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Secretin: Structure of the precursor and tissue distribution of the mRNA

(intestine/preprohormone/cDNA/polymerase chain reaction)

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ABSTRACT **Secretin is a 27-amino acid gastrointestinal hormone that stimulates the secretion of bicarbonate-rich pancreatic fluid. The unusually high number of serine, leucine, and arginine residues in secretin has precluded the use of oligonucleotides to screen cDNA libraries to isolate a secretin cDNA. In the present study, a short cDNA encoding porcine secretin was amplified from duodenal mucosal first-strand cDNA template by using 16,384- and 4096-fold degenerate primers in the DNA polymerase chain reaction. From the sequence of the amplified cDNA, an unambiguous oligonucleotide probe was designed to screen a cDNA library. Here we report the sequences of cDNAs encoding the porcine and rat secretin precursors. The predicted amino acid sequences reveal that each precursor consists of a signal peptide, an N-terminal peptide, secretin, and a 72-amino acid C-terminal peptide. Secretin has been highly conserved through evolution. Rat secretin differs from its porcine counterpart by a single glutamine-for-arginine substitution at position 14. In contrast, the amino acid sequences of the C-terminal peptides are only 39% conserved between the two species, suggesting that the C-terminal peptide does not have an essential physiologic function. RNA blot hybridizations reveal that the rat secretin gene is expressed throughout the small intestine. Although secretin immunoreactivity has been localized in the central nervous system by some laboratories, we are unable to detect secretin mRNA in tissues of the central nervous system by Northern blot hybridization.**

Secretin is a 27-amino acid hormone produced by specific endocrine cells, S cells, localized in the mucosa of the proximal small intestine. Since its discovery in 1902 by Bayliss and Starling (1), secretin has been known to be a potent stimulus for the secretion of bicarbonate-rich pancreatic fluid. Secretion of secretin is stimulated by the presence of either acidic pH or fatty acids in the duodenum. Although the amino acid sequence of this hormone was elucidated in 1970 (2), little is known about regulation of its biosynthesis or structure of its precursor. As with other regulatory peptides, secretin appears to be synthesized as a larger precursor. Although Gavelin *et al.* (3) have identified an incompletely processed precursor in which secretin is extended at its C terminus by Gly-Lys-Arg, other peptides derived from its precursor remain to be identified.

The localization of secretin-producing cells in the proximal small intestine is well established (4–8). However, the identification of extraintestinal secretin-producing tissues by immunochemical techniques has been the subject of considerable controversy. Secretin immunoreactivity has been reported to be widely distributed in the central nervous system (CNS) (9). The apparent secretin content per gram of tissue for pituitary and pineal was reportedly higher than in duodenum, while the levels reported for hypothalamus, thalamus, and olfactory lobe were comparable to those in small intestine (9). Others report that brain contains either small amounts (10, 11) or no secretin-like immunoreactivity (12).

In the present study, we have isolated cDNAs encoding the rat and porcine secretin precursors.† The deduced amino acid sequence includes a signal peptide, an N-terminal peptide, secretin, and a 72-amino acid C-terminal peptide. We have used the secretin cDNA as a probe in Northern blot hybridizations to address unresolved questions regarding the tissue localization of secretin biosynthesis in the rat CNS and gastrointestinal tract. We demonstrate that the small intestine is the major site of secretin gene expression.

MATERIALS AND METHODS

Polymerase Chain Reaction (PCR). Oligo(dT)-primed first-strand cDNA was reverse-transcribed from poly(A)$^+$ porcine duodenal mucosal RNA for use as template in the PCR. The primers for PCR were purified over oligonucleotide purification cartridges (Applied Biosystems). The 5′ and 3′ primers contained all possible nucleotide sequences that could potentially encode the amino acid sequences near the N and C termini of porcine secretin, respectively (Fig. 1). Nine additional nucleotides were included at the 5′ end of the primers to generate restriction sites for subcloning the amplified product. For the first round of amplification, 100 pmol of each primer mix was used to amplify 500 ng of template. The reaction mixture was heated to 95°C for 5 min prior to the addition of 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Cetus/Perkin–Elmer). The reaction mixture was prepared according to the manufacturer's recommendations and was cycled in a Cetus/Perkin–Elmer DNA thermal cycler. Each cycle of PCR included DNA denaturation at 94°C for 30 sec followed by primer annealing for 15 sec. For the first round of amplification, the annealing temperature was gradually increased: 45°C for cycles 1–5, 50°C for cycles 6–10, and 55°C for cycles 11–35. Nascent chains were extended at 72°C for 30 sec to complete each cycle. Following the final cycle, the amplified product was extended for 7 min at 72°C. The product from the first amplification was used as template for an additional 30 cycles. For the second round of PCR, 500 pmol of each primer was added to the reaction mix. Conditions for the second 30 cycles were identical except that the annealing temperature was 45°C throughout. The product was examined in an ethidium bromide-stained 1.8% agarose gel. The expected 101-base-pair (bp) fragment was isolated by electroelution onto NA45 membrane (Schleicher & Schuell). The DNA was digested with *Bam*HI and *Sph* I, phenol/

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Abbreviations: CNS, central nervous system; PCR, polymerase chain reaction.

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†The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M31495 (rat secretin) and M31496 (pig secretin)].

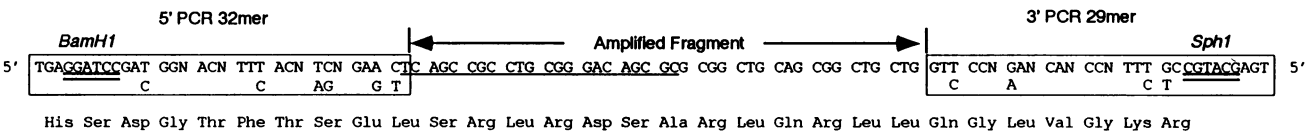

FIG. 1. Strategy for cloning a cDNA encoding porcine secretin. Primers used for PCR are shown above the amino acid sequence of Gly-Lys-Arg-extended porcine secretin. Amino acids, designated by three-letter abbreviations, were reverse-translated and the corresponding primers, enclosed in boxes, were designed to include all possible codons. The 3' primer corresponds to the antisense strand of the cDNA and is shown in reverse orientation. The 5' and 3' PCR primers were 16,384-fold and 4096-fold degenerate, respectively. In positions where the primers were 2-fold degenerate, the second nucleotide is indicated below the first. Positions where all four nucleotides were introduced are indicated by N. Nine additional bases were added at the 5' end of each primer to generate either a *Bam*HI or *Sph* I restriction endonuclease cleavage site (doubly underlined) for subsequent cloning. The nucleotide sequence of a porcine secretin cDNA generated by PCR is noted above the corresponding amino acids. A synthetic oligonucleotide (underlined) was used to screen a porcine duodenal cDNA library.

chloroform-extracted, and subcloned into bacteriophage M13 for nucleotide sequencing.

To detect the presence of secretin transcripts in different tissues by PCR, oligo(dT)-primed first-strand cDNA template was synthesized from either 4 μg of total cellular RNA or 40 ng of poly(A)$^+$ RNA (pituitary). PCR primers (100 pmol) (5'-ATGGAGCCTCTACTGCCCACG-3'; 5'-ACAACCAATCCCTACTCCAGTCA-3') were used to amplify a 454-bp fragment by denaturing at 94°C for 45 sec, annealing at 60°C for 45 sec, and extending at 72°C for 45 sec for 30 cycles. Products were analyzed by DNA blot hybridization (see Fig. 4*C*).

Rat Duodenal Mucosal cDNA Library. Oligo(dT)-primed first-strand cDNA was synthesized from rat duodenal mucosal poly(A)$^+$ RNA with Moloney murine leukemia virus reverse transcriptase (BRL). The second strand of the cDNA was produced as described by Gubler and Hoffman (13). Blunt ends were generated with T4 DNA polymerase prior to addition of *Eco*RI/*Sal* I linker-adapters. cDNA longer than 300 bp was isolated by electrophoresis in a 5% acrylamide gel and ligated to dephosphorylated, *Eco*RI-digested λgt11 phage arms (Promega). The DNA was packaged (Gigapack Plus; Stratagene) into phage particles and amplified. The library contained ≈1.5 × 10^6 primary recombinants; >95% of the phage contained inserts.

Isolation of cDNAs Encoding Porcine and Rat Secretin. To isolate a full-length secretin cDNA, a porcine duodenal cDNA library (14) in bacteriophage λgt11 was screened with an end-labeled synthetic oligodeoxynucleotide probe, 5'-TCAGCCGCCTGCGGGACAGCGC-3', designed from the sequence of the secretin cDNA generated by the PCR (Fig. 1). Filters were hybridized in 5× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) at 58°C and washed in 2× SSC/0.1% SDS at the same temperature. Positive clones were plaque-purified by sequential low-density plating (15) and bacteriophage DNA was isolated from plate lysates. The cDNA inserts were excised with *Eco*RI and subcloned.

The rat duodenal mucosal library was screened (15) with the near-full-length porcine secretin cDNA hybridization probe labeled by priming with random hexamers (16). Filters were hybridized in 5× SSC at 55°C and then washed in 2× SSC/0.1% SDS at the same temperature. Inserts from plaque-purified recombinant DNA either were excised with *Sal* I or *Eco*RI or were amplified with *Taq* polymerase by using primers complementary to sequences flanking the *Eco*RI site in λgt11. Single-stranded template was prepared from M13 and sequenced by the chain-termination method using Sequenase (United States Biochemical) according to the manufacturer's instructions. A highly G+C-rich region of the porcine cDNA was sequenced by the method of Maxam and Gilbert (17). Computer analysis of nucleotide and protein sequences was performed as described by Devereux *et al.* (18).

Northern Blot Hybridization. Following electrophoresis in denaturing agarose gels, RNA was transferred to Nytran membranes (Schleicher & Schuell) by capillary blotting. Antisense RNA hybridization probes were constructed by cloning a 395-bp *Sal* I restriction fragment of the rat secretin cDNA into plasmid pIBI31 (IBI). Radiolabeled antisense transcripts were generated with T7 RNA polymerase and [α-^{32}P]UTP. Hybridization and washing were carried out at 72°C. Other conditions were as described (19).

RESULTS

Isolation of a Porcine Secretin cDNA by PCR. The strategy used to isolate a porcine secretin cDNA is illustrated in Fig. 1. Sense (5') and antisense (3') PCR amplification primers were designed to include all possible nucleotide combinations that could encode the amino acids at either the N or the C terminus of porcine secretin. An earlier report (3) describing the isolation of a precursor form of secretin extended at its C terminus by Gly-Lys-Arg allowed us to include these three additional amino acids in designing the primers. Nine bases were added to the 5' end of each primer (Fig. 1) to generate *Sph* I and *Bam*HI restriction endonuclease sites in the amplified product to facilitate cloning into M13 vectors. The sense PCR primer included a mixture of the 16,384 possible nucleotide sequences encoding the N terminus of porcine secretin, and the antisense primer included the 4096 possible nucleotide sequences encoding the C-terminal amino acids. After the first round of amplification, a broad smear, rather than the predicted 101-bp fragment, was visualized in an ethidium bromide-stained agarose gel. After the second round of amplification, in which 5-fold more primer and a reduced annealing temperature (45°C) were used, the predicted fragment was visualized as a broad band (not shown). Three out of eight M13 subclones encoded amino acids corresponding to the midportion of secretin (Fig. 1). Based on the nucleotide sequence of the amplified material, a 22-base synthetic oligonucleotide probe (Fig. 1) was designed and used to screen 800,000 recombinants from a porcine duodenal cDNA library. The probe hybridized to 2 recombinants with inserts 450 and 550 nucleotides in length. The nucleotide sequence of the shorter cDNA did not contain ≈100 bp found at the 5' end of the longer clone but was otherwise identical.

The nucleotide and corresponding amino acid sequences of the porcine secretin cDNA are shown in Figs. 2 and 3, respectively. The cDNA includes an open reading frame of 393 nucleotides (Fig. 2) encoding a 131-amino acid peptide (Fig. 3). At the N terminus is a hydrophobic region characteristic of a signal peptide. Rules to predict the most likely site of cleavage of the signal peptide from the mature protein (20) cannot unambiguously identify this cleavage site in the porcine secretin precursor. On the basis of the amino acid sequence of the signal peptide, cleavage of the signal peptide is predicted to occur following residue 16, 17, 18, 19, or 20. Comparison with the amino acid sequence of the rat secretin precursor suggests that the porcine signal peptide is most likely cleaved following residue 16, 17, or 18. Each of these sites fulfills the requirement for amino acids with short, neutral side chains (Ala, Gly, Cys) in positions −1 and −3

FIG. 2. Optimum alignment of the rat and porcine secretin cDNA nucleotide sequences. Corresponding functional domains of the protein-coding regions are indicated above the sequences, as are amino acids corresponding to posttranslational processing sites. Colons denote conserved nucleotides and dashes indicate gaps inserted to generate this alignment. Codons for the initiator methionine and translation termination are singly and doubly underlined, respectively. The polyadenylylation signal (AATAAA) is underlined.

(20). The porcine secretin cDNA we isolated did not appear to encode the N terminus of the precursor. An N-terminal peptide, rich in proline and arginine, separates the signal peptide from secretin. Secretin is presumably cleaved from this N-terminal peptide following a single arginine residue. The amino acid sequence of porcine secretin, predicted from the cDNA, is identical to sequence of the purified peptide reported by Mutt *et al.* (2). Secretin is followed in its precursor by the sequence Gly-Lys-Arg. The glycine residue presumably serves as the amide donor for the amidation of the C-terminal valine residue of secretin. The Lys-Arg sequence serves as a dibasic trypsin-like cleavage site for removal of the 72 amino acid C-terminal peptide encoded by the remainder of the open reading frame of the cDNA. Inspection of the C-terminal peptide reveals no obvious potential cleavage sites. Within the 3′ untranslated region of the mRNA, a polyadenylylation signal, AAUAAA, precedes the poly(A) tract.

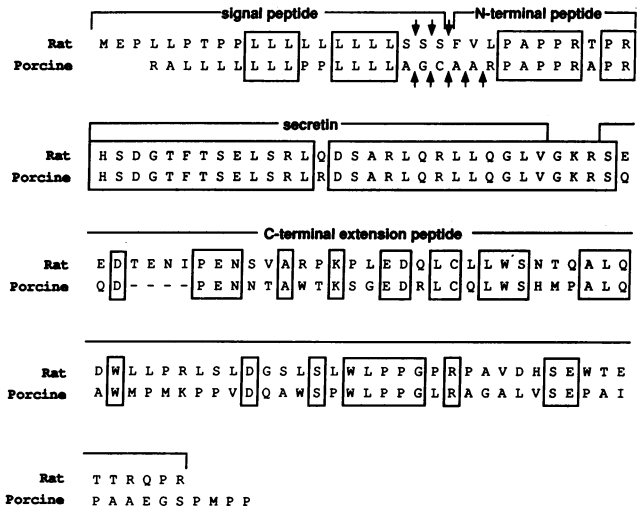

FIG. 3. Comparison of the predicted amino acid sequences (one-letter symbols) for the rat and porcine secretin precursors. Identical amino acids are boxed. Dashes indicate gaps introduced to optimize the alignment. Predicted functional domains are indicated above the amino acid sequences. Potential signal-peptide cleavage sites are indicated by arrows.

Isolation of a Rat Secretin cDNA. To isolate a cDNA encoding the rat secretin precursor, we screened a rat duodenal mucosal cDNA library with the porcine cDNA as a hybridization probe. The probe identified 4 recombinants out of 10^6 examined. The nucleotide and corresponding amino acid sequences of the rat secretin cDNA are shown in Figs. 2 and 3, respectively. The structural organization of the rat and porcine secretin precursors are similar. The rat secretin precursor consists of 134 amino acids and has a molecular weight of 15,074. Like the porcine precursor, rat preprosecretin consists of a signal peptide, an N-terminal peptide, secretin, and a C-terminal peptide. At the N terminus of the signal peptide of rat secretin is a potential initiator methionine residue. The nucleotide sequence flanking this codon, ACCATGG, has been proposed as a consensus sequence surrounding methionine codons that serve as translational startpoints (21). The signal peptide of rat preprosecretin is most likely cleaved following a serine residue in position 19, 20, or 21. Like the porcine secretin precursor, a short N-terminal peptide rich in proline and arginine precedes secretin. The predicted amino acid sequence of rat secretin is identical with the amino acid sequence of the peptide isolated from rat intestine (22) and differs from porcine secretin by the substitution of a glutamine for arginine in position 14 (Fig. 3). Rat secretin, like porcine secretin, is followed in its precursor by an unusually long, 72-amino acid C-terminal peptide. Neither the porcine nor the rat C-terminal peptide has significant amino acid similarity to any proteins listed in the National Biomedical Research Foundation (20.0) or translated GenBank (59.0) data base.

Comparison of the rat and porcine secretin precursor shows that the nucleotide sequences encoding the signal peptide, N-terminal peptide, secretin, and C-terminal peptide are 61%, 67%, 90%, and 58% conserved, respectively, between the two species (Fig. 2), suggesting that each of these domains arose from a common ancestral gene. The corresponding amino acid sequences are 39%, 64%, 96%, and 39% conserved (Fig. 3). Although optimum alignment of the C-terminal peptides of rat and porcine preprosecretin requires the introduction of a 4-amino acid insertion/deletion near the N terminus, the divergence in the C-terminal peptide appears to have arisen primarily from the accumulation of point mutations in codon positions 1 and 2.

Tissue Distribution of Secretin mRNA. RNA from different rat tissues of both the gastrointestinal tract and the CNS was examined for the presence of secretin mRNA by Northern blot hybridization under conditions of high stringency (Fig. 4 *A* and *B*). The secretin antisense RNA probe identified a single mRNA of ≈750 bases in all regions of the rat small intestine examined. Secretin mRNA is most enriched in the ileum and is less abundant in the more proximal regions of the small intestine. Secretin mRNA also appears to be present in lesser amounts in the colon. The probe failed to detect appreciable levels of secretin mRNA in gastric antrum, stomach, cerebral cortex, olfactory lobe, brainstem, hypothalamus, and pituitary (Fig. 4*B*) and in pancreas (data not shown). With oligonucleotide primers specific for rat secretin, amplification of first-strand cDNA prepared from hypothalamus, brainstem, and cortex, but not pituitary, generated the predicted 454-bp secretin cDNA product (Fig. 4*C*), suggesting that secretin mRNA may be present in these tissues at levels below the detection limit of Northern blot analysis.

DISCUSSION

We have isolated cDNAs encoding the precursors of rat and porcine secretin. The isolation of cDNAs encoding low-abundance peptides like secretin has been problematic. The ability to detect a target sequence by conventional filter hybridization techniques using degenerate mixtures of oligonucleotide probes depends on the probe specific activity and the actual amount of probe bound to the target sequences. The abundance of serine, leucine, and arginine residues in secretin would require an unusually complex mixture of probes to include all possible sequences encoding secretin. At the high degree of degeneracy required, the actual amount of the correct oligonucleotide in the mixture may be too small to provide an adequate signal on the filters. In addition, greater probe complexity increases the likelihood of detecting cDNAs other than the target sequence.

In this report, we describe the use of mixed oligonucleotide primers containing either 4096 or 16,384 unique sequences in the PCR. Other reports suggest that degeneracies of 10^5 (23) and 10^6 (24) can be tolerated for DNA amplification. Annealing of amplification primers may be carried out at lower stringencies, allowing for more mismatched base-pairing than is generally possible when screening libraries directly with mixed oligonucleotides. Prior knowledge of the amino acid sequence of porcine secretin, and therefore, the predicted size of the desired amplification product, enabled us to readily identify the target DNA sequences. Generation of a short portion of a secretin cDNA by the PCR enabled us to design an unambiguous oligonucleotide probe to screen a cDNA library.

From the nucleotide sequence of the rat and porcine secretin cDNAs presented here, we deduced the amino acid sequences of the corresponding precursor peptides. The functional domains of rat and porcine preprosecretin are similar, consisting of a signal peptide, an N-terminal peptide, secretin, and a C-terminal extension peptide (Fig. 3). The present study confirms an earlier report (22) that rat secretin differs from its porcine counterpart by a single substitution, glutamine for arginine, at position 14. This single amino acid substitution may explain the slightly longer retention time on HPLC noted previously for rat secretin (9). Comparison of the amino acid sequences of secretin from rat, human, porcine, canine, and bovine species reveals variability in positions 14–16 (2, 22, 25–27). The functional significance, if any, of the glutamine substitution at position 14 of rat secretin is unknown.

Unusually long, 72-amino acid C-terminal peptides are encoded by the mRNAs for both rat and porcine preprosecretin. It is not clear whether these peptides undergo further posttranslational cleavages, although neither the porcine nor the rat C-terminal peptide contains potential dibasic trypsin-like cleavage sites. Neither of the 72-amino acid sequences

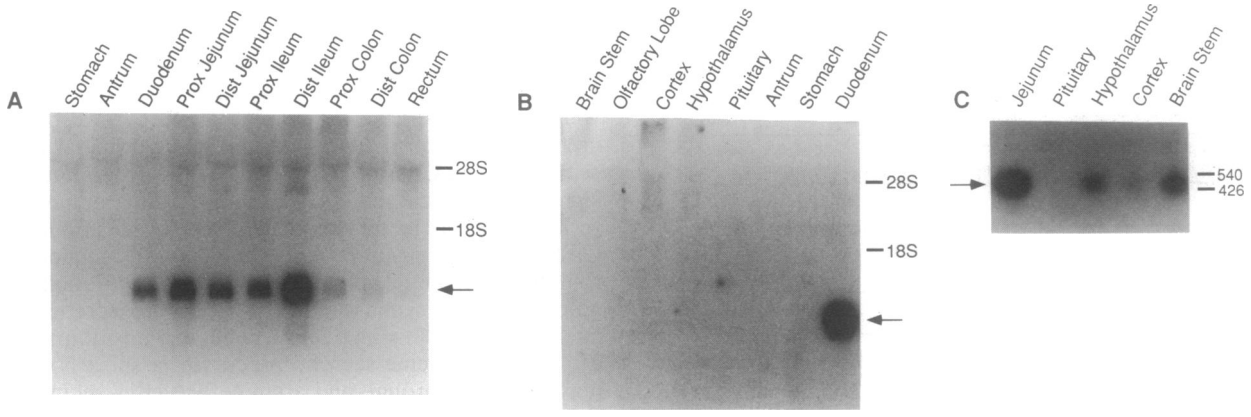

FIG. 4. Analysis of secretin mRNA from regions of the rat gastrointestinal tract and CNS by Northern blot hybridization and cDNA amplification. (*A*) Northern blot hybridization of RNA from gastrointestinal tissues. Twenty micrograms of total cellular RNA from the indicated tissue (Prox, proximal; Dist, distal) was loaded. The autoradiograph was exposed for 4 days. Arrow denotes the position of secretin mRNA. Positions of 28S and 18S ribosomal RNA are indicated at right. (*B*) Northern blot hybridization of RNA from CNS tissues. Twenty-five micrograms of total cellular RNA was loaded in each lane with the exception of the pituitary lanes, where 2 μg of poly(A)$^+$ RNA was loaded. The autoradiograph was exposed for 3 days with one intensifying screen. Samples of total RNA from stomach and duodenum were included as negative and positive control, respectively. As a control for possible variation in loading and transfer, the blot was reprobed for β-actin mRNA; a hybridization signal of appropriate size was detected in all lanes. (*C*) Amplification of secretin cDNA from various rat tissues. First-strand cDNA was amplified as described in *Materials and Methods* and analyzed by DNA blot hybridization using the rat secretin cDNA as a probe. Arrow marks the position of the 454-bp amplified secretin cDNA. DNA size markers (bp) are indicated at right.

shares significant similarity with other peptides represented in either the translated GenBank or the National Biomedical Research Foundation data base.

During evolution, physiologically important peptides are conserved, particularly between closely related species. The amino acid sequences of the rat and porcine preprosecretin C-terminal peptides are far less conserved than secretin itself, suggesting that the C-terminal peptide is not physiologically important. Alignment of the rat and porcine C-terminal peptides reveals only 39% amino acid identity (Fig. 3), with a maximum of five consecutive conserved amino acids. Although there is less amino acid conservation in the C-terminal peptide of preprosecretin relative to secretin itself, a comparison of the rat and porcine cDNA sequences (Fig. 2) clearly shows that all functional domains of both cDNAs arose from a common ancestral gene. The divergence at the peptide level arises for the most part from point mutations in codon positions 1 and 2. This observation implies that the rat and porcine secretin genes arose from a common ancestral gene and that selective pressure to conserve secretin exceeded the requirement to conserve the other functional domains of the precursor.

The existence and significance of secretin-producing cells outside of the small intestine has not been well established. The conflicting reports of secretin immunoreactivity in the CNS (9–11, 28) and gastric antrum (29) may be a reflection of the relative sensitivity and specificity of the different radioimmunoassays employed. A number of laboratories have reported secretin-like immunoreactivity in brain with chromatographic properties similar to those of the purified peptide (9–11), although its abundance is controversial. In the present study, we have determined the tissue distribution of secretin gene expression by Northern blot hybridization carried out at high stringency. The specificity of nucleic acid hybridization techniques under these conditions makes cross-detection of related peptides unlikely. Using these techniques, we were unable to detect secretin-specific mRNA in several regions of the CNS and in the gastric antrum. Our ability to amplify a secretin cDNA from hypothalamus, brainstem, and cortex suggests that trace levels of secretin mRNA may be expressed in these tissues. The observation that secretin mRNA can be detected in CNS by cDNA amplification must be interpreted with some caution, due to reports of amplification of "illegitimate" transcripts of tissue-specific genes from any tissue (30). Our finding that levels of secretin mRNA in the small intestine far exceed levels in the CNS is consistent with previous reports demonstrating that secretin peptide is present in the CNS at levels either much lower than in the gastrointestinal tract (10, 11) or below the limit of detection by radioimmunoassay (12). The physiological significance of low levels of secretin mRNA in brain is uncertain and awaits further anatomic characterization.

For most species studied thus far, the levels of secretin immunoreactivity are highest in the duodenum and decline markedly in the jejunum and ileum (4–8). In rat small intestine, the level of secretin immunoreactivity has been reported to be equal in duodenum and ileum (7), suggesting that the ileum is also an important site of secretin production in the rat. Others have reported that the levels of secretin are severalfold lower in rat ileum than in duodenum (4, 8). The reasons for these discrepancies in the reported distribution of secretin in the rat intestine are unclear. We have found that secretin mRNA levels in the ileum are higher than the levels in the duodenum. For each tissue, our results have been normalized to the ribosomal RNA content, a reflection of cell number, rather than tissue weight or protein content as reported earlier for the peptide measurements.

In addition to finding high levels of secretin mRNA in ileum, we have also observed that the rat secretin gene is expressed at low levels in the colon. Although the occurrence of immunoreactive secretin in porcine colon has been reported (5), colon is not widely regarded as a secretin-producing tissue. Our data suggest that the distal small intestine and colon may be important sites of secretin synthesis in the rat. Determination of the physiologic function of secretin in the distal alimentary tract awaits further study.

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