CELL-FREE TRANSLATION OF THE RAT PITUITARY MESSENGER RNA CODING FOR THE PRECURSORS OF α and β SUBUNITS OF LUTROPIN

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1. Introduction

Lutropin is a glycoprotein belonging to a group of pituitary glycoprotein hormones which includes also follitropin and thyrotropin. It is composed of two dissimilar, noncovalently linked subunits, α and β. The amino acid sequences of α and β subunits of glycoprotein hormones from many species have been established and are well known. Within a species, the sequence is identical or nearly identical for α subunits of all pituitary hormones. In the human being, it is also identical to that of a placental glycoprotein hormone, chorionic gonadotropin. The amino acid sequence of the hormone-specific β subunits differs, although considerable homology exists in some portions of their primary structure (reviewed in [1]). Each subunit of LH and hCG contains two *N*-asparagine-linked oligosaccharides. A structure has been proposed for asparagine-linked oligosaccharides of human LH and CG; these might be identical [2,3].

Messenger RNA has been extracted from placental tissue and has been translated in vitro by systems derived from wheat-germ embryo, ascites tumors or reticulocyte lysates [4–7]. The first evidence for the cell-free synthesis of LHα and LHβ from bovine origin by separate mRNAs appeared in [8,9].

Abbreviations. LH, lutropin; FSH, follitropin; TSH, thyrotropin; CG, chorionic gonadotropin; PRL, prolactin; GH, growth hormone; the prefixes b, h, o, r, signify bovine, human, ovine, rat, respectively; mRNA, messenger RNA; poly(A), polyadenylated; Hepes, *N*-2-hydroxyethylpiperazine–*N'*-2-2-ethanesulfonic acid; RCXM, reduced and *S*-carboxymethylated (subunits); SDS, sodium dodecyl sulfate; EGTA, ethylene glycol tetraacetic acid; EDTA, ethylene diamino tetraacetic acid; M_r, relative molecular mass

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Here, we have isolated poly(A)-containing RNA from rat anterior pituitaries and translated it in a wheat-germ system in the presence of [^{35}S]methionine. Using immunoprecipitation with specific antisera and electrophoresis on SDS–polyacrylamide gels, we have identified two products with larger M_r-values than the apopeptides, LHα and LHβ. These products may represent the precursor forms of the rLH subunits.

2. Materials and methods

[^{35}S]Methionine (spec. act. 800–1200 Ci/mmol) was purchased from the CEA (Saclay). Nucleotides were supplied by Boehringer (Mannheim) and micrococcal nuclease, by Worthington. Oligo(dT)-cellulose was from Collab. Res., Inc. (Waltham, MA). X-ray films were obtained from Kodak (RP royal X-O-mat). All other reagents used were A-grade.

2.1. *Preparation of rat pituitary messenger RNA*

Anterior pituitary glands were excised from Wistar rats (laboratory strain) without distinction of age and sex. They were quick-frozen and stored in liquid nitrogen. Total RNA was prepared by phenol extraction of a pituitary homogenate according to the procedure detailed in [10] and poly(A)-mRNA was isolated by two successive passages over oligo(dT)-cellulose [11]. The yields currently obtained were ~2 A_{260} units (or 80 μg) mRNA/g wet tissue, which represented ~2% (w/w) of the total ribonucleic acid.

2.2. *Cell-free protein biosynthesis*

A wheat-germ S-30 extract, prepared according to [12], was nuclease-pretreated under conditions usually employed for depleting rabbit reticulocyte lysate of endogenous mRNA [13]. Cell-free translation of pitu-

itary mRNA was achieved in optimized conditions: final vol. 25–150 μl contained 30 mM Hepes (pH 7.4), 2 mM dithioerythritol, 0.2 mM GTP, 2 mM ATP, 8 mM creatine phosphate, 3.5 U/ml creatine phosphokinase, 0.4 mM spermidine, unlabelled amino-acids except methionine, at 40 μM 1.2 mCi [^{35}S]methionine/ml, 106 mM potassium acetate, 3.5 mM magnesium acetate and 100 μg/ml mRNA. Addition of 30% (v/v) wheat-germ extract initiated the reaction, which was allowed to proceed for 60 min at 30°C. Protein synthesis was monitored by trichloroacetic acid-precipitation of aliquots on Whatman 3 MM filters [14].

2.3. *Immunoprecipitation of polypeptides*

Precipitation of LH subunits synthesized in vitro was investigated using a number of antisera. Only antisera directed against reduced, *S*-carboxymethylated (RCXM) subunits of LH gave reliable results and we routinely used either anti-bovine RCXM-LHα and RCXM-LHβ provided by Dr J. G. Pierce or antisera against ovine RCXM-LHα and RCXM-LHβ [15] raised in rabbits in our laboratory. Anti-rat prolactin was a gift from Dr A. Tixier-Vidal and anti-rat growth hormone was supplied by Dr A. F. Parlow (NIAMDD). Translation products, freed from ribosomes by centrifugation (150 000 × *g*, 60 min), were precipitated with trichloroacetic acid (10%, w/v) solubilized in 1% SDS, 10 mM Tris–HCl (pH 8) and denaturated by heating 2 min in boiling water. Aliquots were then submitted to immunoprecipitation in 25 mM Tris–HCl (pH 8), 10^{-5} M bacitracine, 10 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS. Complete reduction and alkylation [15] prior to trichloroacetic acid-precipitation of the translation products improved the immune reaction and reduced the non-specific radioactivity attached to immunoprecipitates. Antigen–antibody complexes were precipitated with Pansorbin [16].

2.4. *Electrophoretic analysis of polypeptides*

Peptides in translation mixtures, total trichloroacetic acid-precipitable peptides and immunoprecipitable peptides were analyzed by electrophoresis on SDS–15% polyacrylamide slab gels [17] and revealed by fluorography [18].

3. Results

The use of the wheat-germ cell-free system, pretreated with nuclease to lower endogenous mRNAs [13], allowed translation of rat pituitary mRNA which stimulated [^{35}S]methionine incorporation into trichloroacetic acid-insoluble protein 140–160-fold over the background (table 1). In the case of the nuclease-pretreated extracts, endogenous wheat-germ proteins represented <1% of total translated proteins, instead of 10–20% as seen with untreated extracts. Conditions of translation were investigated and optimized as indicated in section 2.

Table 1
Stimulation of [^{35}S]methionine incorporation in response to rat pituitary messenger RNA in wheat-germ cell-free system pretreated or not with nuclease[a]

Wheat-germ extract	mRNA (μg/50 μl)	Incorp. (10^3 cpm/50 μl)	Stimulation (-fold)
Untreated	0	550	
	5	6 200	11.3
Nuclease -treated	0	35	
	5	5 700	162.8
Untreated	0	450	
	5	4 600	10.2
Nuclease -treated	0	31	
	5	4 280	138

[a] Wheat-germ cell-free extract was incubated for 10 min at 22°C in the presence of micrococcal nuclease (75 U/ml) + 1 mM $CaCl_2$

At the end of the incubation period, medium was made 2 mM in EGTA. Other conditions were as in section 2

Fig.1 shows a fluorograph of ^{35}S-labelled polypeptides synthesized in response to mRNA prepared from rat pituitaries. Several bands were observed. The first upper band, migrating with M_r ~25 000, was identified as pre-PRL.

When aliquots of the translation media were immunoprecipitated with specific antisera, one major band appeared in each case (fig.2). Anti-rat PRL (lane 2) and anti-rat GH (lane 3) each precipitated a product of app. M_r 27 000 and 25 000, respectively. Antiserum to RCXM-bLHα precipitated a main product with app. M_r 17 000 (lane 4) which did not precipitate in the presence of an excess of unlabelled oLHα (lane 5). Antiserum to RCXM-oLHβ precipitated a single product with an app. M_r 18 500 (lane 6) which did not precipitate when the medium was saturated with an excess of cold oLHβ.

Table 2 shows a quantitative evaluation of the

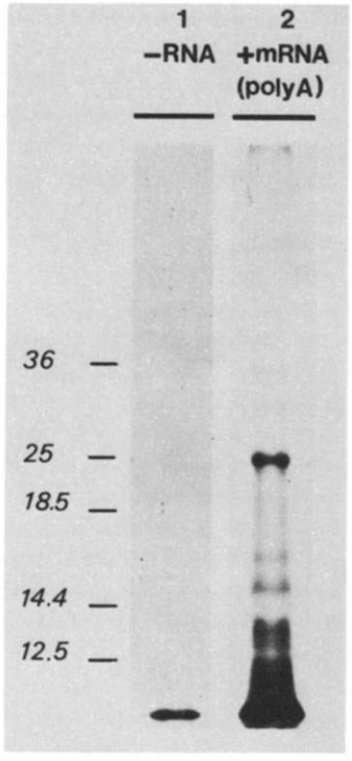

Fig.1. Fluorograph of ^{35}S-labelled polypeptides synthesized in a wheat-germ cell-free system, pretreated with nuclease [13], in response to rat pituitary mRNA: (1) blank without RNA, trichloroacetic acid-precipitate. (2) translation in presence of RNA (5 μg/50 μl), trichloroacetic acid-precipitate. The amount of radioactivity applied was ~50 000 cpm in lane (2). The SDS–polyacrylamide gels (15%) were exposed to fluorography for 6 days. Relative positions of protein marked with their M_r values × 10^{-3} are given on the left of the figure. The following protein markers were used (from top to bottom): lactate dehydrogenase, chymotrypsinogen, ferritin, α-lactalbumin, cytochrome *c*.

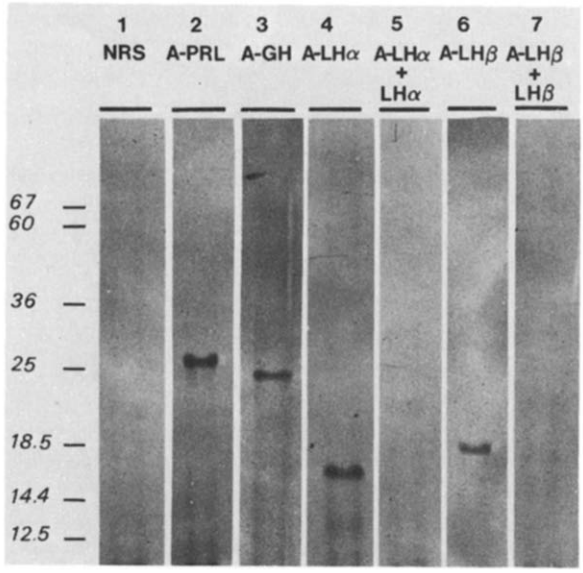

Fig.2. Fluorograph of ^{35}S-labelled polypeptides synthesized in a wheat-germ cell-free system, pretreated with nuclease [13] in response to rat pituitary mRNA. Polypeptides synthesized in vitro, were reduced and *S*-carboxymethylated, precipitated with trichloroacetic acid and submitted to specific antisera after their dissolution. Control, normal rabbit serum; (2) anti-rat prolactin serum; (3) anti-rat growth hormone serum; (4) anti-RCXM-bovine LHα serum; (5) as in lane (4) + excess of ovine LHα; (6) anti-RCXM-ovine LHβ serum; (7) as in lane (6) + excess of ovine LHβ. The radioactive material which bands in lanes (2–4,6) represented ~4000 cpm/lane. It was applied on the SDS–polyacrylamide gels at 15% and revealed by fluorography in 15 days. Apparent M_r-values were calculated from linear plots of the logarithm of protein marker M_r values *vs* the distance covered by the standards (M_r × 10^{-3} on the left of the figure). Protein markers, reduced and *S*-carboxymethylated, were as in legend to fig.1, + bovine serum albumin (67 000) + catalase (60 000).

radioactivity incorporated into proteins in the presence or absence of rat pituitary poly(A)-RNA. Antisera against RCXM-bLHα and RCXM-oLHβ precipitated ~0.30% and ~0.05%, respectively of the radioactivity as compared to total, trichloroacetic acid-precipitable radioactivity. Thus, the amount of radioactivity precipitated with anti-LHα serum was ~6-times higher than in the case of anti-LHβ.

4. Discussion

Extraction and partial purification of rat anterior pituitary RNAs by affinity chromatography on oligo-(dT)-cellulose yielded mainly poly(A)-RNAs, containing most of the mRNAs [11]. The yield was ~80 μg RNA/g wet tissue and we have shown that 3–5 μg of this RNA increases 8–12-times the incorporation of [^{35}S]methionine into polypeptides generated in a wheat-germ cell-free system. In order to deplete endogenous wheat-germ RNAs and thus lower the proportion of endogenous proteins in the translation media, wheat-germ extracts were pretreated with micrococcal nuclease [13]. Using nuclease-pretreated extracts, the increase in incorporated radioactivity was 140–160-fold over the control media. As shown in fig.1, translation media with nuclease-pretreated extract contained very low, often undetectable amounts of endogenous wheat-germ proteins (lane 1),

Table 2
Quantitative evaluation of [^{35}S]methionine incorporation into protein in the presence or absence of rat pituitary poly(A)-RNA[a]

RNA	[^{35}S]Met incorporated (10^3 × cpm/50 μl)		
	TCA[b]	Anti-bLH[c]	Anti-oLH[c]
Control (no RNA)	43 000	0	0
Poly(A)-RNA (5 μg)	3 720 000	11 300 (0.31%)[d]	2 200 (0.06%)[d]
Control (no RNA)	36 000	0	0
Poly(A)-RNA (3.5 μg)	2 764 000	7 600 (0.28%)[d]	1 350 (0.05%)[d]

[a] Translation proceeded using wheat-germ cell-free extracts pretreated with nuclease (see table 1). Other conditions are described in section 2
[b] Trichloroacetic acid-precipitate (total protein)
[c] Trichloroacetic acid-precipitate redissolved and immunoprecipitated with specific antisera against bovine RCXM-LHα and ovine RCXM-LHβ
[d] Percent of radioactivity precipitated with trichloroacetic acid

while in the presence of rat pituitary mRNA, many new polypeptides appeared, mainly in the area of M_r <25 000–26 000.

The fidelity of translation of the rat pituitary RNA preparations was demonstrated by the biosynthesis of pre-PRL and pre-GH (fig.2, lanes 2,3). Both of these hormones have been identified by others as cell-free products translated by RNA from rat and bovine pituitaries and from rat pituitary tumor cells. The app. M_r-values of the precursors of these hormones have been reported as being 25 000–28 000 for pre-PRL and 24 000 for pre-GH [19–21].

We have demonstrated the cell-free biosynthesis, in response to rat pituitary mRNA, of two polypeptides which are immunoprecipitable with specific anti-LHα and anti-LHβ sera. On SDS–polyacrylamide gel electrophoresis (fig.2) these peptides had app. M_r 17 000 for LHα and 18 500 for LHβ. As the M_r-values of the authentic apoprotein forms of rat LHα and LHβ are ~11 000 and ~12 500, respectively [22], our data suggest that we are dealing with precursors of these subunits and that the two subunits might be encoded by separate mRNAs. The specificity of immunoprecipitation was confirmed by the fact that the translation products did not precipitate when the media were treated with an excess of the corresponding unlabelled subunits. On the other hand, treatment of aliquots of translation media with an excess of oLHβ in the case of precipitation with anti-bLHα and with an excess of oLHα in the case of precipitation with anti-oLHβ did not inhibit specific immunoprecipitation (not shown), also suggesting that LHα and LHβ might be encoded by separate mRNAs.

It should be noted that among the several antisera tested, only those raised against RCXM subunits of bovine or ovine LH gave reliable results. Indeed, it has been shown that the conformations of precursor and processed forms of TSHβ differ and that pre-TSHβ does not crossreact with antisera to native TSHβ [23].

The M_r-value of the precursors of rLHα and rLHβ obtained here differ somewhat from the data published by others. An app. M_r 14 000 has been reported for bovine pre-LHα [8,24] and for pre-TSHα synthesized by mRNA from mouse thyrotropic tumor [25,26]. However, comparing pre-α subunits synthesized in cell-free conditions by RNA from bovine pituitaries and from mouse pituitary tumor [27], in both cases M_r 17 000 was found. A somewhat lower M_r (16 000–18 000) than that determined by us for pre-rLHβ has been determined by others for bovine pre-LHβ [9,24].

It is rather difficult to establish at present whether discrepancies between M_r-values of the precursors of LH subunits found here and those reported by others are real or apparent. It is significant that the differences in the M_r-values of both α and β apopeptides and their precursors are in our case 6000, suggesting a common extension sequence. This extension sequence would be somewhat larger than that which would be expected from the known sequences of the signal peptides of gonadotropin subunits [27,28]. We have verified that the observed discrepancies are not due to the relative positions of the different protein markers used here and those used by others (not shown). These discrepancies may be due to anomalous mobilities exhibited by the precursors of rat LH subunits on SDS–polyacrylamide gels, as shown for other proteins [29]. However even if the absolute values for M_r-values of both α and β precursors still remain to be confirmed, it should be noted that the relative difference in size between α and β apopeptides (~1500) is reflected in the size of their precursors.

The last point to be discussed is the quantitative evaluation of the radioactivity incorporated into pre-rLHβ and pre-rLHα. Our data indicate that:

(i) The amount of pre-rLHα synthesized in response

to RNA is ~6-times higher than that of pre-rLHβ, as shown for the precursors of subunits of other glycoprotein hormones [28];

(ii) The proportion of precursors of rLH subunits encoded by rat pituitary RNA is rather low as compared to other pituitary proteins. We will show (in preparation), that the use of RNA from pituitary cells enriched in gonadotrophs increases the yield of these precursors 6–7-times as compared to the present data.

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