Cloning and sequence analysis of a cDNA encoding rat preprocholecystokinin

(recombinant DNA/DNA sequence/oligonucleotide probe)

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ABSTRACT Poly(A) RNA was isolated from a rat medullary thyroid carcinoma that exhibited high levels of immunoreactive cholecystokinin (CCK). Double-stranded cDNA was synthesized from the poly(A) RNA and inserted into the *Pst* I site of pBR322. Bacterial colonies containing CCK cDNA were identified using the hybridization probe d(T-C-C-A-T-C-C-A-N-C-C-C-A-T-G-T-A-G-T-C). The sequence of the probe was deduced from the known amino acid sequence of porcine CCK-8, Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH₂. The nucleotide sequence of the cDNA complementary to the mRNA of rat preprocholecystokinin was determined. The cDNA contains 33 nucleotides in the 5'-noncoding region, 199 nucleotides in the 3'-noncoding region, and 345 nucleotides coding for a precursor to CCK, which is 115 amino acids (*M_r*, 12,826). Examination of the rat CCK gene revealed a suggested transcriptional control sequence analogous to the "TATA" sequence located 33 nucleotides upstream from a proposed transcriptional start site. The amino acid sequence of CCK-39 is flanked by both amino-terminal and carboxyl-terminal extensions. Analysis of CCK mRNA showed that it is ≈750 nucleotides long. CCK mRNA of the rat brain and intestine appeared to be identical in size to the CCK mRNA of the carcinoma.

Medullary thyroid carcinomas (MTCs) are known to synthesize multiple neuroendocrine peptides in addition to producing calcitonin (1). Examples of such neuropeptides include neurotensin (2), somatostatin (3, 4), cholecystokinin (CCK) (5), and proopiomelanocortin-derived peptides (6). Several of these peptides are synthesized initially as larger precursor proteins, which are in turn processed to biologically active peptide(s) (7, 8). Peptides synthesized by MTCs are also found in the brain but generally in low concentrations. Medullary rat tumor lines show a dramatic variability in the levels of expression of some of these neuroendocrine peptides (3). Identification of a specific tumor line that produces elevated levels of a neuropeptide establishes a potential system for examining the structure and precursor processing of that peptide and its related gene products. In addition, MTCs may represent an attractive model system for understanding neuroendocrine gene expression.

We have examined the levels of immunoreactive CCK in 10 rat medullary tumor lines (9). Levels of immunoreactive CCK in 2 MTC lines (denoted here as WD or 1-1-4 and WE or 1-1-4A) were 20 to 40 times higher than the concentrations reported for porcine brain or gut CCK-8 (10). Poly(A) RNA was isolated from one of the MTC lines, and by using recombinant DNA techniques a cDNA library was prepared. Identification of a CCK-specific clone was facilitated by the use of an oligonucleotide probe, the sequence of which was de-

duced from the amino acid sequence of porcine CCK-8. Clones identified by this method have been sequenced, and the nucleotide sequence predicts the complete amino acid sequence for rat preprocholecystokinin.

EXPERIMENTAL PROCEDURES

Synthesis of the Icosadeoxynucleotide (T-C-C-A-T-C-C-A-N-C-C-C-A-T-G-T-A-G-T-C). The icosadeoxynucleotide was synthesized by the solid support phosphotriester method on a derivatized polystyrene resin (11).

The icosamer was characterized by sequence analysis using two-dimensional electrophoresis-homochromatography (12) and by the chemical degradation method of Maxam and Gilbert (13). Both gave results consistent with the structure of the icosamer.

Preparation of mRNA and RNA Blot Analysis. Total RNA was extracted from frozen MTC tumors, rat cerebral cortex, and duodenal intestine by the method of Chirgwin *et al.* (14). A detailed description of this procedure, as carried out in this laboratory, is given by Minth *et al.* (15). Yields of poly(A) RNA were 0.25–1.1% of the total RNA. RNA blot analysis of the tumor RNA was carried out as follows: 5 µg of poly(A) RNA prepared from a WD tumor and 5 µg of poly(A) RNA from a VF tumor (not exhibiting immunoreactive CCK) were subjected to electrophoresis on a 1.7% agarose gel in the presence of 5 mM CH₃HgOH (16). The RNA was transferred to a nitrocellulose filter by the method of Thomas (17). The filter was prehybridized for 4 hr at 45°C in modified Denhardt's solution [6× standard saline citrate (0.9 M NaCl/0.09 M Na citrate)/0.2% Ficoll/0.2% polyvinylpyrrolidone/0.2% gelatin] containing *Torula* RNA at 200 µg/ml. Hybridization with the 4 × 10⁶ cpm/ml (1 × 10⁵ cpm/ng) of 5'-³²P-labeled icosadeoxynucleotides was carried out in the above solution at 30°C for 24 hr [details of the labeling and purification of the radiolabeled oligomers are as outlined by Taylor *et al.* (18) and Minth *et al.* (15)]. The filter was washed twice with 6× standard saline citrate at 0°C and twice with 6× standard saline citrate at 25°C, blotted dry, and subjected to autoradiography for 3 days with an intensifying screen.

Rat tumor, brain, and intestine poly(A) RNA (5–10 µg) were compared on CH₃HgOH gels and transferred to nitrocellulose filters. Restriction fragments from CCK clones were used as hybridization probes for CCK mRNA. After prehybridizing in modified Denhardt's solution containing *Torula* RNA (200 µg/ml) at 65°C for 4 hr, hybridization of the filters with 5 × 10⁵ cpm/ml (1 × 10⁸ cpm/µg) of nick-translated restriction fragments was carried out for 12–16 hr. Filters were washed with three 15-min washes with 3× stan-

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Abbreviations: MTC, medullary thyroid carcinoma; CCK, cholecystokinin.

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dard saline citrate (65°C), followed by two 15-min washes with 1× standard saline citrate (65°C).

Construction and Screening of Recombinant Plasmids. Preparation and cloning of double-stranded cDNA in the plasmid pBR322 has been described (15). Plasmid-containing colonies were plated onto nitrocellulose filters and screened by the method of Grunstein and Hogness (19) using the ³²P-labeled icosadeoxynucleotides as a hybridization probe. After hybridizing for 4 hr at 65°C in modified Denhardt's solution, the filters were hybridized with 5 × 10⁶ cpm/ml (1 × 10⁵ cpm/ng) of the labeled icosadeoxynucleotides at 37°C for 12 hr. The filters were washed as described above.

RESULTS

Design and Synthesis of the Icosadeoxynucleotides d(T-C-C-A-T-C-C-A-N-C-C-C-A-T-G-T-A-G-T-C). To facilitate the identification of a clone for CCK, the mixed sequence oligonucleotide complementary to porcine CCK-8, Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH₂, was synthesized. The amino acid sequence from which the nucleotide sequence was deduced was chosen because it distinguishes between gastrin and cholecystokinin mRNA (the COOH-terminal pentapeptides are identical in the two hormones). The degenerate nature of the code would necessitate synthesis of a mixture of 16 oligonucleotides if all possible codon selections were to be used. Alternatively, because guanine has been shown to be preferred in the third position of codons for both asparagine and tyrosine (20), and, theoretically, a guanine pairing with a uracil does not destabilize a duplex significantly (21), selection of this nucleotide would limit the number of oligonucleotides in the mixture to only four. This strategy was chosen. All four possible nucleotides were incorporated in the third position of the glycine codon, and only the first two nucleotides of the penultimate asparagine codon were used.

Immunoreactive CCK in Rat MTCs. Immunoreactive CCK levels were determined in 10 different transplantable rat MTC series as described (5, 8). The immunoreactive CCK levels in these tumor series are shown in Table 1. These levels ranged from <1 ng of immunoreactivity per mg of protein to ≈43 ng per mg of protein. Two transplantable tumors, WE and WD, with high levels of immunoreactive CCK were selected for further study.

Hybridization Analysis of Rat Medullary Carcinoma RNA. Poly(A) RNA from a WD tumor having 20 ng of immunoreactive CCK per mg of protein and a VF tumor (which had no detectable CCK immunoreactivity) was prepared and subjected to electrophoresis on an agarose gel containing CH₃HgOH. The RNA was transferred to nitrocellulose paper and hybridized under a number of experimental conditions with the ³²P-labeled icosamers. A poly(A) RNA from the WD tumor of ≈750 nucleotides hybridizes specifically to the ³²P-labeled icosamers (Fig. 1). No hybridization is observed to poly(A) RNA from the VF tumor.

Screening and Sequence Analysis of cDNA for Cholecystokinin. Identification of a positive clone was achieved by screening ≈1,000 bacterial colonies with the ³²P-labeled icosamers. Of the 1,000 colonies initially screened, 20 were selected as potential CCK-containing clones. On rescreening, only 5 colonies gave a positive signal. Plasmid DNA was prepared from these 5 bacterial colonies and analyzed by electrophoresis on an agarose gel. Plasmid DNA was transferred to nitrocellulose and hybridization analysis was carried out as described by Southern (22). Two of the 5 colonies gave a positive signal under these conditions. The strongest signal corresponded to pCK2AB5. This plasmid was chosen for DNA sequence analysis and was shown to contain a sequence encoding preprocholecystokinin as well as sequence complementary to the 5'-untranslated region of the RNA. The inserted cDNA of the plasmid pCK2AB5 was nick-translated (23) and ≈2,000 additional colonies were

Table 1. Immunoreactive CCK in various lines of rat MTC

Tumor series	n	CCK immunoreactivity
2-2-1	2	12
2-2-6	4	<1
2-2-7 (VF)	2	<1
2-2-7A	2	7
2-2-10	3	<1
2-3-3	2	<1
1-1-4 (WD)	4	13
1-1-4A (WE)	2	43
1-2-4	2	<1
1-2-9	2	<1

See ref. 9 for details of tumor transplant series. "A" indicates tumor series derived by alternate *in vitro/in vivo* passage of the parent series. n = number of tumors analyzed. CCK immunoreactivity is in ng per mg of protein.

screened. Five positive colonies were identified. One of these colonies, pCK16AB5, had a cDNA insert encoding the carboxyl-terminal region of preprocholecystokinin and a sequence complementary to the 3'-untranslated region of the RNA. The composite restriction map obtained from plasmids pCK2AB5 and pCK16AB5 is shown in Fig. 2.

The results of DNA sequence analysis for clones pCK2AB5 (nucleotides 1–380) and pCK16AB5 (nucleotides 289–577), and the deduced amino acid sequence of preprocholecystokinin are shown in Fig. 2. Both strands of the cDNA were sequenced by the method of Maxam and Gilbert (13) and confirmed by the dideoxy chain-termination procedure of Sanger *et al.* (26). The DNA sequence encoding preprocholecystokinin contains only one open-reading frame flanked by a 33 nucleotide 5'-untranslated region and 199 nucleotides in the 3'-untranslated region.

FIG. 1. Hybridization analysis of RNA from WD and VF rat medullary thyroid tumors. Five micrograms of poly(A) RNA from WD and VF tumors was subjected to electrophoresis on a 1.7% agarose gel containing 5 mM CH₃HgOH, transferred to nitrocellulose, and hybridized with the ³²P-labeled icosamers. Size markers are in nucleotides.

FIG. 2. The composite restriction enzyme map (*Upper*) and nucleotide sequence (*Lower*) of cloned rat preprocholecystokinin cDNA. The nucleotide sequence was derived from pCK2AB5 (nucleotides 1-380) and pCK16AB5 (289-577). The bars under the map indicate the location of the *Pvu* II/*Pvu* II and *Ava* II/*Pvu* II restriction fragments used to hybridize with mRNA on RNA gels (see Fig. 4). The deduced amino acid sequence of rat preprocholecystokinin is written above the nucleotide sequence. The numbering of the preprohormone begins at the putative initiator methionine (residue 1) and terminates at serine (residue 115). Amino acids in parentheses represent substitutions found in canine CCK [the sequence reported for this peptide (24) corresponds to residues 46-83 of the rat preprohormone]. Amino acid substitutions found in porcine CCK (25) are bracketed (the sequences reported for this peptide correspond to residues 65-103 of the rat preprocholecystokinin). The amino acid substitutions common to porcine and canine CCK sequences are noted as < >. Cleavage sites that would result in forms of CCK reported by others (24, 25) are indicated by arrows, and the resulting peptides are numbered using their nomenclature.

Rat CCK Gene. To obtain additional sequence information in the 5'-untranslated region, a rat CCK gene was isolated from a *Hae* III rat liver DNA library in Charon 4A, kindly provided by J. Bonner. (Details of the gene isolation will be published in a separate report.) The CCK gene contains a suggested transcription control sequence analogous to the "TATA" sequence located 33 nucleotides upstream from a proposed transcriptional start site (Fig. 3). This evidence is based on homology with a consensus TATA sequence and transcriptional initiation sequence (27). The 5'-untranslated sequence of the CCK cDNA is identical to the gene DNA sequence and suggests that pCK2AB5 lacked ≈ 27 nucleotides from having a complete 5'-untranslated region. No other codons for methionine (AUG) are found in any reading frame between the TATA box and the proposed initiator me-

cagcactgggtaaacagatgactggctgcgtaccggcggggdcattttaaaggaggatgcgcctgccgctgccctcaACTT

AGCTGGACAGCAGCCGCTTGAAACCCGCAAGCCAGCTGACTCCGATCCGAAGATATGAATGCGGCGTGTGTCTGTGCGT

pCK2AB5 cDNA

FIG. 3. Partial nucleotide sequence of the 5'-untranslated region of the rat *CCK* gene. The portion of the sequence also determined from the cDNA is indicated. A potential TATA sequence is boxed. The proposed initiator methionine is underlined. A possible transcription initiation sequence is underlined with a broken line. The triangle indicates the location of an intervening sequence in the gene of approximately 1 kilobase pair.

thionine. Also preceding the putative initiator methionine is a sequence complementary to the 18S rRNA (underlined in Fig. 2). It has been suggested that interactions in this complementary region are involved in correct initiation of translation (28). However, there are cases in which this sequence is absent without any impairment in translation efficiency (29).

The open-reading frame encodes preprocholecystokinin, a protein of 115 amino acids with a molecular weight of 12,826. The amino terminus of the preprohormone contains a peptide rich in hydrophobic amino acids. This peptide has characteristics similar to the amino-terminal signal sequence of other secreted proteins (30). The proposed initiator methionine (residue 1) is followed by a lysine residue and a hydrophobic core sequence. Cleavage of the preprohormone by a signal peptidase is predicted after alanine-20. This prediction is based on comparison with known signal sequence cleavage sites (31). Assuming the signal sequence is cleaved in this region, a procholecystokinin of 95 residues would be formed.

The sequence of the preprocholecystokinin also predicts a 12 amino acid carboxyl-terminal extension following the sequence corresponding to CCK. It should be noted that the carboxyl-terminal residue of CCK is amidated (25). The three amino acids following the amidated residue of CCK are Gly-Arg-Arg. The presence of glycine following a potential COOH-terminal amidation site has been observed in several prohormones and appears to be a general feature associated with amidation (32). The presence of the two basic amino

FIG. 4. Hybridization analysis of poly(A) RNA from WD tumor, rat brain, and rat intestine. Five micrograms of poly(A) RNA from tumor (lane a), 10 μ g of poly(A) RNA from brain (two preparations) (lanes b, d, e, and g), and 10 μ g of poly(A) RNA from rat small intestine (lanes c and f) were subjected to electrophoresis on a 1.7% agarose gel containing 5 mM CH_3HgOH . After transfer to nitrocellulose the RNA was hybridized with the *Pvu* II/*Pvu* II probe derived from pCK2AB5 (lanes a–d) or the *Ava* II/*Pvu* II probe derived from pCK16AB5 (lanes e–g). Size markers are in nucleotides.

acid residues adjacent to the glycine suggests that this is a cleavage site for processing of the precursor polypeptide (33–35).

Hybridization Analysis of CCK mRNA in Other Tissues. CCK immunoreactivity has been detected in the small intestine as well as in the brain (25). It is not known whether tissue-specific forms of CCK exist or how they compare to the tumor CCK mRNA reported here. Using cDNA hybridization probes corresponding to 5' and 3' regions of the CCK mRNA, we have investigated the presence of CCK mRNA in several tissues. One probe, a *Pvu* II/*Pvu* II fragment (nucleotides 14–162; Fig. 2), is homologous to the 5' region of the mRNA. The second probe, an *Ava* II/*Pvu* II fragment (nucleotides 292–543), is homologous to the portion of the mRNA coding CCK-8 and 3'-untranslated sequences. The results of hybridizing poly(A) mRNA from WD tumor, rat cerebral cortex (two different preparations), and rat intestine mRNA with these CCK probes is shown in Fig. 4. In all three tissues, an RNA of ≈ 750 bases hybridizes with both probes. Although there are differences in the amount of mRNA present (the brain and intestine blots were exposed for 2.5 days, the tumor for 1 day with an intensifying screen), the same RNA hybridizes in all three tissues. This result was obtained with either hybridization probe and thus suggests that there are no apparent differences in the number of nucleotides in the various tissue-specific forms of CCK mRNA. This experiment does not reveal subtle differences in base sequence nor does it rule out the possibility that other CCK mRNAs exist that are not sufficiently homologous to be detected by these probes.

DISCUSSION

CCK was originally isolated from the small intestine (36) and later shown to occur in the brain (37). In the gut, release of CCK causes contraction of the gallbladder and release of pancreatic enzymes (25). The role of CCK in the brain is much less clearly understood. It has been shown that CCK injected into sheep results in decreased feeding, and injection of antibody to CCK stimulates feeding above normal levels (38). The satiety effect observed in sheep has not been observed universally. Similar experiments in rats have failed to elicit a response (39). CCK has also been shown to antagonize morphine-induced analgesia (40). The presence of different molecular forms of brain and gut CCK observed in numerous laboratories has recently been reviewed by Dockray (41). Understanding the physiological roles of CCK may be aided by a detailed understanding of the molecular forms of the hormone.

The amino acid sequence of rat preprocholecystokinin deduced from the cloned mRNA is highly homologous to the sequence of porcine gut (36) and canine gut CCK (24) (Fig. 2). The amino acids of rat preprocholecystokinin have been numbered by assigning the putative initiator methionine as residue 1 and the COOH-terminal residue of the preprohormone as residue 115. Porcine CCK-39 thus corresponds to residues 65–103 of the rat preprohormone. The sequence of porcine CCK-39 and the corresponding sequence of the rat preprohormone differ by only three amino acids. When the partial sequence of canine CCK (24) is compared to the corresponding sequence in the rat, one finds amino acid substitutions at residues 47–52 and at residue 57, 69, and 80. The divergence among these three forms of CCK could represent species or tissue differences. The CCK cDNA cloned from the MTC tumor does not appear to be unique to this tissue. mRNA from rat brain and intestine hybridizes with two probes derived from the cloned tumor cDNA (Fig. 4). If there are tissue-specific forms of CCK, the differences between the CCK mRNAs are such that a detailed analysis of their sequences will be required to resolve these differences. It should be noted that when the predicted rat carcinoma CCK sequence is compared with a canine intestinal CCK, only the region between amino acids 47 and 52 show an appreciable divergence. By cloning a rat intestine and brain CCK cDNA, the nature of the apparent divergence in amino acid sequences found in this region could be addressed.

The processing of preprocholecystokinin results in peptides of various sizes (25). Biologically active forms of CCK that differ in amino acid chain lengths have been reported (24, 36). Very little is known about the processing events that lead to these peptides or whether intermediates have different physiological functions. The cleavage sites of several prohormones occur after adjacent basic amino acid residues (33). The sequence of rat preprocholecystokinin contains 14 arginines and 3 lysines. Cleavage on the carboxyl side of arginine-91 and arginine-95 would produce peptides that have been referred to in the literature as CCK-8 and CCK-12 (Fig. 2). These represent the major forms of CCK observed in this tumor (unpublished results). These cleavage sites follow single basic amino acids. Other potential cleavage sites also exist that could give rise to CCK of 33, 39, and 58 amino acids—the major forms reported for the canine and porcine gut hormones. It should be noted that to form porcine CCK-33, cleavage would occur between arginine-70 and lysine-71 of the rat preprohormone. If the processing of preprocholecystokinin proceeds as described above, then it differs from the commonly observed cleavage on the carboxyl side of two adjacent basic amino acids noted in many prohormones (33). Knowing the sequence of the rat preprohormone will allow the use of region-specific immunochemical reagents to follow the details of post-translational processing events.

MTCs exhibit several features of interest for the study of hormone gene expression (42). These tumors produce many peptide hormones, and the level of expression of a particular hormone can vary in different tumor lines. The reasons for the variation in hormone levels are not understood, but they could include differential gene expression in one cell type or changes in the cell population favoring growth of one cell over another (43). The implication of these variations in the study of transformed cells and metastasis is obvious. Establishing cell lines from MTC tumors and having cDNAs and antibodies to follow changes in expression of these genes provides the tools necessary to study these questions in more detail.

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