage of the fragile intermediate lobe.

The RNA isolated was translated in the cell-free protein-synthesizing system derived from reticulocytes. The translation product was subjected to indirect immunoprecipitation by incubation with either anti-

β-endorphin or anti-ACTH and then with anti-IgG. The immunoprecipitates were analyzed by electrophoresis on dodecylsulfate–polyacrylamide slab gel. Figures 1 and 2 represent the autoradiograms of the electrophoretic analysis of the product formed with RNA from the anterior lobe and from the neurointermediate lobe, respectively. Essentially the same patterns were obtained with RNA from both sources. The immunoprecipitate formed with anti-β-endorphin yielded a heavy radioactivity band (D), which

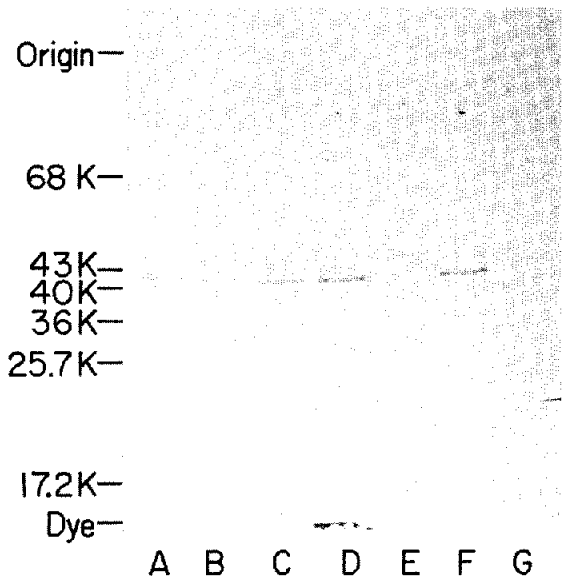

Fig.1. Dodecylsulfate–polyacrylamide gel electrophoresis of the immunoprecipitates derived from the [^{35}S]methionine-labelled translation product formed with RNA from the anterior lobe. Aliquots of the translation product corresponding to 50 μl reaction mixture were subjected to indirect immunoprecipitation with the additions indicated. The amount of radioactivity present in each immunoprecipitate, determined with duplicate aliquots, was as indicated: (A) anti-ACTH and 10 μg porcine β-endorphin, 4177 cpm; (B) anti-ACTH and 10 μg ACTH-(1-24), 2553 cpm; (C) anti-ACTH, 4332 cpm; (D) anti-β-endorphin, 4254 cpm, (E) anti-β-endorphin and 10 μg porcine β-endorphin, 1042 cpm; (F) anti-β-endorphin and 10 μg ACTH-(1-24), 4408 cpm; (G) control IgG, 2478 cpm. Other details were as described in Materials and methods. The marker polypeptides used were bovine serum albumin (68 000 daltons), chicken ovalbumin (43 000 daltons), rabbit muscle aldolase (40 000 daltons), rabbit muscle lactate dehydrogenase (36 000 daltons), bovine pancreas chymotrypsinogen (25 700 daltons) and equine muscle myoglobulin (17 200 daltons). Bromphenol blue served as the tracking dye.

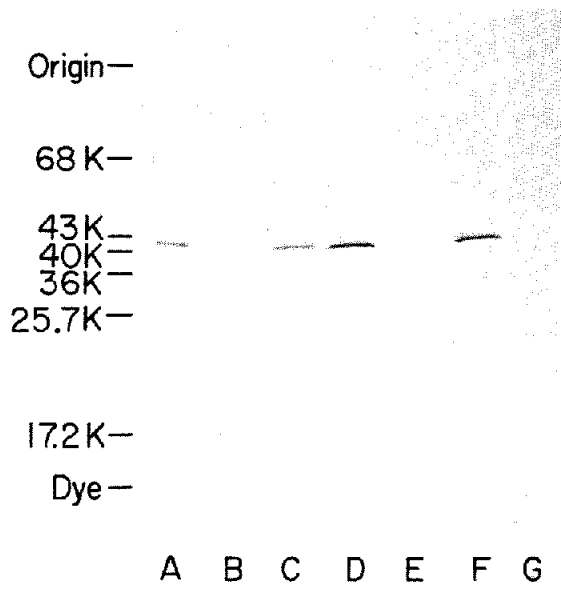

Fig.2. Dodecylsulfate–polyacrylamide gel electrophoresis of the immunoprecipitates derived from the [^{35}S]methionine-labelled translation product formed with RNA from the neurointermediate lobe. Experimental details were as described in the legend to fig.1, except that aliquots of the translation product corresponding to 40 μl of the reaction mixture were used for D, E. and F, and that the amount of radioactivity present in each immunoprecipitate was as indicated: (A) 21 605 cpm, (B) 2601 cpm; (C) 21 059 cpm; (D) 25 588 cpm; (E) 1318 cpm; (F) 25 200 cpm; (G) 2224 cpm.

migrated with a mobility identical to that of the radioactivity band derived from the immunoprecipitate formed with anti-ACTH (C). By comparison with marker peptides, the molecular weight of this labelled product was estimated to be approx. 41 000. The immunoprecipitate formed by incubation of the same translation product with control IgG exhibited no such radioactivity band (G). In our previous work, the molecular weight of the translation product of ACTH mRNA from the anterior lobe of bovine pituitary was estimated to be approx. 35 000 [1]. This value was based on electrophoretic analysis on dodecylsulfate–polyacrylamide gel according to the procedure [19]. The difference observed in the molecular weight of the translation product is apparently due to the different systems of electrophoresis used, as reported for other proteins [20,21].

The specific recognition site of the anti-β-

endorphin used is the 20–27 amino acid sequence of β-endorphin [14], while the anti-ACTH used is directed to ACTH-(1–24). Because the amino acid sequences of the recognition sites of the two antibodies are completely different, it was expected that anti-β-endorphin reacted with the 41 000 dalton product by recognizing β-endorphin but not ACTH. In fact, the radioactivity band observed for the immunoprecipitate formed with anti-β-endorphin was completely abolished by the addition of an excess of β-endorphin to the translation product prior to the addition of the antibody (E). In contrast, prior addition of excess ACTH-(1–24) did not affect this band at all (F). Similarly, the radioactivity band observed for the immunoprecipitate formed with anti-ACTH was abolished by prior addition of an excess of ACTH-(1–24) (B) but not of β-endorphin (A). These results indicated that both anti-β-endorphin and anti-ACTH specifically reacted with the 41 000 dalton product, thus supporting the view that this product contains both the β-endorphin and the ACTH sequence.

In an attempt to verify this concept, we examined whether the translation product isolated by immunoprecipitation with anti-ACTH, after dissociation from the antibody, reacted with anti-β-endorphin. RNA from the neurointermediate lobe was translated, and the immunoprecipitate formed with anti-ACTH was electrophoresed as in the experiment represented in fig.2. The [^{35}S]methionine-labelled 41 000 dalton product, which was freed from anti-ACTH, was eluted from the gel and dialyzed as described in Materials and methods. An aliquot of the dialyzed sample (3810 cpm) was subjected to indirect immunoprecipitation with anti-β-endorphin. Thirty-four percent of the radioactive material was precipitated. In contrast, only 3% of the labelled material was found in the immunoprecipitate formed with control IgG instead of anti-β-endorphin. Although the recovery of the radioactive material in the immunoprecipitate formed with anti-β-endorphin was rather low, this result can be regarded as significant because, on the basis of the competition experiments (see legend fig.2), the material isolated by immunoprecipitation with anti-ACTH is considered to be exclusively the product precipitated through recognition of ACTH but not of β-endorphin. The low recovery of the second immunoprecipitation with anti-β-endorphin was probably due to denaturation of the product during electrophoresis and elution in the presence of dodecylsulfate.

Further evidence for the structural identity of the translation product containing the β-endorphin sequence with that containing the ACTH sequence was provided by comparing the cyanogen bromide cleavage peptides derived from the immunoprecipitate formed with anti-β-endorphin and that formed with anti-ACTH with the use of dodecylsulfate–urea–polyacrylamide slab-gel electrophoresis. Figure 3 shows the fluorogram of the electrophoretic analysis. Identical patterns were observed for both immunoprecipitates; some of the radioactivity bands were shown to represent fragments arising from incomplete cleavage (unpublished results).

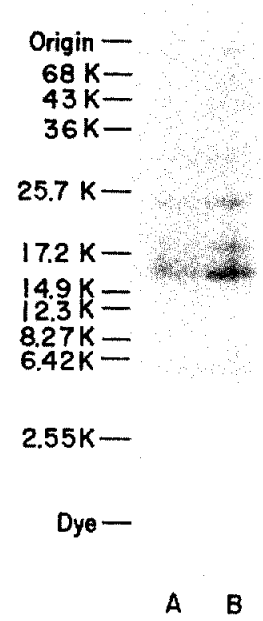

Fig.3. Dodecylsulfate–urea–polyacrylamide gel electrophoresis of the fragments resulting from cyanogen bromide cleavage of the immunoprecipitate formed with anti-ACTH (A) or with anti-β-endorphin (B). RNA from the neurointermediate lobe was translated in the presence of [^{3}H]leucine. The immunoprecipitates derived from aliquots of the translation product corresponding to 50 μl reaction mixture were used; the amounts of radioactivity present in the immunoprecipitates were 13 400 cpm (A) and 20 520 cpm (B). Other details were as described in Materials and methods. The markers used were equine heart cytochrome *c* (12 300 daltons) and cyanogen bromide cleavage products of equine muscle myoglobin (14 900, 8270, 6420 and 2550 daltons) in addition to those given in the legend to fig.1.

4. Discussion

The present investigation with the use of a cell-free protein-synthesizing system has indicated that mRNA not only from the anterior lobe but also from the intermediate lobe of bovine pituitary directs the synthesis of a mol. wt 41 000 product containing both the ACTH and the β-endorphin sequence. The structural identity of the products encoded by mRNA from the two lobes is strongly suggested by the identical molecular weight as well as by the presence of both peptides. By pulse-chase experiments with radioactive amino acids in cultured mouse pituitary tumor cells, Mains et al. [22,23] have recently presented evidence indicating that a glycoprotein with mol. wt 31 000 is a common precursor of ACTH and β-endorphin. Studies by Lowry et al. [24] on human pituitary extracts have also suggested that antigenic determinants for ACTH and β-LPH occur in the same molecule. Thus, it can be concluded that the large translation product formed in the cell-free system represents a precursor which is capable of producing various biologically active peptides, i.e., ACTH, α-MSH, CLIP, β-LPH, γ-LPH, β-MSH, endorphins and methionine-enkephalin, depending on its localization in the pituitary.

Our previous work has shown that glucocorticoids are involved in the regulation of the level of ACTH mRNA in the pituitary [25]. The present investigation raises the question, how the production of each biologically active peptide from the common precursor is regulated in the different regions of the pituitary.

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