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Elucidation of the nucleotide sequence of chicken calcitonin mRNA: direct evidence for the expression of a lower vertebrate calcitonin-like gene in man and rat

F.Lasmoles, A.Jullienne, F.Day, S.Minvielle, G.Milhaud and M.S.Moukhtar

Laboratoire d'Endocrinologie Moleculaire, LA 163 CNRS, U113 INSERM, CHU Saint-Antoine, 27 Rue Chaligny, 75012 Paris, France

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Calcitonin shows considerable divergence in amino acid sequence between lower vertebrates and higher vertebrates. Immunoreactive salmon-like calcitonin molecules are present in the thyroid of man and rat. Elucidation of the almost complete sequence of chicken calcitonin mRNA revealed that the calcitonin precursor in chickens had the same organisation as in higher vertebrates (man and rat) but showed considerable differences in amino acid sequence. cDNA probes specific for chicken calcitonin mRNA hybridized to poly(A)-rich RNA extracted from a case of medullary carcinoma of the thyroid and from murine thyroid. These results suggest the expression in man and rat of a gene coding for an avian calcitonin-like precursor.

Key words: amino acid sequence/calcitonin precursor/evolution/medullary carcinoma of the thyroid/ultimobranchial gland

Introduction

Calcitonin, the hypocalcemic hypophosphatemic hormone (Copp *et al.*, 1962; Hirsch *et al.*, 1963), is involved in the regulation of calcium metabolism. The hormone is secreted by the C cells, localized in the thyroid in mammalia and in the ultimobranchial glands in lower vertebrates. Calcitonin is also the specific marker (Milhaud *et al.*, 1968) of a human cancer, medullary carcinoma of the thyroid, which exists in two forms, sporadic and familial (autosomal dominant). Radioimmunoassays of the human hormone are routinely used for the detection and monitoring of established and infra-clinical forms of the disease. They are the only method for detecting individuals at high risk of developing the cancer in familial forms of the illness. Calcitonin is considered as an example of unusual evolution (Staehelin, 1972) because the molecule shows considerable variation in sequence between teleosts and mammalia, and in mammals between artiodactyls on the one hand and primates and rodents on the other. For instance, human and porcine calcitonins differ in 18 amino acids out of 32, whereas the salmon and human molecules differ by only 15 amino acids. Immunoreactive salmon-like molecules are present in extracts of normal human thyroid, medullary carcinoma (Fischer *et al.*, 1983; Tobler *et al.*, 1984) and in murine thyroid (Perez Cano *et al.*, 1982). This could result from the expression of a gene coding for a salmon-like hormone or from the presence of a molecule sharing only common antigenic determinants with salmon calcitonin. The human (Craig *et al.*, 1982; Le Moullec *et al.*, 1984) and murine (Jacobs *et al.*, 1981; Amara *et al.*, 1982) calcitonin mRNAs show a high homology in nucleotide sequence and consequently a strong conservation of amino acids present in the precursor. Elucidating the nucleotide sequence of a lower vertebrate calcitonin mRNA would be of great help in studying the evolution of the calcitonin gene and in searching for a gen expressing a salmon-like molecule. To our knowledge no se quence of the precursor of a lower vertebrate calcitonin has s far been reported. We have recently reported the isolation of clone containing a partial sequence of calcitonin mRNA in a lowe vertebrate, the chicken, which allowed us to deduce the amin acid sequence of calcitonin in this species (Lasmoles *et al.*, 1985 We report here the isolation of a clone containing an almost com plete sequence of chicken calcitonin mRNA. Using cDNA fro this clone we show that a gene coding for a lower vertebrate typ calcitonin-like molecule is expressed in man and rat.

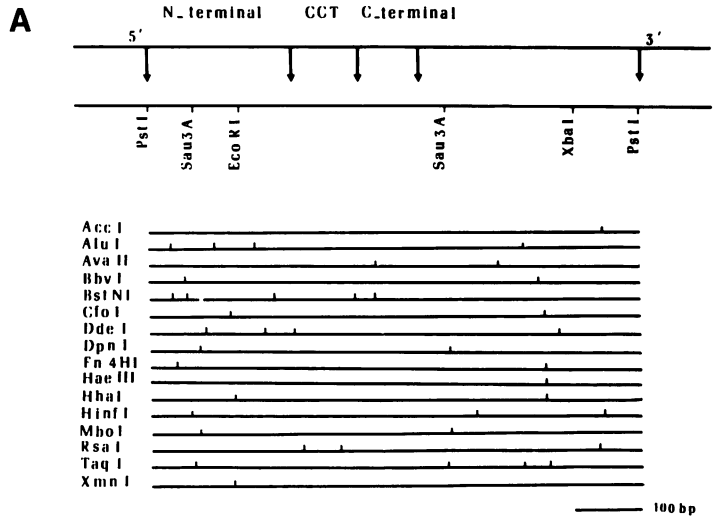
Results

Sequence of chicken calcitonin mRNA

Figure 1A shows the restriction map of the cDNA cloned i pCCT22 and the sequencing strategy. The restriction map is quit different from that of human (Le Moullec *et al.*, 1984), in par ticular the following restriction sites present in the human messer ger are absent: *Bcl*I, *Bgl*II, *Hae*III, *Msp*I, *Sac*I, *Sph*I and *Tha* Restriction enzyme sites for *Eco*RI and *Taq*I were present in th chicken mRNA but not in the human molecules. The nucleotid sequence and the deduced amino acid sequence of this insert i shown in Figure 1B. This 731-bp cDNA comprises the poly(A tail, the 3′ region of the calcitonin messenger and the coding se quence for the C-terminal peptide, the calcitonin molecule, th N-terminal peptide and part of the signal peptide. The chicke precursor shows the same organisation as human or murine calc tonin precursors: an amino-terminal peptide preceding calcitoni and a carboxy-terminal peptide following the calcitonin molecule The sequence of chicken calcitonin mRNA shows a limited ove all homology (37%) with human calcitonin mRNA and (31% with the human calcitonin gene-related peptide (CGRP) (Jona *et al.*, 1985). The region of highest homology (68%) with huma calcitonin mRNA encompasses residues 156–320, which cod for the last 16 amino acids of the N-terminal peptide, the protec lytic cleavage site, the calcitonin molecule and the proteolyti and amidation C-terminal cleavage site. The important differenc in amino acid sequence found between chicken and human calci tonin (53% substitution in amino acids) is also found in the amino terminal (48%) and carboxy-terminal (68%) regions of the mol ecule. The probable signal peptide cleavage site: Ala-Ala-Pro the first proteolytic site: Lys-Arg and the proteolytic amidatio sequence: Gly-Lys-Lys-Arg are completely identical.

Hybridization of human and murine calcitonin mRNA to chicke cDNA calcitonin probe

Results of hybridization experiments using nick-translated probe from pCCT22 to poly(A)-rich RNA extracted from human o murine medullary carcinoma of the thyroid are shown in Figur 2. Hybridization of the probe was specific; a positive signal wa still detected when the filters were washed at high stringency (0. SSC, temperature ranging from 56° to 77°C). No hybridizatio of the chicken cDNA probe to cDNA specific for the huma messenger, also immobilized on GeneScreen, was observed.



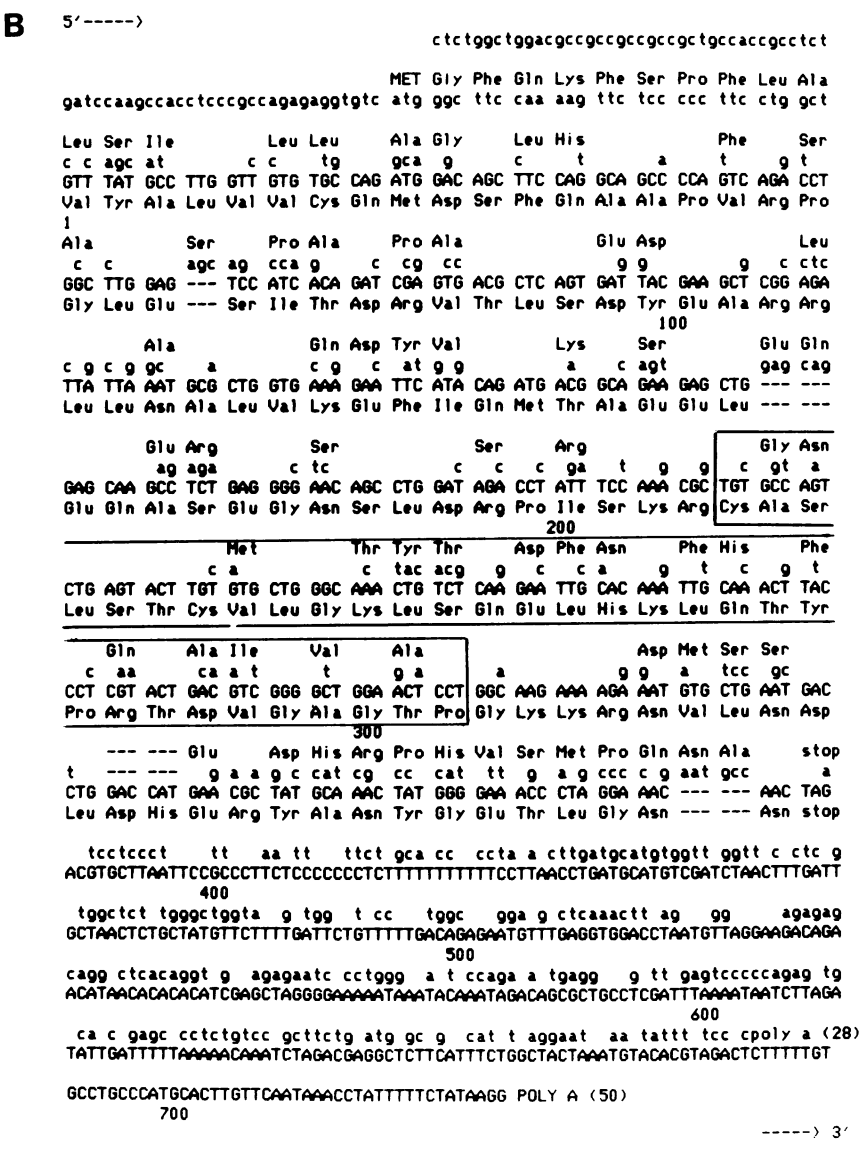


Fig. 1. **(A)** Restriction map of insert from pCCT22. Restriction sites used to generate DNA fragments for sequencing and subcloned in the corresponding sites of phage M13 (mp10, mp11) are shown in the upper panel. **(B)** Nucleotide sequence of chicken calcitonin (CCT) mRNA derived from pCCT22. The figure compares the known nucleotide sequence of human calcitonin (HCT) mRNA with gaps (---) introduced to maximize homology. The nucleotide sequence of chicken calcitonin is shown in capital letters (amino acid sequence below) and of human calcitonin in lower case letters. Blanks in the sequence of the human molecule indicate the same nucleotide base or amino acid as in chicken. Numbers below the chicken nucleotide sequence denote the relative number of bases from the 5' end.

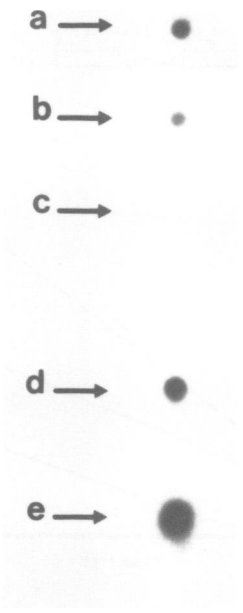


Fig. 2. Autoradiography of different poly(A)-rich RNA dotted on GeneScreen after hybridization with a ^{32}P-labelled chicken probe: the *Pst*I insert of plasmid pCCT22 was labelled with [α-^{32}P]dGTP and dCTP using DNA polymerase I (BRL) in the presence of excess cold dATP and dTTP. Hybridization was performed for 16 h at 42°C and the filter washed at 72°C. **(a)** 5 μg poly(A)-rich RNA extracted from medullary carcinoma of the thyroid. **(b)** 5 μg poly(A)-rich RNA extracted from murine tumour. **(c)** 0.1 μg of cDNA to human calcitonin mRNA. **(d)** 0.1 ng of cDNA to chicken calcitonin mRNA. **(e)** 1 ng of cDNA to chicken calcitonin mRNA.

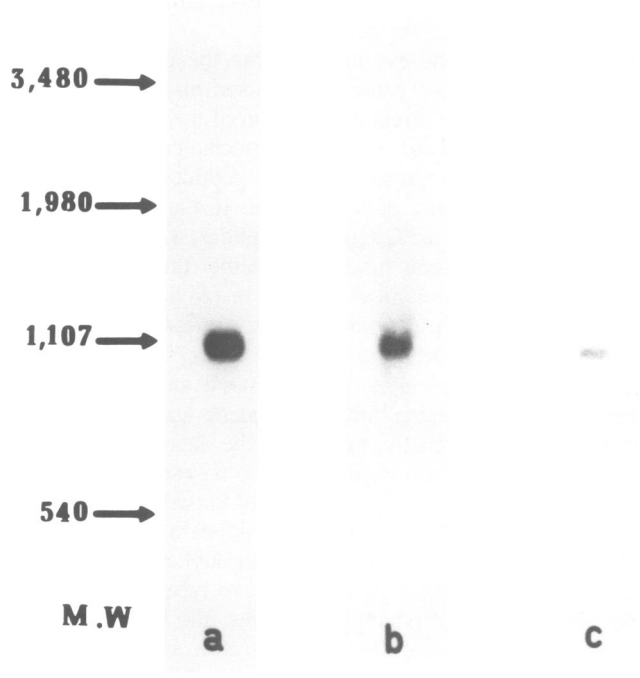


Fig. 3. Northern analysis of poly(A)-rich RNA extracted from medullary carcinoma tissues. 10 μg aliquots were denatured with glyoxal, electrophoresed on 1% (w/v) agarose gel and transferred to GeneScreen. Hybridization was performed using human or chicken calcitonin probes labelled as in Figure 2. **(a)** *Pst*I/*Sac*I restriction fragment of cDNA for human calcitonin. The membrane was washed at 67°C. **(b)** *Pst*I/*Xba*I restriction fragment of cDNA for chicken calcitonin. The membrane was washed at 57°C. **(c)** Same as in **(b)** but washed at 67°C. Autoradiographs for **(a)** were exposed for 1 day at −80°C and for **(b)** and **(c)** 7 days at −80°C.

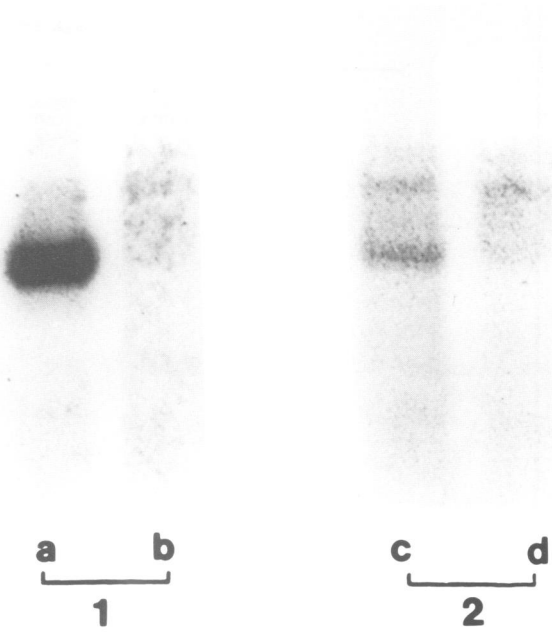


Fig. 4. SDS-polyacrylamide gel analysis of immunoprecipitated polypeptides synthesized in cell-free system (rabbit reticulocyte lysate) by poly(A)-rich RNA, extracted from a case of medullary carcinoma. Immunoprecipitation was carried out with anti-human calcitonin antibodies **(1)** or anti-salmon calcitonin antibodies **(2)**. **(a)** Anti-human calcitonin antibody. **(b)** Anti-human calcitonin antibody saturated with 5 μg of synthetic human calcitonin. **(c)** Anti-salmon calcitonin antibody. **(d)** Anti-salmon calcitonin antibody saturated with 5 μg of synthetic salmon calcitonin.

When poly(A)-rich RNA extracted from medullary carcinoma tissues was subjected to a Northern blot and probed with labelled human or chicken cDNA for calcitonin mRNA, an mRNA species co-migrating with human calcitonin mRNA, hybridized to the chicken probe. The concentration of this species of mRNA was ~500-fold less than the mRNA species hybridizing to the human probe (Figure 3).

Identification of a lower calcitonin-like translation product

Messenger RNA extracted from a case of human medullary carcinoma directed the synthesis of a radioactive protein precipitated by antibodies against synthetic salmon calcitonin (Figure 4). In the presence of excess salmon calcitonin no such protein was detected in autoradiographs of the polyacrylamide gel. No displacement of ^{125}I-labelled salmon calcitonin by an excess of human calcitonin was observed in a radioimmunoassay using the same anti-salmon calcitonin antibody (Figure 5).

Evolution of calcitonin precursors

Figure 6 shows the percentage of divergence in replacement sites for the N-terminal, calcitonin and C-terminal peptides. The percentage of divergence of the flanking peptides increases with a comparable linear rate down to birds. The divergence rate of the calcitonin molecule is very low down to rodents and then increases at a rate comparable with the flanking peptides down to birds.

Discussion

The presence of a second calcitonin gene in rat (Perez Cano *et al.*, 1982) and man (Fisher *et al.*, 1983; Tobler *et al.*, 1984) encoding a salmon-like calcitonin molecule is suggested by immunochemical data. The direct demonstration of the existence of transcripts of salmon-like genes has been hampered by the lack

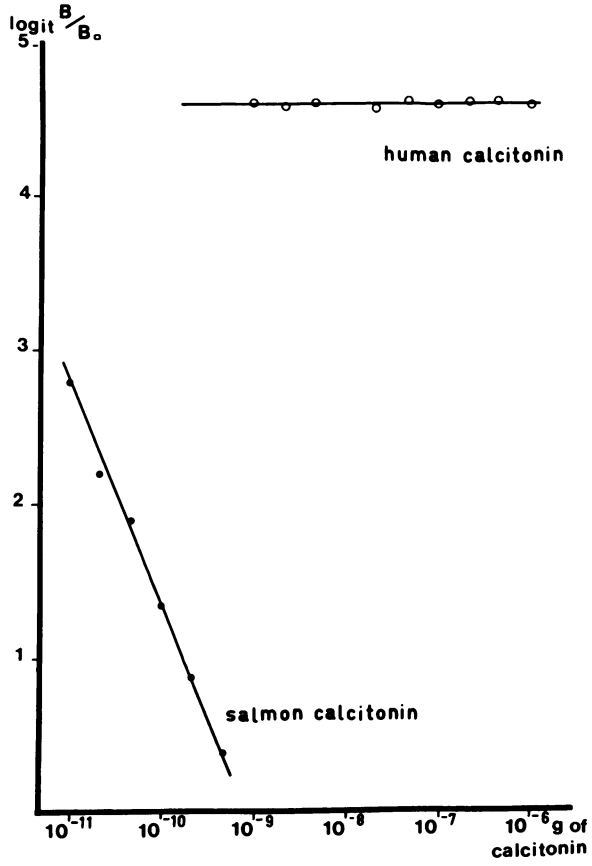


Fig. 5. Displacement of [^{125}I]salmon calcitonin bound to anti-salmon calcitonin antibodies used in the immunoprecipitation studies (—●—) by salmon calcitonin or (—○—) by human calcitonin. Curves were linearized by using logit of B/Bo = log [1/(1−B/Bo)] function of log calcitonin, where B = counts bound to antibody in the presence of × dose of unlabelled calcitonin, Bo = counts bound in the absence of unlabelled calcitonin.

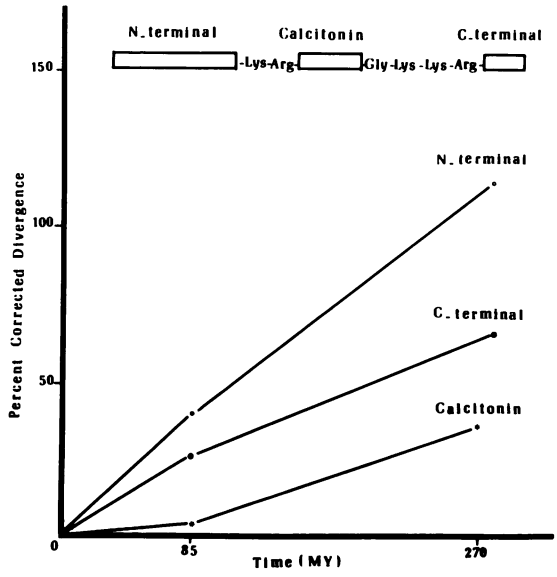


Fig. 6. Plot of the sequence divergence of replacement substitutions against the divergence time of the three peptides present in the calcitonin precursor. The chicken/mammal divergence occurring some 270 × 10^6 and rodent/mammal 85 × 10^6 years ago.

of suitable probes. We report here what is, to our knowledge, the first sequence of a lower vertebrate calcitonin precursor. This precursor shows the same organisation as human or murine precursors: an amino-terminal peptide preceding calcitonin and a carboxy-terminal peptide following the calcitonin molecule. This lower vertebrate calcitonin mRNA codes for a calcitonin molecule (Lasmoles *et al.*, 1985) which shows a high amino acid sequence homology with salmon (84%) and eel (94%) calcitonins. The important difference in amino acid sequence found between chicken and human calcitonin (53% substitution in amino acids) is also found in the amino-terminal (48%) and carboxy-terminal (68%) regions of the molecule. The most conserved amino acid sequences are those involved in the post-translational modifications of the precursor, i.e., signal peptide, proteolytic cleavage and amidation sites.

The hybridization of the cDNA probe specific for chicken calcitonin with mRNAs extracted from a case of medullary carcinoma of the thyroid or from murine thyroid indicates that an mRNA species, showing a high homology with chicken calcitonin mRNA and having the same size, is expressed in these tissues. Furthermore, translation studies confirm that the mRNA species detected by the chicken calcitonin probe directed in an *in vitro* system the synthesis of a protein specifically immunoprecipitated by antibodies against salmon calcitonin. The apparent mol. wt. of this molecule is similar to human (Goodman *et al.*, 1979; Desplan *et al.*, 1980), murine (Jacobs *et al.*, 1979; Desplan *et al.*, 1980) and avian (Lasmoles *et al.*, 1983) calcitonin precursors. Our results thus demonstrate that the immunoreactive salmon-type molecules reported in murine thyroid or in cases of human medullary carcinoma are probably the result of the persistence in mammals of a calcitonin gene which is transcribed into biologically active mRNA, albeit at a much lower level than the gene coding for the mammalian hormone.

An analysis of gene evolution during the 250−300 × 10^6 years in which the divergence of birds and mammal took place can be undertaken by direct comparison of the sequence of this mRNA with mRNAs for human and murine calcitonin precursors. The divergence rates of the three peptides comprising the calcitonin precursor are quite different. In the case of the N-, and to a lesser extent the C-terminal peptides, a particular amino acid is not vital for their function. Whether these two flanking peptides have a physiological function or play a role in conferring to the calcitonin precursor a proper configuration during its post-translational processing remains to be established. The divergence rate of the calcitonin molecule is much more limited in the time span separating birds from rodents and then is severely constrained. These results, as well as the data from the amino acid sequence of the seven calcitonins so far established, favour the hypothesis of a strong conservation of the calcitonin sequence during evolution up to the time of the appearance of the mammalia or perhaps of the reptiles; thereafter duplication of this gene and sequence divergence gave rise to two types of mammalian genes: human and artiodactyl. However, the lower vertebrate ancestral gene, or a slightly divergent molecule, was retained in the human and murine genomes and is still transcribed.

Our results, and the immunological studies so far reported (Perez Cano *et al.*, 1982; Fischer *et al.*, 1983; Tobler *et al.*, 1984), raise several important questions. Is the lower vertebrate-type calcitonin biologically active and, if so, what is its physiological role? Do cases of medullary carcinoma of the thyroid, in which the gene coding for the human molecule is transcribed at a low rate while the gene coding for the lower vertebrate type is actively transcribed, exist? Such cases would not be diagnosed as most radioimmunoassays for human calcitonin do not cross-

react with salmon calcitonin and therefore would not detect the pathological increases of salmon-type calcitonin.

The availability of a probe specific for a lower vertebrate calcitonin will certainly be useful in isolating the calcitonin gene of a lower vertebrate, in checking if exons coding for CGRP-like molecules exist in this gene and in studying the evolution of the calcitonin gene. This probe will also be useful in isolating and sequencing the human gene which transcribes an mRNA showing a high degree of homology with chicken calcitonin mRNA.

Materials and methods

Construction and screening of chicken ultimobranchial cDNA library

The isolation of chicken ultimobranchial mRNA and the construction and screening of the cDNA library have previously been reported (Lasmoles *et al.*, 1985). Forty clones from this library were further screened using a 141-bp ^{32}P-labelled cDNA to chicken calcitonin mRNA (Lasmoles *et al.*, 1985).

Isolation of plasmid DNA

DNA of selected plasmids was prepared from bacterial cells by SDS extraction, purified by centrifugation on a CsCl gradient and cDNA inserts recovered by digestion with the restriction enzyme *Pst*I.

DNA sequence analysis

An insert (~800 bp) from a clone (pCCT22) showing maximal hybridization with the ^{32}P-labelled cDNA calcitonin probe was subjected to restriction map analysis. Fragments of this insert obtained by digestion with *Sau*3A, *Xba*I and *Eco*RI were subcloned in the corresponding restriction sites of single-stranded DNA cloning vectors M13 mp10 and mp11 (Messing *et al.*, 1981). Both DNA strands were sequenced using the dideoxy method (Sanger *et al.*, 1977).

Hybridization and translation studies

mRNA, from human or murine medullary carcinoma of the thyroid, was extracted with phenol chloriform and subjected to either dot hybridization or Northern analysis.

Aliquots of poly(A) RNA (2 μg) were denatured by glyoxal (Thomas, 1980) and spotted on GeneScreen membranes (New England Nuclear) previously washed and equilibrated with 20 × SSC. The membranes were dried and baked for 2 h at 80°C. Pre-hybridization (4 h at 42°C) was carried out in 50% formamide, 5 × SSC, 5 × Denhardt (1 × Denhardt = 0.02% bovine serum albumin, 0.02% Ficoll and 0.02% polyvinyl pyrrolidone), 50 mM sodium phosphate buffer, pH 6.5, 100 μg/ml of denatured herring sperm DNA and 1 μg/ml of poly(A) (PL Biochemicals). Membranes were hybridized for 18 h at 42°C in a modified hybridization buffer (i.e., 1 × Denhardt, 20 mM sodium phosphate buffer) containing 0.3 μCi of nick-translated (Rigby *et al.*, 1977) [^{32}P]cDNA denatured by heating. The membranes were washed three times in 2 × SSC, 0.1% SDS for 5 min at room temperature, followed by two washes in 0.1% SSC, 0.1% SDS for 30 min at temperatures varying between 57° and 72°C. Autoradiographs of the dried membranes were exposed at −80°C to Royal X-OMAT AR5 film (Kodak).

Glyoxal-denatured RNA was electrophoresed on 1% agarose and RNA fragments transferred to GeneScreen (Thomas, 1980) and pre-hybridization and hybridization carried out as above, with the exception that second and third washes were carried out at 57° or 67°C.

Aliquots of 2 μg of poly(A)-rich RNA extracted from the medullary carcinoma tissue were dissolved in 2 μl of bi-distilled water and added to 20 μl of rabbit reticulocyte lysate (Amersham) containing 50 μCi of [^{35}S]methionine (Amersham). Incubation was carried out for 60 min at 30°C. Radioactivity incorporated into synthesized proteins was estimated by tricholoroacetic acid precipitation. Aliquots of translation products were incubated for 24 h at 4°C with sheep anti-human or anti-salmon calcitonin antisera (10 μl of a 1/15 dilution) in the presence or absence of 5 μg of synthetic human or salmon calcitonin, respectively, and further incubated with anti-sheep anti-globulin antiserum. Immunoprecipitates were collected by centrifugation over a 1 M sucrose cushion, washed with phosphate buffer (0.1 M phosphate buffer pH 7.4, 0.2% heat-denatured human albumin, 0.1% L-methionine and 0.25% Triton X-100) dissolved and heat denatured in 25 μl of sample buffer (60 mM Tris-HCl, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, 0.4% bromophenol blue). Aliquots were counted using beta spectrometry. Specificity of the immunoprecipitates was checked by electrophoresis on 0.1% SDS polyacrylamide gel (20%) (Laemmli, 1970).

Computer analysis

The rate of divergence (Perler *et al.*, 1980) was estimated by computer programs developed by Dr C.Mugnier at C.I.T.I. CITI 2.

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