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Characterization of rat calcitonin mRNA

(cDNA clone/precursor protein/nucleotide sequence/gene expression)

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ABSTRACT **A chimeric plasmid containing cDNA complementary to rat calcitonin mRNA has been constructed. Partial sequence analysis shows that the insert contains a nucleotide sequence encoding the complete amino acid sequence of calcitonin. Two basic amino acids precede and three basic amino acids follow the hormone sequence, suggesting that calcitonin is generated by the proteolytic cleavage of a larger precursor in a manner analogous to that of other small polypeptide hormones. The COOH-terminal proline, known to be amidated in the secreted hormone, is followed by a glycine in the precursor. The cloned calcitonin DNA was used to characterize the expression of calcitonin mRNA. Cytoplasmic mRNAs from calcitonin-producing rat medullary thyroid carcinoma lines and from normal rat thyroid glands contain a single species, 1050 nucleotides long, which hybridizes to the cloned calcitonin cDNA. The concentration of calcitonin mRNA sequences is greater in those tumors that produce larger amounts of immunoreactive calcitonin. RNAs from other endocrine tissues, including anterior and neurointermediate lobes of rat pituitary, contain no detectable calcitonin mRNA.**

Many small polypeptide hormones are synthesized initially as larger precursors (1–13). Processing of precursor molecules involves removal of an NH_2-terminal hydrophobic leader sequence (14) and excision of secretory peptides. The excised peptides, which may or may not contain hormonal information, are generally flanked by basic dipeptides which appear to direct the proteolytic processing mechanism. In some well studied molecules, such as preopiocortin, multiple hormones are generated from the precursor by post-translational proteolytic processing (15–17). Calcitonin, a small polypeptide hormone (M_r = 3500), is synthesized in the C cells of the thyroid gland (18). Based upon cell-free translation of mRNA purified from rat thyroid tumors of C-cell origin, calcitonin appears to be a component of a protein precursor of either 15,000 (19) or 17,500 (20) daltons. The calcitonin precursor protein is significantly larger than the secretory form of calcitonin and, therefore, could contain peptides that have additional biological functions.

In this report, we describe the cloning of calcitonin cDNA and its use in the characterization of calcitonin mRNA and the structure of the precursor it encodes. In addition, this cloned cDNA was used as a probe to assay the expression of the calcitonin gene.

MATERIALS AND METHODS

Materials. Oligo(dT)-cellulose was obtained from Collaborative Research (Waltham, MA). Avian myeloblastosis virus reverse transcriptase containing minimal RNase activity was kindly provided via the National Institutes of Health Viral Distribution Program by Joseph Beard (Life Science, Inc.). *Escherichia coli* DNA polymerase I, all restriction enzymes used, and terminal transferase were obtained from Bethesda Research Laboratories (Rockville, MD). Dextran sulfate 500 was purchased from Pharmacia; [α-^{32}P]dCTP, [γ-^{32}P]ATP (2900 Ci/mmol), and [^{35}S]methionine were purchased from Amersham. Oligo(dT)$_{12-18}$ and polynucleotide kinase were obtained from P-L Biochemicals. Deoxyribonucleotide [α-^{32}P]triphosphates (>400 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) were purchased from New England Nuclear.

Cloning of Calcitonin cDNA. Total cellular poly(A)-rich RNA was prepared from a high calcitonin-producing line of rat medullary thyroid carcinoma as described (20, 21). The RNA was centrifuged through 15–30% linear sucrose density gradients containing 10 mM Tris·HCl (pH 7.4), 20 mM NaCl, and 0.1% $NaDodSO_4$. The RNA fraction centered at 11.5 S was selected and reextracted with phenol/chloroform, 1:1 (vol/vol). After serial ethanol precipitations, this poly(A)-rich mRNA was used as template for cDNA synthesis with reverse transcriptase as described (22). The cDNA was subsequently centrifuged through 5–20% linear sucrose density gradients containing 0.1 M NaOH and 0.9 M NaCl. The region of the gradient containing DNA 800–1500 nucleotides long was made double stranded with *E. coli* DNA polymerase I (23). The reaction mixtures were adjusted to 0.1% $NaDodSO_4$ and 25 mM EDTA, extracted with phenol/chloroform, and passed over a Sephadex G-200 column. The excluded portion was reprecipitated and subjected to nuclease S1 digestion in order to break the 5′ hairpin loop (23). The double-stranded DNA was extracted with phenol/chloroform and again purified by Sephadex G-200 column chromatography.

Homopolymeric extension of the 3′ termini of the DNA was accomplished with terminal deoxynucleotidyltransferase and dATP (24). Plasmid pBR322 DNA was cleaved with *Pst* I (23, 25) and homopolymeric extension was performed with dTTP. [^{3}H]dATP and [^{3}H]dTTP served as tracers to monitor the extent of reactions. Hybridization was carried out as described (26) except that samples were initially heated to 65°C for 10 min, incubated at 41°C for 2 hr, and allowed to cool slowly at 25°C.

These chimeric plasmids were used to transform *E. coli* K-12 strain SF8 as described (26) in accord with the biological and physical containment procedures approved under the National Institutes of Health Guidelines for Recombinant DNA Research. Transformants found to be tetracycline resistant were tested for ampicillin sensitivity. Bacterial clones containing cDNA inserts were identified by an *in situ* colony hybridization assay (27). Plasmids from selected colonies were amplified with 125 μg of chloramphenicol per ml and purified by the cleared lysate technique (28). Plasmid C-1 was selected for sequence analysis and use in the subsequent study on the basis of restriction digests and ability to arrest the translation of calcitonin precursor mRNA by hybridization (29).

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Terminal Labeling of the DNA Fragment. Plasmid C-1 was cleaved at a single *Bgl* II site within the insert (30). The linearized plasmid was labeled by filling the resultant 5′ overhangs with [^{32}P]deoxyribonucleotides by using reverse transcriptase. Reaction mixtures of 50 μl, containing 50 mM Tris acetate (pH 8.3), 9 mM $MgCl_2$, 2 mM dithiothreitol, 50 mM KCl, 20 μg of DNA, 40 μCi of each of four [α-^{32}P]deoxyribonucleotides, and 22 units of reverse transcriptase, were incubated for 30 min at 42°C. Two additional 15-min incubations were performed after the deoxyribonucleotide concentrations were adjusted to 2.5 μM and 100 μM; 11 units of additional enzyme were added prior to the second incubation. Mixtures were extracted with phenol followed by ether, and the aqueous phases were passed over a P-60 column in 10 mM Tris·HCl, pH 7.4/0.1 mM EDTA to separate free nucleotides. After ethanol precipitation from 0.35 M LiCl, the labeled fragment was digested with *Hpa* II and chromatographed on a 0.5-mm-thick 5% polyacrylamide gel with a 1:20 ratio of bisacrylamide to acrylamide. Labeled fragments were cut and eluted from the gel and subjected to sequencing analysis. The 5′ ends were labeled with [γ-^{32}P]ATP and polynucleotide kinase as described (31). Labeled fragments were digested with *Hpa* II, chromatographed on a 5% gel, and eluted as described above.

Determination of Nucleotide Sequence. Sequence analysis of both strands of one eluted fragment (520 base pairs) was performed as described (31). Two alternative purine cleavage reactions were substituted for the dimethylsulfate guanine-specific cleavage. The modified protocol uses pyridinium formate to cleave at adenine and guanine residues and methylene blue to cleave at guanine residues as described (32).

Analysis of RNA After Transfer to Diazobenzyloxymethyl-Cellulose. Poly(A)-rich RNA was prepared from various rat tissues that had been rapidly frozen in liquid nitrogen, and aliquots of 0.5–35 μg of RNA were denatured by reaction with glyoxal (33). After denaturation, the samples were subjected to electrophoresis on 1.5% agarose slab gels buffered with 50 mM Tris·HCl/20 mM sodium acetate/1 mM EDTA, pH 7.9. After electrophoresis, the gel was shaken gently for 20 min in 50 mM NaOH in the presence of acridine orange, photographed, then neutralized with two washes of 0.2 M sodium acetate (pH 4.3). The gel was then transferred to diazobenzyloxymethyl-paper and prehybridization washes were performed (34, 35). ^{32}P-Labeled probe was generated by "nick-translation" of the purified plasmid (C-1) to a specific activity of 1–2 × 10^8 cpm/mg (36). Labeled plasmid (2–5 × 10^5 cpm/ml) was hybridized to the blot, washed, and radioautographed.

RESULTS

mRNA purified from rat medullary thyroid tumors directs the cell-free synthesis of a 17,500-dalton putative calcitonin precursor. When this mRNA was fractionated by size on a linear sucrose density gradient, the A_{260} profile revealed a peak of RNA sedimenting at 12 S, coincident with the peak of translatable calcitonin mRNA (Fig. 1). The fractions corresponding to the 9.5–13.5S region of the gradient were pooled and used as template for synthesis of cDNA. Alkaline agarose gel analysis (37) of the single-stranded cDNAs synthesized showed a range of products with a prominent species approximately 900 nucleotides long. We have described the strategy for constructing recombinant DNA clones by insertion of the double-stranded cDNA into the *Pst* I site of the plasmid pBR322 and transformation of the weakened *E. coli* SF8 host in *Materials and Methods*.

Tetracycline-resistant colonies showing ampicillin sensitivity were subjected to *in situ* filter colony hybridization (27) by using [^{32}P]DNA complementary to the mRNA used for generating cloned material. Significant hybridization was detected in 180 of the 950 colonies analyzed. These colonies were rescreened by using DNA complementary to RNA highly enriched for calcitonin sequences (fraction 15 in Fig. 1). Twelve of the resultant positive colonies were selected for analysis on the basis of signal strength. Two of the plasmids, designated C-1 and C-3, inhibited the translation of the mRNA encoding the calcitonin precursor in a hybridization-dependent manner (data not shown).

Nucleotide sequence analysis of the C-1 insert was initiated from the *Bgl* II site. Fig. 2 shows the nucleotide sequence determined for the calcitonin-coding region. The corresponding amino acid sequence is also indicated. Amino acid residues 1–32 correspond to those of the known sequence of rat calcitonin (38), followed by glycine at residue 33. The 33 amino acids are bracketed by basic peptides. Lys-Arg precedes the calcitonin sequence and Lys-Lys-Arg follows Gly residue 33.

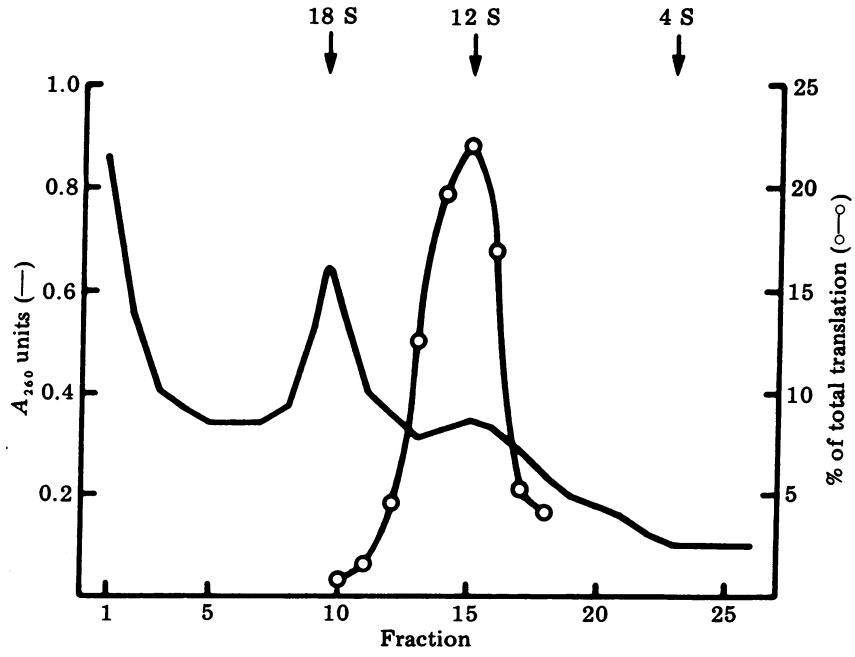

FIG. 1. Sedimentation profile of poly(A)-rich mRNA from rat medullary thyroid carcinoma. The mRNA (2.5 A_{260} units) was fractionated on a 15–30% linear sucrose density gradient containing 20 mM Tris·HCl (pH 7.4), 10 mM NaCl, 0.5 mM EDTA, and 0.1% $NaDodSO_4$. Gradients were centrifuged for 14 hr at 39,000 rpm in a Beckman SW-41 rotor. Each 0.4-ml fraction was precipitated with ethanol, and 0.45 μg of RNA from fractions 10–18 was translated in a 15-μl wheat germ lysate reaction mixture with [^{35}S]methionine. The radiolabeled reaction products were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (20). Gels were radioautographed and the radioautograms were scanned on a Helena gel scanner. For each translation reaction the area contained under the peak corresponding to the 17,500-dalton calcitonin precursor was calculated as a percentage of total area in the scan. Assignment of S values is based on migration of 18S rRNA and 4S tRNA markers, as indicated.

```
                1                5                   10
      Lys Arg Cys Gly Asn Leu Ser Thr Cys Met Leu Gly Thr Tyr

5'--- AAG CGG TGT GGG AAT CTG AGT ACC TGC ATG CTG GGC ACG TAC
3'--- TTC GCC ACA CCC TTA GAC TCA TGG ACG TAC GAC CCG TGC ATG
               1                15                  30

          15                  20                  25
  Thr Gln Asp Leu Asn Lys Phe His Thr Phe Pro Gln Thr Ser Ile

  ACA CAA GAC CTC AAC AAG TTT CAC ACC TTC CCC CAA ACT TCA ATT
  TGT GTT CTG GAG TTG TTC AAA GTG TGG AAG GGG GTT TGA AGT TAA
           45                  60                  75

          30                  35
  Gly Val Gly Ala Pro Gly Lys Lys Arg

  GGG GTT GGA GCA CCT GGC AAG AAA AGG ---3'
  CCC CAA CCT CGT GGA CCG TTC TTT TCC ---5'
           90                  105
```

FIG. 2. Partial nucleotide sequence of cDNA insert in plasmid C-1. The predicted amino acid sequence encoded by residues 1–96 corresponds to that of rat calcitonin. The sequence shown follows a *Bgl* II cleavage site and extends 114 bases to the 3′ end of the coding sequence. (See *Note Added in Proof.*)

The cloning of calcitonin cDNA was undertaken in order to provide a specific probe for studies of expression of the calcitonin gene. ^{32}P-Labeled plasmid C-1 was used as a probe to analyze size-fractionated RNAs from different tissues. As shown in Fig. 3, the C-1 ^{32}P-labeled probe hybridized only to a 1050-base-pair species of cytoplasmic poly(A)-rich RNA from the high calcitonin-producing tumor (Fig. 3, lane 1). In order to determine whether the calcitonin mRNA in the tumor corresponds to that expressed in normal C-cells, poly(A)-rich RNA from rat thyroid glands was analyzed. An RNA species of similar, if not identical, size was detected (Fig. 3, lane 2). No hybridization was detected in pituitary RNAs from neurointermediate lobes (Fig. 3, lane 3), anterior lobes (Fig. 3, lane 4), or other tissues, including hypothalamus and GH_4 and AtT20 cell lines (data not shown). The sensitivity of the RNA blot hybridization as an assay for calcitonin-specific sequences was assessed by hybridization of the probe to a series of dilutions of poly(A)-rich RNA from cytoplasmic medullary thyroid carcinoma estimated to contain 5–10% calcitonin-specific sequences. Hybridization was observed even with the lowest amount tested (0.1 μg), which should contain 5–10 ng of calcitonin mRNA (data not shown).

The calcitonin probe was also used to characterize specific mRNA sequences in rat medullary thyroid carcinomas exhibiting different calcitonin production rates. We previously reported that the translation product of calcitonin mRNA could be correlated with the calcitonin content of the tumor (20). Fig. 4 shows hybridization of probe to RNA from tumors containing radioimmunoassayable calcitonin of 44.6 μg/mg of total protein (Fig. 4, lane A) and 10.1 μg/mg of total protein (lane B). It is apparent that increased hybridization is associated with increased calcitonin production, suggesting that the observed translational differences do reflect actual alteration in the concentration of calcitonin mRNA sequences.

DISCUSSION

The biosynthesis of calcitonin has been suggested to involve initial synthesis of a larger precursor from which the secreted 32 amino acid hormone is generated by post-translational processing (39, 40). This view was given further support by the immunological identification of a protein of M_r of 17,500 (20) or 15,000 (19) which was proposed to represent the cell-free translation product directed by rat calcitonin mRNA. Unequivocal identification of this mRNA as calcitonin mRNA has now been provided by molecular cloning technology. A chimeric plasmid was constructed and shown to contain the

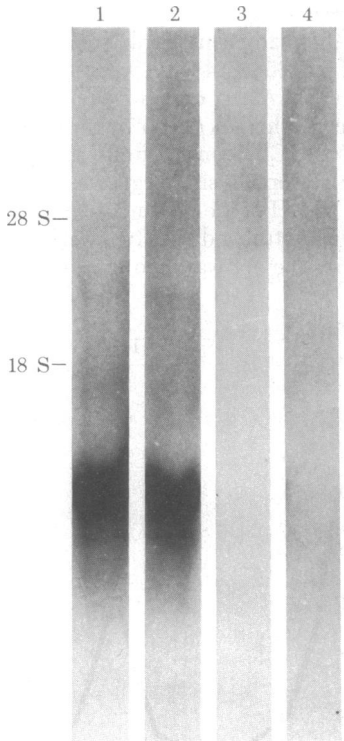

FIG. 3. Tissue distribution of calcitonin-hybridizable RNA sequences. Poly(A)-rich mRNA from various tissues was denatured, subjected to electrophoresis, transferred to diazobenzyloxymethyl-paper, hybridized against ^{32}P-labeled plasmid C-1, and radioautographed. Poly(A)-rich RNA was from: lane 1, rat medullary thyroid carcinoma cytoplasm (5 μg); lane 2, normal rat thyroid glands (33 μg); lane 3, normal rat neurointermediate lobe pituitaries (30 μg); lane 4, normal rat anterior lobe pituitaries (30 μg). Migration of denatured 18S and 28S rRNA is indicated.

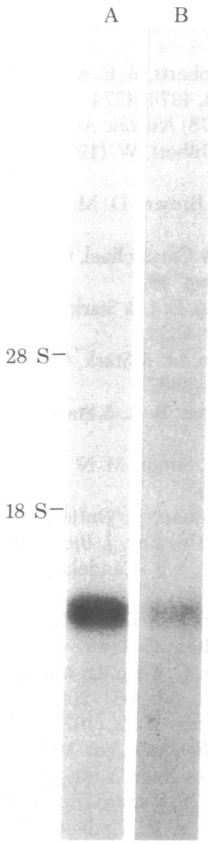

FIG. 4. Calcitonin mRNA levels in different calcitonin-secreting tumors. Equal amounts of total cell poly(A)-rich RNA (10 μg) from one tumor with a calcitonin content of 44.6 μg/mg of total protein (lane A) and a second tumor with a calcitonin content of 10.1 μg/mg of total protein (lane B) were subjected to denaturation, electrophoresis, transfer to diazobenzyloxymethyl-paper, hybridization against ^{32}P-labeled plasmid C-1, and radioautography. Migration of 18S and 28S rRNAs is indicated.

complete nucleotide coding sequence for rat calcitonin (38). The predicted amino acid sequence indicates that calcitonin is flanked by basic peptides. This suggests that calcitonin is generated by proteolytic excision from a larger precursor by processing mechanisms similar to those documented for other small peptide hormones (1–7, 15–17). Mature calcitonin contains an amidated carboxyl-terminal proline (38); nucleotide sequence predicts that Pro-Gly will be at the carboxyl terminus of the hormone. This is similar to promelittin and to α-melanotropin, in which a glycine also follows the residue that is amidated in the native peptides (15, 41). Although the precise mechanism for carboxyl-terminal amidation has not been determined (41–43), these data are consistent with the requirement for an adjacent carboxyl-terminal glycine residue.

Generation of the cloned calcitonin probe permitted the characterization of calcitonin mRNA sequences both in rat medullary thyroid tumors and in other tissues. The size of the calcitonin mRNA, which migrated as 12 S on rate zonal centrifugation under nondenaturing conditions, was found to be 1050 ± 50 nucleotides in both the tumor tissue and in normal rat thyroids. The initial translation product of 17,500 apparent M_r would be encoded by approximately 450 nucleotides. Therefore, a noncoding region of approximately 600 nucleotides, including the poly(A) tract, would be predicted. The nature of the large noncoding sequence and the possible existence of coding sequences for other hormones in the mRNA will be resolved most effectively by determining the complete nucleotide sequence.

Because of the report that some cells in rat pituitary contain material histochemically immunoreactive with calcitonin antiserum (44), poly(A)-rich RNA from several tissues was assayed for the possible presence of calcitonin RNA sequences. The hybridization conditions were modified with respect to amount of poly(A)-rich RNA used and of labeled probe added until 5–10 ng of calcitonin mRNA could be detected. The absence of detectable calcitonin-hybridizable sequences in hypothalamus, GH_4 cells, or the anterior or neurointermediate lobes of the pituitary can therefore be interpreted to mean that any calcitonin sequences present would have to represent less than 0.02% of poly(A)-rich RNA. In contrast, C cells represent less than 3% of total cells in the thyroid gland, and yet the calcitonin mRNA is readily detectable by the assay used. These data suggest that pituitary cells to not express the calcitonin gene at levels implied by the histochemistry, and other explanations for the observed histochemical staining must be considered.

Finally, the calcitonin clone was used to initially define the correlation between calcitonin biosynthesis and calcitonin mRNA levels. In the "high-producer" calcitonin lines of rat medullary thyroid carcinoma, calcitonin content varies from 1% to 5% of total protein as assayed by radioimmunoassay (45). Hybridization demonstrated that calcitonin mRNA sequences were significantly increased in tumors containing larger amounts of calcitonin. The correlation between calcitonin synthesis and its mRNA concentration is therefore similar to that described in many other mRNA species (46).

Note Added in Proof. The following additional nucleotide sequence of the cDNA in plasmid C-1 has now been determined (see Fig. 2).

```
                          40                                  45
Asp  Met  Ala  Lys  Asp  Leu  Glu  Thr  Asn  His

GAT  ATG  GCC  AAG  GAC  TTG  GAG  ACA  AAC  CAC
CTA  TAC  CGG  TTC  CTG  AAC  CTC  TGT  TTG  GTG
               120                            135

                     50
His  Pro  Tyr  Phe  Gly  Asn  stop

CAC  CCC  TAT  TTT  GGC  AAC  TAG  ---3'
GTG  GGG  ATA  AAA  CCG  TTG  ATC  ---5'
               150
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