CELL FREE TRANSLATION OF mRNA CODING FOR HUMAN AND MURINE CALCITONIN

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1. Introduction

Calcitonin (CT), the hypocalcemic hypophosphatemic hormone, is secreted by the thyroidal C-cells in mammals and by the ultimobranchial gland in submammalian species. We have reported the presence of several immunoreactive forms [1–3] larger than calcitonin monomer in human medullary carcinoma of the thyroid (MCT), a differentiated calcitonin secreting tumour [4]. These forms could represent higher molecular weight biosynthetic precursors of human CT. So far studies on the biosynthesis of calcitonin in 3 animal species have provided evidence for the presence of a prohormone of M_r 7000 (trout) [5], 13 500 (chicken) [6] and 11 800 (human) [7]. Using mRNA extracted from MCT and a murine transmissible tumour, primary translation product was reported to be a molecule of M_r 65 000 [8]. In [9] M_r 15 000 was reported for the primary translation product of mRNA extracted from codfish or from a murine transmissible tumour, while in [10] M_r 15 000 also for the primary translation product of mRNA extracted from MCT was reported. We report here studies on the primary translation product of mRNA extracted from MCT. As work concerning mammalian mRNA was done with tumoral tissue, we have also extracted and translated mRNA from a normal tissue (rat thyroid) as in this species a considerable sequence homology with human CT (hCT) exists [11].

2. Materials and methods

Tissues from a patient suffering from MCT were obtained during operation and immediately frozen in liquid nitrogen. Thyroid glands were obtained from normal male rats (killed by a sharp blow on the head) and collected also in liquid nitrogen. All tissues were stored at −80°C until extracted.

2.1. *RNA extraction*

The tissues were ground in a waring blendor in the presence of powdered dry ice for 2 min and then extracted for 1 min with 20 vol. of Tris 50 mM, NaCl 150 mM, EDTA 50 mM and 1% SDS buffer (pH 7.4) at room temperature. The homogenate was then incubated for 10 min at 20°C in the presence of proteinase K (Boehringer) 5 mg/100 ml and then extracted with a mixture of phenol/chloroform/pentanol 2 (50/50/1 by vol.). After centrifugation 6000 rev./min at 4°C, the aqueous phase was separated and reextracted twice with the above mixture. The aqueous phase was made 0.2 M with CH_3COONa (pH 5) and nucleic acids precipitated by the addition 2 vol. ethanol at −20°C overnight. After centrifugation the precipitate was dissolved in distilled water and precipitated by 3 vol. CH_3COOK 4 M, EDTA 10 mM (12). Total RNA was collected by centrifugation redissolved in distilled water and precipited by ethanol.

2.2. *Purification of poly(A)-RNA*

Poly(A)-RNA was separated by affinity chromatography using oligo(dT) cellulose T3 (Collab. Res.) and collected by ethanol precipitation [13].

2.3. *Reticulocyte lysate preparation and incubation conditions*

Rabbits were made anaemic by injections of *N*-acetylphenylhydrazine according to [14]; the mRNA-dependent reticulocyte lysate was prepared as in [15] with minor modifications [16] and stored

in small aliquots in liquid nitrogen. Incubations were performed in 50 μl containing 25 μl reticulocyte lysate and 1 mM $MgCl_2$, 100 mM KCl, 19 amino acids (except methionine) at 20 μM each, 3 μg rat liver tRNA the mRNA and 25 μCi L-[^{35}S]methionine (NEN). Incubations were usually done at 30°C for 90 min. After incubation 1 μl aliquots were removed, spotted on Whatman 3 MM disks and treated as in [17].

2.4. *Immunoprecipitation*

Translation products (50 μl) were diluted to 250 μl with phosphate buffer 0.1 M containing 0.2% human albumin, 0.1% sodium azide, and 1% Triton X-100; 1 μl pure rabbit anti-hCT sheep antiserum was added and incubation carried out for 24 h at 4°C. 50 μl Ig anti-sheep IgG (rabbit) (Miles) were added and after a further incubation for 24 h the precipitate was collected by centrifugation, washed with the same buffer and dissolved in 50 μl sample electrophoresis buffer according to [18]. The anti-hCT antiserum used showed complete cross reaction with murine CT and partial crossreaction with fragments 11–32 and 17–32 of the human molecule implicating an N and C specificity. The following controls were routinely performed: saturation of the translation products with 3 μg synthetic hCT or omission of the anti-hCT antibody. Iodinated hCT was also identically processed.

2.5. *Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)*

Molecular weight determination was performed using a 17% acrylamide as a running gel and a 5% stacking gel. All samples were denatured in the presence of SDS and β-mercaptoethanol. Molecular weights were determinated by comparison with ^{14}C-labelled proteins purchased from NEN: phosphorylase *b*, 92 500; bovine serum albumin, 69 000; ovalbumin, 46 000; carbonic anhydrase, 30 000; cytochrome *c*, 12 300 and with immunoprecipitated ^{125}I-hCT (3400). The gel slabs were dried in vacuo with the aid of heat and autoradiographed for 1 week at 80°C with LKB ultrofilm or Kodak Royal O Mat.

2.6. *Determination of immunoreactive forms*

An aliquot of the medullary carcinoma tissues was extracted in cold 0.1 N HCl, centrifuged and the supernatant freeze dried.

The lyophylised extracts were dissolved in standard radioimmunoassay (RIA) buffer (0.1 M phosphate containing 0.2% human heat denatured albumin, and 0.1% sodium azide) and applied to an affinity column of anti-hCT sheep antibodies (Ig fraction) coupled to Sepharose 4B by cyanogen bromide. The bound hormone was eluted using 1 M acetic acid. All these steps were performed at 4°C. The acetic acid extracts were lyophylised. Aliquots of the affinity extract were denatured and alkylated with iodoacetamide [19] and then subjected to SDS electrophoresis using a 5% running gel. The gel was sliced using a mechanical gel slicer and the fractions extracted for 24 h in RIA buffer. CT content was estimated using the same antibody used in the immunoprecipitation studies and the affinity chromatography. In brief, 100 μl of several dilutions for each fraction were incubated in 500 μl total vol. containing 50 pg labelled hCT and antibody at a final dilution of 1/200 000. All samples and reagents were diluted in RIA buffer. Standard curves were established using serial dilutions of cold synthetic hCT, and controls for unspecific binding were also included in the assay.

3. Results

Table 1 shows the total amount of mRNA extracted from both human and murine tissues, the total amount of labelled methionine incorporated in the cell-free translation system, and the total amount of immunoprecipitable material. In the absence of mRNA no significant incorporation of labelled amino acid was observed. Human and murine mRNA increased incorporation of radioactive label 10-fold. Maximum incorporation was achieved with 2 μg human mRNA and 0.5 μg murine mRNA. When the total translation products were subjected to SDS–PAGE, several mRNA-dependent products were revealed (not shown). However after specific immunoprecipitation was performed, PAGE revealed the presence of a single band in both translation products of human or murine tissues (fig.1). This molecule has est. M_r 14 500. No such band was observed when the translation products were incubated in the presence of synthetic hCT or when the specific antiserum was replaced with normal sheep serum.

The profile of immunoreactive material present in the tissues used for the separation of mRNA is shown in fig.2. Trace amounts of immunoreactive material are present in the first fractions of the gel. Two small fractions of M_r 55 000 and 11 500, respectively, in

Table 1
Amounts of mRNA extracted from human MCT or rat thyroids

Tissue	MCT (human)	Rat thyroid
Weight	1.5 g	13.2 g
Total RNA	1.3 mg	18.5 mg
Poly(A)-RNA/mg total RNA	31 μg/mg	21 μg/mg
[^{35}S]Methionine incorp./mg	20 × 10^6 cpm/mg	34 × 10^6 cpm/mg
[^{35}S]Methionine immunoprecipitated/mg	1.5 × 10^6 cpm/mg	1.25 × 10^6 cpm/mg
% Immunoprecipitated	8.0%	3.6%

Rates of incorporation of [^{35}S]MCT in proteins (trichloroacetic acid-precipitable) and in calcitonin precursors (immunoprecipitable) expressed in cpm/mg total RNA

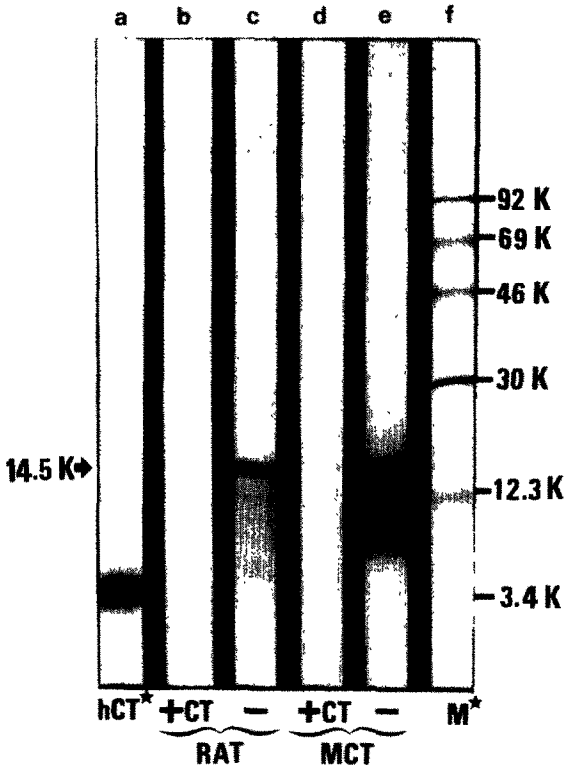

Fig.1. 7 days autoradiography of a 17.5% polyacrylamide-electrophoresis slab: (a) ^{125}I-hCT immunoprecipitated by anti-hCT antiserum and Ig fraction of anti-sheep IgG as double antibody; (b) immunoprecipitation of translation product of poly(A)-RNA from rat thyroid incubated in the presence of 3 μg synthetic hCT; (c) as in (b) without hCT; (d) immunoprecipitation of translation product of poly(A)-RNA from medullary carcinoma of the thyroid, incubated in the presence of 3 μg synthetic hCT; (e) as in (d) without hCT; (f) ^{14}C calibration proteins.

addition to an important peak of immunoreactivity comigrating with synthetic calcitonin are also observed.

4. Discussion

Our results clearly indicate that the primary translation product for human CT of tumoral origin or normal rat CT has relative M_r 14 500, as PAGE analysis

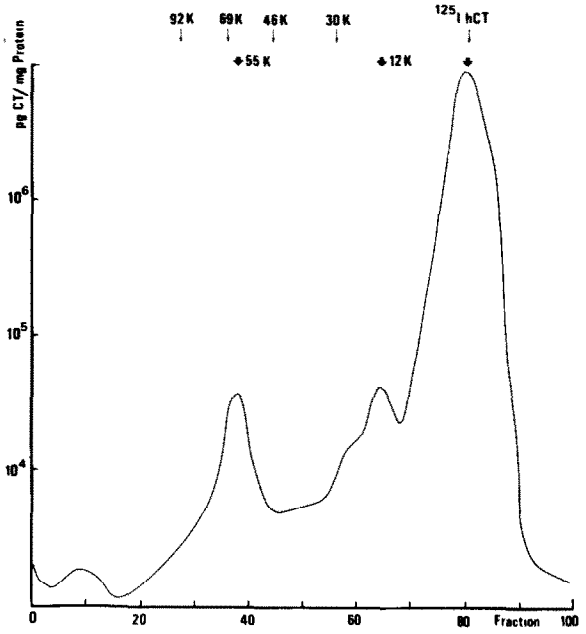

Fig.2. Profile of immunoreactive material present in human MCT tissue extract. Separation by 5% polyacrylamide gel electrophoresis sliced, eluted and assayed by specific hCT radioimmunoassay. Results are expressed in pg equiv. hCT by mg tissue extract before affinity chromatography.

revealed in both species a single band after specific immunoprecipitation, and saturation of the antibodies with synthetic hCT abolished the reaction. Our results are similar to those in [10] for MCT and [9] for the transmissible murine tumour. We have not observed any translation product with larger molecular weight such as that in [8] M_r 65 000, for both MCT and the rat transmissible tumour. The reasons for this discrepancy could be due to:

1. The use of a different translation system as the above work was carried out with frog oocytes while we used rabbit reticulocyte system.
2. Fragmentation of the mRNA during the extraction; however when we analysed the translation product prior to specific immunoprecipitation several large translation products were found in both the MCT and the rat extracts, and therefore our extraction procedure did not tend to fragment large size mRNA.
3. Differences in the MCT tumours studied, reflecting the degree of dedifferentiation of the cancer cell.

The M_r of the primary product of translation 14 500 is much smaller than the app. M_r of the 55 000 peak present in the tissues. Thus it is probable that this large form does not represent a biosynthetic precursor for CT. In view of our results the 11 500 M_r material could represent a prohormone cleaved from this sequence after the deletion of a M_r 3000 leach, as translation of the mRNA in the presence of dog pancreatic membrane lead to the appearance of a 12 000 M_r protein [9,10] and we have shown in incubation studies the incorporation of labelled cysteine in a M_r 11 800 immunoreactive form [7]. As the tissues extracts were both denatured and alkylated before electrophoretic separation the 55 000 M_r material could represent post-translational modifications of the hormone or its precursors perhaps involved in the formation of the storage granules. However in view of the very small amounts detected, a few ng, incomplete dissociation cannot be excluded. Thus the genesis of immunoreactive CT in plasma and tissues having $M_r > 12\,000$ is still unsolved.

So far, two normal tissues have been studied, quite far apart in evolution, i.e., codfish [9] and normal rat thyroids. More so, though the primary amino acid sequence of codfish is not known, it is quite likely to differ considerably from hCT in the 11–32 portion of the molecule, as salmon CT does, while rat CT has a primary amino acid structure quite similar to hCT, differing by only 2 amino acids [11]. It is therefore quite remarkable that in both species a similar MW for the primary translation product is found, 15 000 [9] codfish, and 14 500 for the rat. It is therefore our conclusion that the primary translation product for human CT is likely to have M_r 14 500 and not 65 000.

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