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IN VITRO TRANSLATION OF DISTINCT mRNAs CODING FOR THE PRECURSORS OF PORCINE LH SUBUNITS

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Conditions are described to characterize and estimate the precursors of porcine LH α and β subunits and indirectly their specific mRNAs. Poly(A) RNAs extracted from castrated male pig anterior pituitaries were translated in a wheat-germ system in the presence of [^{35}S] cysteine and [^{35}S] methionine. The translation products were precipitated by antisera directed against reduced and carboxymethylated LH α and β subunits and analyzed by high resolution electrophoresis.

It is shown that the precursors of pLH α and β subunits are located in two distinct ≈ 15 K proteins and represent – on the basis of the incorporation of the [^{35}S] labeled aminoacids into proteins – ≈ 0.12 % and 0.05 % respectively of the total translation products.

It is suggested that in the pig, as in other species, the LH α and β subunits are encoded by two distinct mRNAs, and at variance with other species the leader sequence of LH α mRNA is longer than that of LH β mRNA.

Gonadotropin concentrations in plasma and in anterior pituitary vary with physiological status and pharmacological manipulations. Attempts are currently made to determine the molecular level at which modulating factors are acting. In order to determine whether or not the transcription of the gonadotropin subunit genes is involved in gonadotropin regulation, cell-free translation assays for the corresponding mRNAs have been developped. Precursors of the subunits of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were detected among the translation products of poly(A) RNAs extracted from anterior pituitaries of different species : bovine (1), rat (2, 3) and ovine (4).

We report here for the first time, a characterization by electrophoresis and a tentative estimate of the translation products of mRNAs coding for LH subunits from porcine anterior pituitary. Another point of interest in this paper is to use together the best conditions available for the analysis of minute amounts of the precursors of LH subunits : labelling with a mixture of [^{35}S] cysteine-[^{35}S] methionine (5), immunoprecipitation with antisera directed against the denatured LH subunits (1-4) and separation of proteins by a high resolution electrophoresis (6).

Abbreviations used : LH : luteinizing hormone ; FSH : follicle-stimulating hormone ; poly(A) RNA : polyadenylic acid rich RNA ; SDS : sodium dodecyl sulfate ; TCA : trichloracetic acid ; RCM : reduced and carboxymethylated ; pLH : porcine LH ; bLH : bovine LH ; K : kilodaltons ; hCG : human chorionic gonadotropin ; TSH : thyreostimulating hormone.

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MATERIAL AND METHODS

Reagents : [^{35}S] methionine and [^{35}S] cysteine (specific activities ≈ 1000 Ci/mmole) were purchased from Amersham. Micrococcal nuclease was supplied by Worthington, oligo dT type T3 cellulose by Collaborative Research, X-ray-films (XAR-5) by Kodak and wheat germ by "Les Grands Moulins de Paris". All the other reagents were of analytical grade.

Preparation of poly(A) RNAs : Male pigs were castrated at one month old (Large White x Landrace) and killed at seven month old at the local slaugther-house by electric schock. The anterior pituitaries were excised within 30 min. following the electric schock and frozen immediately in liquid nitrogen. Total RNA was extracted as previously described (7, 8). Poly(A) RNAs were prepared by oligo dT chromatography (9). RNA concentrations were measured by spectrometry at 260 nm (1 OD = 50 µ g/ml). DNA was measured by fluorimetry using ethidium bromide (10).

The cell-free translation assay : Wheat germ extracts (11) treated with micrococcal nuclease (12) were used. The volume of assay was 100 µl. Final concentrations were : 30 % (v/v) wheat germ extract, 20 mM HEPES pH 7.4, 1 mM ATP, 0.2 mM GTP, 2 mM dithioerythritol, 8 mM creatine phosphate, 1.7 UI/ml creatine phosphokinase, 0.5 mM spermidine, 40 µM of all unlabeled amino acids excepted [^{35}S] methionine and [^{35}S] cysteine added at 500 µ Ci/ml each. [^{35}S] methionine alone was used for determining the optimal conditions of translation. Poly(A) RNAs were heated at 60°C for 3 min. before they were added to the translation mixture. The reaction was allowed to proceed for 60 min. at 30°C.

Measure and characterization of translation products : For the assay of the total translation products, the newly synthetized proteins were reduced and alkylated (13) and thereafter precipitated by trichloracetic acid (TCA 10 % v/v). The precipitate was collected on glass filter (GFC Whatman), digested in NaOH 1 N, 0.5 ml, for 1 h. at 60°C and neutralized. Finally 0.5 ml water was added before counting in 5 ml of 399 Packard scintillating mixture.

For the electrophoretic analyses, the translation products were either precipitated by 10 % TCA or immunoprecipitated according to the method of Alexander et al (4) slightly modified. Only one immunoprecipitation was performed. The samples were incubated with antisera in the presence of Aprotinin (Sigma) 2.8 U/ml for 18 h at room-temperature and thereafter for 1 h at 37°C with Staphylococcus Aureus Cowan. The antiserum directed against the reduced and carboxymethylated porcine LH β subunit (RCM-pLHβ) was prepared in New-Zealand rabbits and used at 1:200 final dilution. The pLHβ was kindly provided by Dr. Y.Combarnous (INRA, Nouzilly, France) and was reduced and alkylated (14). The antiserum against RCM-bLHα was supplied by Dr. J. Pierce and used at 1:200 final dilution. The TCA precipitates and the immunoprecipitates were heated for 1 h at 37°C and for 3 min. at 100°C in Laemmli's lysing buffer (15) and analyzed by polyacrylamide gel electrophoresis in denaturing conditions. The Laemmli's system was used with a 10-18 % polyacrylamide 0-10 % sucrose gradients in the presence of 7 M urea (6).

Radioactive polypeptides were revealed by exposure of dried gels to X Ray films at −70°C. The areas of the gel containing the immunoprecipitated material (≈ 15 K area) were excised and solubilized in 1 ml of 10 % ammonium hydroxyde and counted for radioactivity after neutralization. The counting efficiency of this extract was similar to that of the TCA precipitate counting. The relative abundance of each polypeptide was calculated by comparing the radioactivity of each area to the radioactivity precipitated by TCA in conditions known to decrease the non specific binding of [^{35}S] cysteine i.e. after reduction and carboxymethylation (13).

RESULTS

The RNA and the cell-free translation assay. The amounts of RNA extracted from six different pools of anterior pituitaries of castrated pigs were 3.38 ± 0.74 mg/g of wet tissue (mean ± SD). The A 260/A 280 ratio was higher than 2, and the contamination by

DNA of the RNA preparations was lower than 1 %. The percentages of poly(A) RNA recovered from the oligo dT cellulose columns were 4.16 ± 0.55 % of the total RNA (mean ± SD, n = 6).

The translation efficiencies of poly(A) RNAs were determined by measuring the TCA precipitable proteins by varying the concentrations of K^+ acetate and Mg^{++} acetate. The optimal efficiency for the wheat-germ extract was obtained with 70 mM K^+ and 1.5 mM Mg^{++} (results not shown). In these conditions, the amount of newly synthetized proteins was directly proportional to the concentrations of RNA in the translation assay up to 30 µg/ml (Fig. 1). Further studies were done at this concentration. In this condition and using both [^{35}S] cysteine and [^{35}S] methionine the blank level – i.e. the radioactivity precipitated by TCA when exogenous RNA was omitted – was multiplied by a factor of 10. This apparent low stimulation is due to the type of labeled aminoacids used since the factor of multiplication was ≈ 70 when they were replaced by [^{3}H] leucine.

Characterization and estimate of the precursors of LH subunits. Antisera directed against the mature pLH α or pLH β subunits did not precipitate any of the translation products. Thus, immunoprecipitation have been done with antisera directed against reduced and carboxymethylated subunits. Fig. 2 shows the autoradiograph of the cell-free translation products of poly(A) RNA from the anterior pituitaries of castrated male pigs analyzed by polyacrylamide gradient gel electrophoresis. When the products were previously precipitated by TCA (lane 1) the most prominent bands correspond to ≈ 23, 21 and 15 K proteins. According to the results obtained in different species, the first two bands contain very likely pre-PRL and pre-GH respectively. The analysis of the products immunoprecipitated by antisera directed against the RCM-bLHα or the

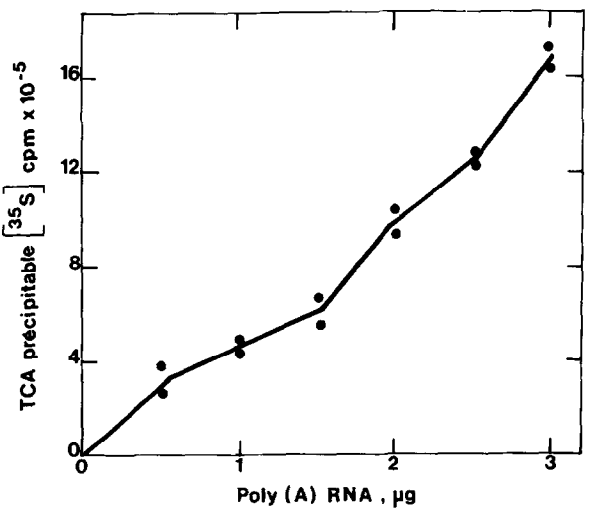

Figure 1. Translation assay for increasing concentrations of poly(A) RNA. Increasing concentrations of poly(A) RNA were translated in the wheat-germ system in the presence of [^{35}S] methionine. Newly synthetized proteins in the assay were measured after trichloracetic acid precipitation.

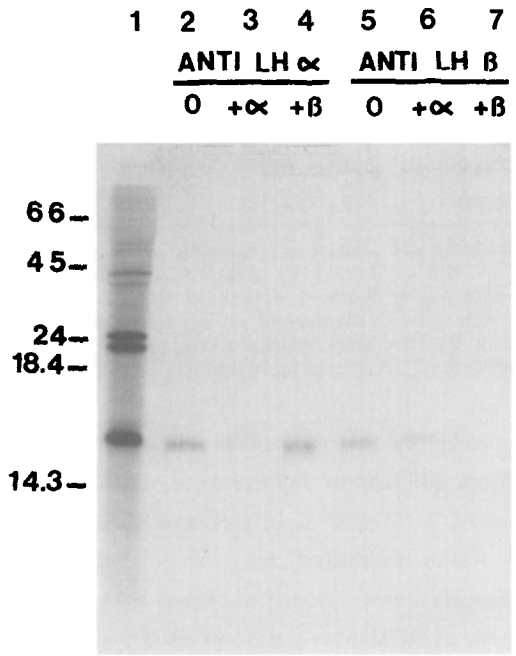

Figure 2. Autoradiography of translation products.
Poly(A) RNAs extracted from castrated pig anterior pituitaries were translated in a wheat-germ system in the presence of [^{35}S] methionine and [^{35}S] cysteine. The proteins were precipitated in different conditions and separated by high resolution electrophoresis. The exposure time of the dried film was 4 days.
Lane 1 : TCA precipitate of 8 µl assay. Lanes 2-4 : the material of 15 µl assay was precipitated by RCM bLHα antiserum either in the absence of LH subunit (lane 2) or in the presence of 3 µg RCM-pLH α (lane 3) or in the presence of 2 µg RCM-pLHβ (lane 4). Lanes 5-7 : material from 30 µl assay was precipitated by RCM-pLH β antiserum either in the absence of LH subunit (lane 5) or in the presence of 3 µg RCM-pLH α (lane 6) or in the presence of 2 µg RCM-pLH β (lane 7).

RCM-pLH ß subunits indicates that the antigenic determinants of the two LH subunits are located in two distinct ≈ 15 K proteins. Indeed, the immunoprecipitation by the RCM-bLHα antiserum (lane 2) was totally inhibited by an excess of RCM-pLHα (lane 3) while it was not affected by an excess of RCM-pLHß (lane 4). Likewise, the immunoprecipitation by the RCM-pLH β antiserum (lane 5) was slightly decreased by an excess of RCM-pLHα (lane 6) while it was totally inhibited by an excess of homologous RCM subunit (lane 7).

From a qualitative point of view, in conditions which allowed a good separation of putative pre-GH and pre-PRL, the proteins which could be the precursors of pLH α and β subunits appear of similar molecular weights. When electrophoresis was run in the presence of 0.1 % SDS and 17 % polyacrylamide but in the absence of urea and without sucrose gradient the bands revealed by autoradiography – including those corresponding to the 15K proteins – were larger than those shown here and a smear was seen in the 21-23 K area instead of the distinct 23-21 K bands.

From a quantitative point of view, the material precipitated by RCM-bLHα antiserum, in the absence of exogenous LH subunits, represents in Fig. 2 ≈ 0.12 % of

Table 1. Specificity of the immunoprecipitation

	COMPETITOR		
	0	RCM-pLH α	RCM-pLH β
RCM-bLH α antiserum	0.12 ± 0.05	0.02 ± 0.02	0.12 ± 0.04
RCM-pLH β antiserum	0.05 ± 0.02	0.05 ± 0.03	0

The translation products are immunoprecipitated by two different antisera directed against either RCM-LH α or RCM-LH β subunits. The immunoprecipitation was done either in the absence (0) or in the presence of RCM-LH subunits and analyzed by electrophoresis as described in the legend of fig. 2. The 15 K areas were excised and counted for radioactivity. The amounts of the immunoprecipitates were given as the % of the cpm counted in the TCA precipitable material (mean ± SD, n = 6).

the total translation products ; the material precipitated by RCM-pLH β antiserum in the absence of exogenous LH subunit represents ≈ 0.05 % and only ≈ 0.02 % in the presence of an excess of RCM-pLH α. In five additional analyses of the translation products of poly(A) RNAs extracted from five additional pools of pitutiaries, essentially the same results were obtained excepted that in an half of the experiments the addition of an excess of RCM-pLH α did not affect at all the immunoprecipitation by RCM-pLH β antiserum. These results are shown in Table 1.

We observed that the amount of the synthetized 15 K proteins was negligible in the absence of pituitary poly(A) RNA, and that no protein directed by pituitary poly(A) RNA was precipitated by a normal rabbit serum (results not shown).

DISCUSSION

This paper reports, for the first time, the in vitro translation of porcine pituitary poly(A) RNAs and the analysis by electrophoresis of proteins specifically immunoprecipitated by antisera directed against reduced and carboxymethylated LH subunits.

The yield of RNA extraction and the percentage of poly(A) RNA were essentially the same as those reported in bovine (1), rat (2, 3), or ovine (4) anterior pituitaries. Technical points have to be underlined : First, since pLH subunits were known (16, 17) to contain a low number of methionine residues (only one in the β subunit and 4 in the α subunit) and a high number of cysteine residues (12 in the β subunit and 10 in the α subunit) the translation products were labeled with [^{35}S] methionine and [^{35}S] cysteine. Second, antisera for immunoprecipitation of the translation products had to be prepared against the reduced and carboxymethylated LH subunits. This is in agreement with the large majority of reports concerning the immunoprecipitation of the precursors of glycoproteins : TSH (18), LH (1, 3) and FSH (4, 5).

From the specificity of the immunoprecipitation we conclude that the antigenic determinants of LH α and β subunits are not located in the same protein suggesting that pLH α and β subunits are encoded by two distinct mRNAs. Similar conclusion was proposed for the mRNAs encoding for the subunits of bovine (1) and rat (2, 3) LH, for

bovine TSH (18), for ovine (4, 5) and rat (5) FSH and finally for hCG (19) using either the in vitro translation assay or the hybridization with specific probes (20).

Although a high resolutive electrophoresis was used (6), the apparent molecular weights of the putative precursors of LH α and β subunits are not different. This could be characteristic of the porcine LH since, in the other species, the molecular weight of the LH α precursor was shown higher than that of the LHβ precursor (1-5). The apoproteins of the mature pLHα and β subunits contain 91 and 119 aminoacid residues respectively, corresponding to ≈ 10,800 and 13,300 molecular weights. The comparison of these values with the results reported here suggest that, at variance of what have been reported in other species, the leader sequence of pLHα mRNA would be longer than that of pLHβ mRNA. We cannot definitely exclude a limited proteolysis of LHβ occuring during the transcription assay or the immunoprecipitation period. However, this hypothesis is unlikely, in particular because the immunoprecipitation was performed in the presence of a proteolysis inhibitor.

If we assume that the unknown aminoacid composition of the signal sequences of the precursors of LH α and LHβ subunits are not different from the composition of the mature subunits and since the sum of methionine and cysteine residues is known to be 14 in the mature pLH α and 13 in the mature pLH β subunits (16, 17), our results suggest that, in castrated male pig pitutiaries, the LH αmRNA:LHβ mRNA ratio is approximately equal to 2. This value is slightly smaller than that reported earlier in rats (5), steers and cows (1).

Although the translation assay is time consuming, cumbersome and does not allow to measure accurately mRNAs, it is a usefull method for determining the mRNA activity in pituitary tissue or pituitary cells. Indeed, it had to be used in the absence of specific probes, it has already been fruitful for studying the regulation of gonadotropin synthesis either in vivo (21, 22) or in vitro (22) and finally it is potentially efficient for determining the role of endoplasmic reticulum from different origins in the maturation of the precursors of gonadotropin subunits.

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REFERENCES

1. Keller, D., Fetherston, J., and Boime, I. (1980) Eur. J. Biochem. 108, 367-372.
2. Godine, J.E., Chin, W.W., and Habener, J.F. (1980) J. Biol. Chem. 225, 8780-8783.
3. Counis, R., Ribot, G., Corbani, M., Poissonnier, M., and Jutisz, M. (1980) FEBS Letters 123, 151-155.
4. Alexander, D.C., and Miller, W.L. (1981) J. Biol. Chem. 256, 12628-12631.
5. Counis, R., Corbani, M., Poissonnier, M., and Jutisz, M. (1982) Biochem. Biophys. Res. Commun. 107, 998-1005.
6. Hashimoto, F., Horigome, T., Kanbayashi, M., Yoshida, K., and Sugano, H. (1983) Anal. Biochem. 129, 192-199.

7. Auffray, C., and Rougeon, F. (1980) Eur. J. Biochem. 107, 303-314.
8. Morel, Y., Pascal, O., and André, J. (1983) Biochem. Biophys. Res. Commun. 113, 462-469.
9. Aviv, H., and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408-1412.
10. Karsten, V., and Wollenberger, A. (1977) Anal. Biochem. 77, 464-470.
11. Marcu, K., and Dudock, B. (1974) Nucleic Acids Res. 11, 1385-1397.
12. Pelham, H.R., and Jackson, R.J. (1976) Eur. J. Biochem. 67, 247-256.
13. Hirs, C.H.W. (1967) Methods in Enzymology, Vol. 11, Enzyme Structure (C.H.W. Hirs ed.), pp. 199-203, Academic Press, New York and London.
14. Pierce, J.G., Falth, M.R., and Donaldson, F.M. (1976) Gen. Comp. Endocrinol. 30, 47-60.
15. Laemmli, U.K. (1970) Nature 277, 680-685.
16. Maghuin-Rogister, G., Combarnous, Y., and Hennen, G. (1973) Eur. J. Biochem. 39, 255-263.
17. Maghuin-Rogister, G., and Hennen, G. (1973) Eur. J. Biochem. 39, 235-253.
18. Giudice, L.C., Waxdal, M.J., and Weintraub, B.D. (1979) Proc. Natl. Acad. Sci. USA 76, 4798-4802.
19. McQueen, S.D., McWilliams, D., Birken, S., Canfield, R., Landefeld, T., and Boime, I. (1978) J. Biol. Chem. 253, 7109-7114.
20. Boorstein, W.R., Vamvakopoulos, N.L., and Fiddes, J.C. (1982) Nature 300, 419-422.
21. Counis, R., Corbani, M., and Jutisz, M. (1983) Biochem. Biophys. Res. Commun. 114, 65-72.
22. Alexander, D.C., and Miller, W. (1982) J. Biol. Chem. 257, 2282-2286.