Cell free translation of chicken calcitonin messenger RNA

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The primary step of calcitonin biosynthesis was studied in a normal organ: chicken ultimobranchial gland, a tissue particularly rich in calcitonin secretory cells. Poly(A)-rich RNA was extracted and purified from ultimobranchial organs and translated in a reticulocyte lysate in the presence of labelled methionine. Polyacrylamide gel electrophoresis of specific immunoprecipitates revealed a major band of M_r 14500 and a band of M_r 13300. Thus, in chicken the precursor of calcitonin is a M_r 14500 polypeptide. The minor component of M_r 13300 could represent limited processing by the reticulocyte lysate.

Calcitonin *Messenger RNA* *Translation* *Chicken* *Ultimobranchial gland* *Radioimmunoassay*

1. INTRODUCTION

In [1] we identified the primary translation product of calcitonin mRNA, an M_r 14500 polypeptide, in the rat [1]. However, in mammals the calcitonin-producing cells, C cells, constitute but a small percentage of the thyroidal cells. In birds, the ultimobranchial glands contain large amounts of calcitonin, in cells which form a majority of the cellular population. The ultimobranchial gland in birds constitutes an excellent model for the study of calcitonin biosynthesis and its regulation at the molecular level. As a first step, we have extracted and translated mRNA from chicken ultimobranchial glands and identified for the first time the primary translation product of chicken calcitonin mRNA, an M_r 14500 protein.

Abbreviations: mRNA, messenger RNA; M_r, relative molecular mass; RIA, radioimmunoassay; CT, calcitonin; sCT, salmon calcitonin; UB, ultimobranchial gland; EDTA, ethylene diamine tetracetic acid; TEB, Tris–EDTA buffer; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride

2. MATERIALS AND METHODS

2.1. *Radioimmunoassay*

A heterologous radioimmunoassay [2] for chicken calcitonin was developed using unlabelled synthetic salmon calcitonin (sCT) (spec. act. 4000 U/mg, batch no. 20051, Sandoz) and ^{125}I-sCT labelled by chloramine T [3]. The antiserum was raised in the sheep against unconjugated synthetic sCT. In brief the RIA was performed as follows: the antibody diluted 1/20000 in the RIA buffer (0.1 M phosphate, 0.2% human albumin and 0.1% sodium azide, pH 7.4), 50 pg labelled hormone and increasing amounts of synthetic hormone (0.03–1 μg) were incubated for 4 days at 4°C. Free and bound hormone were separated by dextran–charcoal. The calcitonin content of chicken UB glands, extracted in 0.1 N HCl mixed with a polytron and after lyophilisation dissolved in RIA buffer, was estimated by the above RIA. Displacement curves were linearized by using the logit transform [4] and regression curves estimated by the least square, according to classical statistical methods:

$$\text{logit } B/B_0 = \log_e\left(\frac{1}{B/B_0}\right)$$

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where:

B = counts bound to antibody in the presence of x dose of unlabelled sCT;

B_0 = counts bound to antibody in the absence of unlabelled sCT.

2.2. *Antibody purification*

Antiserum against synthetic salmon calcitonin showing a high cross reaction with chicken CT was purified by affinity chromatography, using sCT coupled to Sepharose 4B (Pharmacia) as in [5].

2.3. *Extraction of messenger RNA*

UB glands were collected from 4-week old chicks and frozen in liquid nitrogen until processed. Total nucleic acids were extracted as in [6]: aliquots of tissue (100–200 glands) were ground (polytron homogenizer) in 10 ml 0.1 M Tris–HCl buffer (pH 9), 0.01 M EDTA (TEB), containing 0.1 M dithiothreitol, 2% SDS and 10 ml phenol/chloroform/isoamyl alcohol (50:50:2), pre-equilibrated with TEB. After centrifugation the phenol phase was reextracted with an equal volume of TEB. The combined aqueous phases were extracted 3 times with an equal volume of phenol/chloroform/isoamyl alcohol and the nucleic acids precipitated (16 h at −20°C) with 0.1 vol. 2 M sodium acetate (pH 5.5) and 2.5 vol. cold ethanol. The precipitates collected by centrifugation were dissolved in sterile distilled water and precipitated by 0.25 vol. 10 M LiCl [7].

2.4. *Purification of poly(A)-rich RNA*

The poly(A)-rich RNA was separated by affinity chromatography on oligo(dT)-cellulose (Collab. Res.) equilibrated with Tris–HCl 10 mM (pH 7.5), 1 mM EDTA, 0.5 M NaCl containing 0.1% SDS.

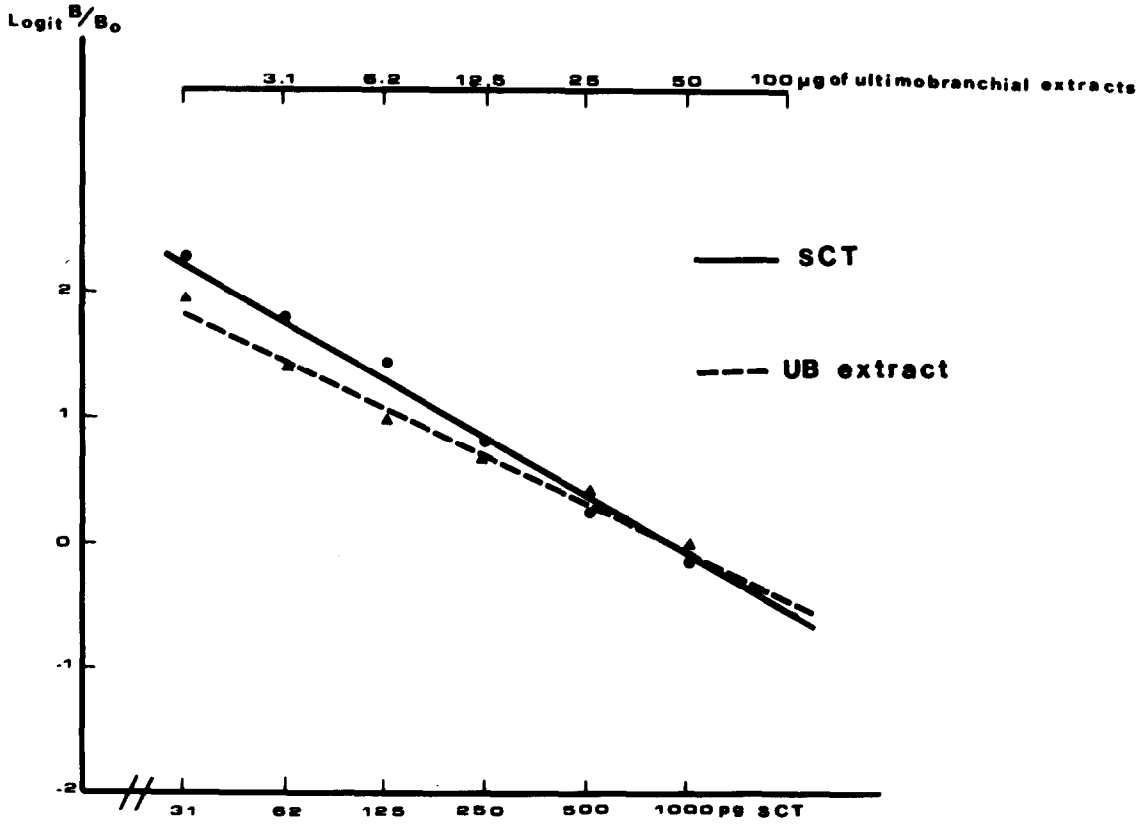


Fig.1. Displacement of ^{125}I-salmon calcitonin bound to anti-sCT antibodies by ultimobranchial extracts and unlabelled sCT. The curve was linearized by using logit B/B_0 function of the log of pg standard sCT; UB extracts are expressed as the equivalent of μg wet wt.

The poly(A)-rich RNA was eluted at 37°C using 10 mM Tris–HCl, 1 mM EDTA, 0.1% SDS and precipitated overnight at −20°C with 0.1 vol. 2 M sodium acetate (pH 5.5) and 2.5 vol. cold ethanol. Precipitates were washed twice with cold 75% ethanol, dried and dissolved in sterile distilled water at 1 μg/μl.

2.5. *In vitro translation*

The RNA (0.25–2 μg) was translated in 25 μl total vol. using a commercial reticulocyte lysate kit (New England Nuclear) in the presence of L-[^{35}S]methionine (2000 Ci/mmol, New England Nuclear), using the procedure described by the producer. Translations were performed in the presence of 0.04% PMSF. After incubation at 37°C for different times (15, 30, 60, 120 min) the reaction was stopped by cooling in an ice bath. Aliquots (1 μl) were spotted onto Whatmann 3MM filter paper, precipitated with 20% trichloroacetic acid, boiled in 10% trichloroacetic acid, washed with ethanol and ether [8], dried and counted in a β-counter, using a scintillation cocktail of 3% Protosol in Econofluor (New England Nuclear).

2.6. *Immunoprecipitation*

Translation products (50 μl) were incubated with 10 μl affinity purified sheep anti-sCT antibodies, in the presence or absence of 5 μg synthetic salmon calcitonin, in 500 μl final vol. of 0.1 M phosphate buffer (pH 7.4), 0.2% human albumin, 0.1% L-methionine and 0.25% Triton X-100, containing 0.04% PMSF, to avoid degradation of translation products by proteases present in the antiserum used (second antibody). After overnight incubation at 4°C, the anti-calcitonin antibodies were precipitated with 25 μl rabbit antiserum to sheep immunoglobulin (Kallestad) (24 h incubation). Immunoprecipitates were collected by centrifugation through a 1 M sucrose cushion and washed 4-times with the immunoprecipitation buffer.

2.7. *Polyacrylamide gel electrophoresis*

The translation products were diluted, and the immunoprecipitate dissolved in 0.625 M Tris–HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 0.001% bromophenol blue and 10% glycerol (sample buffer) and denatured by heating at 100°C for 3 min prior to loading. All samples were subjected to electrophoresis in 15 or 20% acrylamide containing 0.1% SDS as in [9]. M_r-markers labelled with ^{14}C were included in each run. Gels containing immunoprecipitates were vacuum dried, while gels containing total translation products were fix-

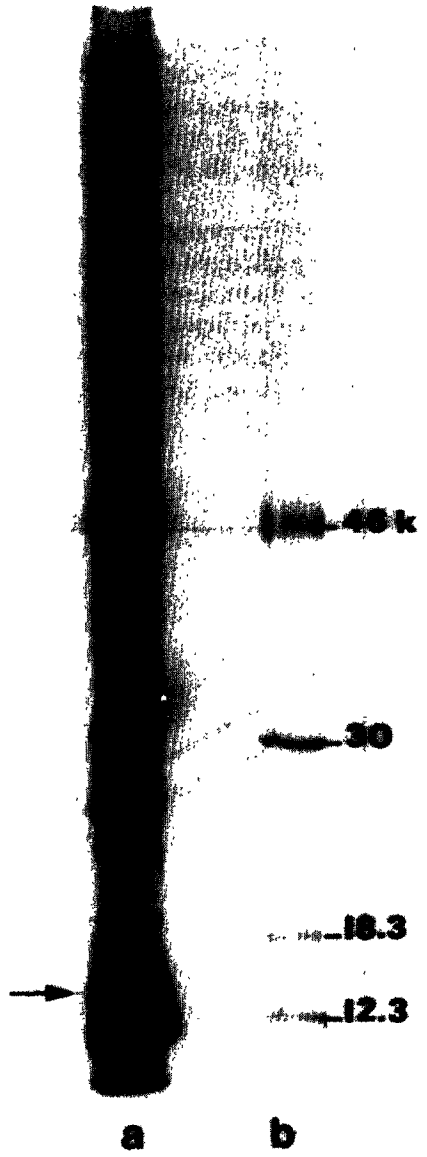


Fig.2. SDS–polyacrylamide gel (15%) electrophoresis of translation products of ultimobranchial mRNA in cell free system: (a) total translation products; (b) ^{14}C-labelled protein M_r standards: cytochrome *c*, 12300; lactoglobulin A, 18367; carbonic anhydrase, 30000; ovalbumin, 46000. Arrow indicates position of major protein precipitated by specific antiserum (cf. fig.3).

ed in 11.4% trichloroacetic acid, 3.4% sulfosalicylic acid, 30% methanol and treated with Enhance (New England Nuclear), prior to drying. Fluorograms were obtained by exposing the gels to X-Omat AR 5 films (Kodak) for 2–8 days at −80°C.

3. RESULTS

Identical displacement of labelled sCT bound to anti-sCT antibodies was achieved with UB extracts or synthetic salmon CT (fig.1). Translation of total poly(A)-rich RNA extracted from the UB glands resulted in the incorporation of labelled methionine into a large number of proteins of different M_r-values (fig.2). In the absence of exogenous mRNA, no significant incorporation into visible protein bands was detected (not shown). After immunoprecipitation with highly purified sCT antibodies, only 2 bands with estimated M_r 14500 for the major band and M_r 13300 for the minor band were detected; the M_r 13300 band increased when incubation was carried out for longer periods (fig.3). When immunoprecipitation was performed in the presence of excess unlabelled sCT, no such bands were observed.

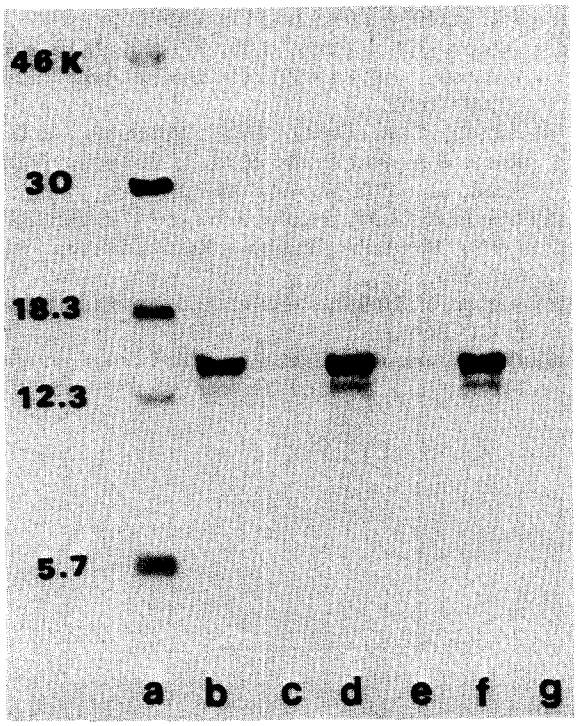


Fig.3. SDS–polyacrylamide gel (20%) electrophoresis of immunoprecipitates of translation products synthesized in reticulocyte cell free system, in presence of 0.04% PMSF and incubated during different times: (a) M_r-markers (as in fig.2, plus insulin 5766); (b,d,f) specific immunoprecipitation of biosynthetic products after translation carried out for 15, 30 and 60 min, respectively; (c,e,g) specific immunoprecipitates in the presence of excess of unlabelled sCT of biosynthetic products after translation carried out for 15, 30 and 60 min, respectively.

4. DISCUSSION

The amino acid composition of chicken calcitonin is quite similar to that of salmon calcitonin [10] and the molecule shares common antigenic determinants with sCT [11,12]. This last fact is confirmed in the present work as the antibodies used cross-react highly with ultimobranchial CT. The detection of a major band after specific immunoprecipitation with anti-sCT antibodies and the absence of this band after saturation of the antibodies with unlabelled sCT, is strong evidence that the primary translation product of CT mRNA is an M_r 14500 polypeptide. The size of this precursor is similar to that reported in normal rat [1] or cod fish [13]. The minor band of M_r 13300, which was also specifically immunoprecipitated could represent limited processing of the major form during translation, as the intensity of this band increased with longer incubation times. Translation of mRNA extracted from normal tissues: rat [1], cod fish [13], or in this work chicken, directs the synthesis of a precursor of M_r ~15000. Messenger RNAs, isolated from murine [13,14], or human [1,15,16] medullary carcinoma of the thyroid, code for precursors showing diversity in M_r-values: for the rat, 15000 [13] and 17555 [14]; and for human, 15000 [1,15] and 21000 [16]. The apparent size stability of the initial precursor during evolution (fish, birds, mammals), in contrast to its M_r-heterogeneity when encoded by mRNA extracted from malignant tissues, suggests that the biosynthesis of calcitonin may vary at the transcriptional and probably post-transcriptional level during the course of malignancy. Further investigations on the structure of the primary translation precursor of calcitonin in chicken will be of value in determin-

ing the molecular events involved in the biosynthesis of the hormone. In addition to the relative abundance of calcitonin producing cells in the chicken ultimobranchial, this animal model is particularly attractive for such studies as isohormones have been reported in this species [17]. This probably implicates splicing events in the transcription of a common gene or alternatively the presence of two distinct genes, as suggested on the basis of immunochemical studies by others [14].

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