The construction and partial characterization of plasmids containing complementary DNA sequences to human calcitonin precursor polypeptide

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(1) Total poly(A)-containing RNA isolated from human thyroid medullary carcinoma tissue was shown to direct the synthesis in the wheat germ cell-free system of a major (M_r 21 000) and several minor forms of human calcitonin precursor polypeptides. Evidence for processing of these precursor(s) by the wheat germ cell-free system is also presented. (2) A small complementary DNA (cDNA) plasmid library has been constructed in the *Pst*I site of the plasmid pAT153, using total human thyroid medullary carcinoma poly(A)-containing RNA as the starting material. (3) Plasmids containing abundant cDNA sequences were selected by hybridization *in situ*, and two of these (pH-T-B3 and pH-T-B6) were characterized by hybridization–translation and restriction analysis. Each was shown to contain human calcitonin precursor polypeptide cDNA sequences. (4) RNA blotting techniques demonstrate that the human calcitonin precursor polypeptide is encoded within a mRNA containing 1000 bases. (5) The results demonstrate that human calcitonin is synthesized as a precursor polypeptide.

Calcitonin is a small peptide hormone consisting of 32 amino acid residues (M_r 3500) synthesized and secreted in mammals by the C-cells of the thyroid (Foster *et al.*, 1964). In lower vertebrates, calcitonin is synthesized in other tissues. Thus the main gland of origin is the ultimobranchial gland, the lung and the thyroid in chicken, lizard and pigeon respectively (Ravazzola *et al.*, 1981). In humans the predominant role of calcitonin is to inhibit osteoclastic destruction of the bone. Thus in abnormal situations of elevated bone turnover (Paget's disease), calcitonin is the treatment of choice (see MacIntyre *et al.*, 1980; Austin & Heath, 1981).

At the molecular level interest in the expression of the calcitonin gene centres on observations that mRNA isolated from rat and cod calcitonin-synthesizing tissues directs the synthesis in cell-free protein synthesizing systems of a calcitonin precursor polypeptide of M_r 15 000–17 000, as judged by immunoprecipitation with antiserum raised against calcitonin, and subsequent identification by SDS/polyacrylamide-gel electrophoresis (Goodman

et al., 1979; Jacobs *et al.*, 1979; Amara *et al.*, 1980a; Desplan *et al.*, 1980). Recent application of recombinant DNA techniques followed by DNA sequence analysis of cloned rat calcitonin DNA has demonstrated that post-translational processing of the calcitonin precursor polypeptide requires removal of *N*-terminal and *C*-terminal peptides (Amara *et al.*, 1980b; Jacobs *et al.*, 1981a). These cryptic peptide sequences may ultimately prove to be of biological significance, a situation similar to the bovine and human corticotropin- β -lipotropin precursors (Nakanishi *et al.*, 1979; Chang *et al.*, 1980).

Here we describe the isolation of a human poly(A)-containing RNA population from a medullary carcinoma of the thyroid, which directs the synthesis in a wheat germ cell-free protein synthesizing system of calcitonin precursor polypeptides. We also describe the construction and identification of recombinant DNA plasmids containing human calcitonin precursor cDNA sequences.

Materials and methods

Materials

Restriction enzyme *Pst*I was obtained from Boehringer and calf thymus terminal deoxynucleo-

Abbreviations used: cDNA, complementary DNA; SDS, sodium dodecyl sulphate; DBM, diazobenzoyloxymethyl-; AMV, avian myeloblastosis virus.

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tidyl transferase from P.L. Biochemicals. All other enzymes were obtained from sources described previously (Craig *et al.*, 1981). L-[³⁵S]Methionine (700–1300 Ci/mmol), deoxy[5-³H]cytidine 5'-triphosphate (18.4 Ci/mmol), deoxy[8-³H]guanosine 5'-triphosphate (11.7 Ci/mmol), adenosine 5'-[γ -³²P]triphosphate (2000–3000 Ci/mmol) and deoxyguanosine 5'-[α -³²P]triphosphate (≥ 400 Ci/mmol) were from Amersham International; AMV reverse transcriptase (lot no. G-91180) was provided by Dr. J. W. Beard, Life Sciences Inc., St. Petersburg, FL 33707, U.S.A. All other chemicals and solvents were obtained from sources previously described (Craig *et al.*, 1976, 1979; Pascall *et al.*, 1981).

Isolation of poly(A)-containing RNA from human medullary carcinoma of the thyroid: the construction, transformation and selection of recombinant plasmids

Total RNA was isolated from frozen human thyroid medullary carcinoma tissue as described by Hall *et al.* (1979). The total RNA was then digested with deoxyribonuclease and the poly(A)-containing RNA isolated by affinity chromatography on oligo-(dT)-cellulose, and a size-selected double-stranded cDNA population was then synthesized from the resulting poly(A)-containing RNA population as described previously (Craig *et al.*, 1981; Hall *et al.*, 1981). The size-selected double-stranded cDNA was then extended at the 3'-hydroxyl termini with dC residues in the following manner. Double-stranded cDNA (1 μ g/ml) was dissolved in 30 mM-Tris/100 mM-cacodylic acid (adjusted to pH 7.5 with 10 M-KOH) containing 2 mM-CoCl₂, 0.1 mM-dithiothreitol, 50 μ g of nuclease-free bovine serum albumin/ml, and 0.1 mM-[³H]dCTP (4 Ci/mmol). Terminal deoxynucleotidyl transferase (300 units/ml) was then added and the mixture incubated at 30°C. When a 30–50 residue long poly(dC) tail had been added (approx. 15 min as determined by trichloroacetic acid precipitation of 1 μ l amounts at 5 min intervals), the reaction was terminated by extraction with phenol/chloroform, and the nucleic acid was recovered by ethanol precipitation. The precipitate was washed twice with ethanol, dissolved in double-distilled water and stored at –70°C.

Highly purified supercoiled pAT153 DNA prepared as described previously (Craig *et al.*, 1981) was digested with the restriction endonuclease *Pst*I, then tailed with poly(dG)₁₂ essentially as described above, except that the DNA was present at 50 μ g/ml, [³H]dGTP (1.7 Ci/mmol) replaced dCTP as the radiolabelled deoxynucleoside triphosphate, and terminal deoxynucleotidyl transferase was present at a final concentration of 800 units/ml. Poly(dC)-tailed cDNA and poly(dG)-tailed pAT153

DNA were then annealed in 10 mM-Tris/HCl, pH 7.6, containing 200 mM-NaCl and 1 mM-EDTA, in polypropylene 0.4 ml 'snap-cap' tubes at a final concentration of 0.4 μ g/ml and 4 μ g/ml respectively. The mixture was heated in a water bath to 70°C for 30 min, the water bath switched off, and the hybridization mixture allowed to cool to room temperature overnight. The amount of poly(dC)-tailed cDNA used for transformations varied from 10 to 20 ng.

The resulting chimaeric plasmid DNA was then used to transform *Escherichia coli* HB101 rec A[–] (Boyer & Roulland-Dussoix, 1969), under conditions of good microbiological practice in accordance with the guidelines laid down by the British Genetic Manipulation Advisory Group, using methodology described elsewhere (Craig *et al.*, 1981). Transformed cells were selected on 1.5% (w/v) agar in L-broth containing 12.5 μ g of tetracycline/ml. Individual colonies were then replica plated onto fresh agar plates containing 12.5 μ g of tetracycline/ml alone or 100 μ g of ampicillin/ml alone. Those colonies found to be sensitive to ampicillin were selected for subsequent experimentation.

Identification of plasmids containing human calcitonin precursor polypeptide cDNA sequences, and characterization of calcitonin precursor polypeptide mRNA

Colony filter hybridization *in situ*, plasmid growth and purification, positive hybridization–translation, purification of individual cDNA sequences, and size estimation of individual mRNA sequences using the 'Northern' transfer technique have been described elsewhere (see Craig *et al.*, 1981; Burditt *et al.*, 1981; Hall *et al.*, 1981). Nick-translation of plasmids was as described by Rigby *et al.* (1977). Unless specifically stated otherwise all restriction enzyme analyses were performed using the ionic conditions recommended by the manufacturers. Size analysis of restricted plasmid DNA was performed by electrophoresis on flat-bed agarose gels as described elsewhere (Craig *et al.*, 1981).

Cell-free protein synthesis and product analysis

mRNA-directed cell-free protein synthesis in the wheat-germ cell-free protein synthesizing system and antibody precipitation procedures were as described by Craig *et al.*, (1976). SDS/polyacrylamide-gel electrophoretic analysis was performed using slab gels as described by Pascall *et al.* (1981). Fluorography followed the procedure of Bonner & Laskey (1974) except that dimethylsulphoxide was replaced by glacial acetic acid (Burckhardt *et al.*, 1979).

Results

Isolation and characterization of a poly(A)-containing RNA population from human medullary thyroid carcinoma tissue

Total human poly(A)-containing RNA isolated from a medullary carcinoma of the thyroid was added to a wheat-germ cell-free protein synthesizing system, and the resulting [^{35}S]methionine-labelled proteins were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. The results (Fig. 1a) demonstrate that a spectrum of proteins up to M_r 60 000 was synthesized. The most prominent of these, a protein of estimated M_r 21 000, proved to be precipitable with antiserum raised against human calcitonin. In addition to this protein, three less abundant peptides of estimated M_r 19 500, 18 000 and 15 000 were also precipitated by calcitonin-specific antiserum. It is possible that these represent partially processed calcitonin precursor polypeptides, a result of post-translational processing activi-

ties present in the wheat-germ cell-free extract (see Pascall *et al.*, 1981). This processing was particularly apparent at Mg^{2+} concentrations which were suboptimal for protein synthesis. Thus at 1.5 mM- Mg^{2+} the predominant polypeptide was the M_r 18 000 calcitonin precursor polypeptide form whilst at 2.5 mM- Mg^{2+} , the optimum for protein synthesis, the M_r 21 000 calcitonin precursor polypeptide form predominated (see Fig. 1b). Immaterial of the Mg^{2+} concentration, the predominant polypeptide remained precipitable with antiserum raised against human calcitonin (Fig. 1c).

Overall, on the basis of the source of tissue and the identification of polypeptides immunoprecipitable with human calcitonin antisera as the predominant products of mRNA-directed cell-free protein synthesis, it seems reasonable to conclude that an abundant poly(A)-containing RNA species present in human thyroid medullary carcinoma tissue directs the synthesis of calcitonin precursor polypeptide(s).

Fig. 1. mRNA-directed cell-free protein synthesis of human calcitonin precursor polypeptides as judged by immunoprecipitation and SDS/polyacrylamide-gel electrophoresis

Total poly(A)-containing RNA (0.2 μg /assay) isolated from human thyroid medullary carcinoma tissue (4 g) was added to a wheat-germ cell-free protein-synthesizing system and the resulting [^{35}S]methionine-labelled proteins were separated by using SDS/polyacrylamide-gel electrophoresis, then visualized using fluorography (see the Materials and methods section). (a) Lane (i), no added mRNA; lane (ii), human thyroid medullary carcinoma poly(A)-containing RNA; lane (iii), as in lane (ii) but proteins were precipitated using antiserum raised against synthetic human calcitonin. (b) Lanes (i), (iii), (v) and (vii), human thyroid medullary carcinoma poly(A)-containing RNA translated at Mg^{2+} concns. of 1.5, 2.0, 2.25 and 2.5 mM respectively. Lanes (ii), (iv), (vi) and (viii), no added RNA at Mg^{2+} concns. of 1.5, 2.0, 2.25 and 2.5 mM respectively. (c) Lanes (i) and (iii), human thyroid medullary carcinoma poly(A)-containing RNA translated at Mg^{2+} concns. of 1.5 and 2.5 mM respectively; lanes (ii) and (iv), as in lanes (i) and (iii) respectively, but proteins precipitated using antiserum raised against synthetic human calcitonin. Arrows represent the relative mobility of the following Coomassie Blue stained marker proteins of known M_r (see Weber *et al.*, 1972): glyceraldehyde-3-phosphate dehydrogenase (36 000); trypsin (23 300); lysozyme (14 300); and cytochrome c (11 700).

Construction of recombinant cDNA plasmids using total poly(A)-containing RNA isolated from a human medullary carcinoma of the thyroid

All manipulations followed the procedures outlined for the construction of recombinant plasmids containing human and guinea-pig milk protein cDNA sequences (see Craig *et al.*, 1981; Hall *et al.*, 1981) except where specifically stated in the Methods section.

Briefly, cDNA representative of the total poly(A)-containing RNA population isolated from the human medullary carcinoma of the thyroid was synthesized by using AMV reverse transcriptase. Size analysis using agarose-gel electrophoresis showed that the bulk of the cDNA synthesized ranged in size from 500 to 1500 nucleotides in length. This was converted to a double-stranded form using AMV reverse transcriptase, followed by S_1 nuclease excision of the resultant hairpin loop. This population was then sized by preparative

agarose-gel electrophoresis and those sequences ranging from 300 to 2500 nucleotides in length were eluted from the gel, and their 3'-hydroxyl termini extended with poly(dC)₅₀ using calf thymus terminal deoxynucleotidyl transferase. This population was then annealed with *Pst*I-restricted pAT153 DNA which had previously been extended at the 3'-hydroxyl termini with poly(dG)₁₂. The resulting chimaeric molecules were used to transform a rec A⁻ strain of *E. coli* HB101 under conditions of good microbiological practice. Transformants were selected on L-agar plates containing tetracycline, and colonies containing recombinant plasmids were then selected by replica plating onto L-agar containing ampicillin alone, or tetracycline alone. In total a single transformation using 20 ng of double-stranded tailed cDNA yielded 107 colonies that had lost sensitivity to ampicillin, and therefore were presumed to contain cDNA sequences inserted at the *Pst*I site of pAT153.

Fig. 2. Characterization of plasmids containing human calcitonin precursor polypeptide cDNA sequences
 (a) Positive hybridization translation. Sequence-specific poly(A)-containing RNA isolated by hybridization to partially restricted, denatured recombinant DNA immobilized on DBM-paper was added to a wheat-germ cell-free system, and the [³⁵S]methionine-labelled cell-free translation products were separated by SDS/polyacrylamide-gel electrophoresis, then visualized by fluorography. Lane (i), no added RNA; lane (ii), total human thyroid medullary carcinoma poly(A)-containing RNA; lanes (iii), (iv) and (v), poly(A)-containing RNA isolated by hybridization to plasmids pAT153, pH-T-B3 and pH-T-B6 respectively. Arrows denote the relative position of marker proteins as described in the legend to Fig. 1. (b) Size analysis of inserted cDNA sequences. Plasmid DNA samples (0.25 µg) were digested with restriction endonucleases, then electrophoresed on a 1.6% (w/v) agarose gel as described previously (see Hall *et al.*, 1981). DNA fragments of known size (see Sutcliffe, 1978a,b) were generated by *Alu*I digestion of pBR322 DNA and *Hae*III digestion of pAT153 DNA. Lane (i), *Alu*I markers of 910, 659, 655, 521, 403, 281, 257, and 226 base pairs; lane (ii), pAT153 DNA digested with *Pst*I; lane (iii), pH-T-B3 DNA digested with *Pst*I; lane (iv), pH-T-B6 DNA digested with *Pst*I; lane (v), *Hae*III markers of 587, 458, 434, 339, 267, 234 and 213 base pairs. (c) Comparative restriction endonuclease maps of the inserted cDNA sequence within recombinant plasmids pH-T-B3 and pH-T-B6.

Identification and characterization of recombinant plasmids containing human calcitonin cDNA sequences

It was apparent from cell-free protein synthesizing studies that the human medullary carcinoma of the thyroid poly(A)-containing RNA population contained an abundant mRNA population which directed the synthesis of calcitonin precursor polypeptide(s). Consequently total base-cleaved ^{32}P -labelled poly(A)-containing RNA from human medullary carcinoma was used to identify, in a preliminary manner using *in situ* hybridization procedures, those colonies most likely to contain calcitonin precursor polypeptide cDNA sequences (see the Methods section). All the ampicillin-sensitive colonies were screened in this manner, and 17 (16%) showed significant hybridization over the background. Of these, 11 were selected and grown in liquid culture, the plasmid DNA was isolated, and the presence of additional DNA sequence determined by digestion with the restriction endonuclease *Pst*I, followed by agarose-gel electrophoresis. This confirmed that all but one contained additional DNA as compared with the parental plasmid pAT153 (results not shown). Although all these plasmids have now been extensively characterized, we describe below the detailed characterization of two plasmids, pH-T-B3 and pH-T-B6.

Identification of the coding sequences present in each plasmid was carried out by SDS/polyacrylamide-gel electrophoresis of the wheat-germ cell-free translation products of mRNA isolated by hybridization under stringent conditions to partially restricted denatured plasmid DNA immobilized on DBM-paper filters (see Craig *et al.*, 1981). This demonstrated that both plasmids contained DNA sequences complementary to the medullary thyroid carcinoma mRNA species which directed the synthesis of the abundant calcitonin precursor protein (Fig. 2a). In each instance, although cell-free protein synthesis was performed in the presence of 2.5 mM- Mg^{2+} , both the M_r 21000 and M_r 18000 calcitonin precursor polypeptide forms were present. Size analysis of the inserted cDNA sequences by agarose-gel electrophoresis after excision of the cDNA by digestion with the restriction endonuclease *Pst*I, showed (Fig. 2b) that pH-T-B3 and pH-T-B6 contained 590 and 490 base pairs of inserted sequence. Subsequent mapping of these sequences using a variety of restriction endonucleases (Fig. 2c) showed that both recombinant plasmids contained cDNA sequence in common, but that pH-T-B3 contained an additional sequence at one end of the common fragment, and pH-T-B6 an additional sequence at the other.

To determine the proportion of the calcitonin precursor polypeptide mRNA sequence within the

Fig. 3. Size estimation of human calcitonin precursor polypeptide mRNA

RNA samples were treated with glyoxal, then loaded onto separate slots on a 1.5% (w/v) agarose gel, electrophoresed and then blotted onto DBM-paper as described previously (Craig *et al.*, 1981). The position of the human calcitonin precursor polypeptide mRNA sequence was determined by hybridization to a ^{32}P -labelled nick-translated pH-T-B3 plasmid DNA hybridization probe (5.6×10^7 c.p.m./ μg). Lane (i), T-47D poly(A)-containing RNA ($1\mu\text{g}$); lane (ii), human placental poly(A)-containing RNA ($1\mu\text{g}$); lane (iii), total human thyroid medullary carcinoma RNA ($40\mu\text{g}$); lane (iv), non-polyadenylated human thyroid medullary carcinoma RNA ($40\mu\text{g}$); lane (v), human thyroid medullary carcinoma total poly(A)-containing RNA ($1\mu\text{g}$). Size estimations were determined by direct comparison with the mobilities of glyoxal-treated linear pBR322 DNA (4362 bases), rabbit reticulocyte 18S RNA (2060 bases), a *Bam*HI/*Pst*I digest of pBR322 DNA (3237 and 1125 bases), and *E. coli* tRNA (80 bases).

characterized recombinants, the size of the mRNA was estimated using RNA blotting techniques (Alwine *et al.*, 1977) after separation of glyoxal-treated poly(A)-containing RNA (McMaster & Carmichael, 1977) by horizontal agarose-gel electrophoresis (Fig. 3). This demonstrated that the human calcitonin precursor polypeptide mRNA was 1000 ± 100 nucleotides in length. Thus the cDNA sequence inserted into pH-T-B3 and pH-T-B6 represented in total 60–65% of the mRNA sequence. A parallel analysis of the non-polyadenylated human thyroid medullary carcinoma RNA also revealed the presence of calcitonin precursor polypeptide mRNA of identical length, but also considerable low- M_r

hybridizable material, presumably partially degraded mRNA. Calcitonin precursor polypeptide mRNA was not present in detectable amounts, under the conditions employed, in total poly(A)-containing RNA isolated from full-term human placental tissue, or from T-47D cells, a human cell line of mammary origin (see Fig. 3).

Discussion

We have described the mRNA-directed synthesis of several high-molecular-weight forms of human calcitonin in the wheat-germ cell-free protein synthesizing system, as judged by immunoprecipitation using a well characterized, highly specific antiserum 336/6 raised against uncoupled human synthetic calcitonin M (see Girgis *et al.*, 1980). Molecular weight estimations of the two predominant forms (M_r 21000 and M_r 18000) may reflect a precursor-product relationship, since the difference of 3000 in M_r is consistent with the removal of the N-terminal signal peptide, known to be present in the primary translation product of most characterized proteins destined for secretion (Blobel & Dobberstein, 1975a,b). Although we have not added microsomal membranes to the wheat-germ cell-free system, it is probable that the M_r 18000 form reflects peptide processing *in vitro*, since we have recently described the ability of the wheat-germ and reticulocyte cell-free systems to perform limited processing of guinea-pig milk protein primary translation products (Pascall *et al.*, 1981). Consequently, under ionic conditions *in vitro* that do not favour protein synthesis, and may favour signal peptidase-like activity, processing appears to go to completion. Recent detailed analyses of a rat mRNA-directed calcitonin precursor polypeptide, synthesized in the presence and absence of microsomal membranes are consistent with this view (Jacobs *et al.*, 1981b).

Two groups have previously described the mRNA-directed synthesis of a human calcitonin precursor polypeptide, as judged by the criteria of immunoprecipitation and SDS/polyacrylamide-gel electrophoresis (Goodman *et al.*, 1979; Desplan *et al.*, 1980). Each used an mRNA preparation isolated from a human medullary carcinoma of the thyroid, and described a primary translation product of M_r 15000. In one instance the processing of the primary translation product to one of M_r 12000 by added microsomal membranes was described (Goodman *et al.*, 1979). Such disparity between our own M_r estimations and those of others may simply reflect differing mobility of the primary/processed translation products due to variations in the conditions used for SDS/polyacrylamide-gel electrophoresis. Alternatively the differences may prove to be real, and reflect polymorphism at the level of the

genome, or, as has been described in the rat thyroid medullary carcinomas, polymorphism at the level of nuclear RNA processing (Rosenfeld *et al.*, 1981).

The concept of polymorphism deserves consideration on two counts. Firstly we have yet to explain the presence of the minor immunoprecipitable calcitonin precursor polypeptide forms of M_r 19500 and M_r 15000, one of which, the M_r 19500 form, is clearly discernible after hybridization-translation analysis (Fig. 2a). This may reflect yet another processed form of the calcitonin precursor polypeptide generated by the wheat-germ cell-free system, or alternatively since it is always a minor band, reflect the translation of an additional less abundant calcitonin precursor polypeptide mRNA species. Secondly, the tissue under examination was obtained from a patient with a significant family history of thyroid cancer. Although an analysis of the immunoprecipitable forms of calcitonin present in this tissue has yet to be performed, it has been established by others that high- M_r forms of human calcitonin, in addition to fully processed calcitonin, are synthesized and secreted by human medullary carcinoma cells in culture, whilst only high- M_r forms are secreted by a human bronchial carcinoma cell-line (Ham *et al.*, 1980; Lumsden *et al.*, 1980). The former observation is consistent with an explanation based on polymorphisms, whilst the latter might be explained by the absence of specific processing enzymes at some point in the secretory pathway.

Immaterial of the diversity of high- M_r forms of calcitonin, it is apparent that calcitonin is synthesized as a precursor polypeptide which requires extensive post-translational proteolytic cleavage. DNA sequence analysis of rat cDNA clones (Amara *et al.*, 1980b; Jacobs *et al.*, 1981a) has demonstrated that the calcitonin peptide is located within a large precursor polypeptide flanked by basic amino acid residues, a situation analogous to the prohormone cleavage sites identified by DNA sequence analysis of the bovine corticotropin- β -lipotropin precursor (Nakanishi *et al.*, 1979). In this paper we describe the construction of recombinant plasmids containing cDNA sequences to human calcitonin precursor polypeptide(s). Out of the 107 recombinant plasmids generated, nine have now been shown to contain calcitonin precursor polypeptide cDNA sequences, suggestive that the mRNA may represent as much as 10% of the total poly(A)-containing RNA (J. Allison & R. K. Craig, unpublished results). Such abundance, which again is reflected at the level of cell-free protein synthesis, accounts for the pre-operative serum calcitonin levels of the patient in question, which were grossly elevated (800-fold) when compared with normal levels. Restriction analysis of two of these recombinants (pHT-B3 and pHT-B6) demonstrates that

we have successfully cloned over 60% of the human calcitonin precursor polypeptide mRNA, when compared with the estimated size of the mRNA (1000 nucleotides). Nucleotide sequence analysis of the cDNA fragments contained within pH_T-B3 and pH_T-B6 is at present in progress. Although incomplete, we have confirmed that, as in the rat, additional peptides flank the *N*- and *C*-terminal ends of the calcitonin sequence, and that the expected flanking basic amino acid residues are also present (R. K. Craig, L. Hall & M. Edbrooke, unpublished results). Thus human calcitonin joins an increasing number of small polypeptide hormones such as vasopressin and neurophysin (Brownstein *et al.*, 1980), somatostatin (Goodman *et al.*, 1980), glucagon (Patzelt *et al.*, 1979), adrenocorticotropin, melanotropin and the endorphins (Nakanishi *et al.*, 1979) which are synthesized as high-*M_r* polypeptides, subsequently cleaved by proteolytic enzymes to the mature hormone form(s), and other cryptic peptides of as yet unknown biological significance.

Complete sequence analyses of the estimated 1000 bases that we have shown to encode the human calcitonin precursor polypeptide mRNA should reveal cryptic peptide sequences of great biological interest and possible practical clinical application.

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