

## Three rat preprotachykinin mRNAs encode the neuropeptides substance P and neurokinin A

(corpus striatum/tachykinin peptides/differential RNA splicing)

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Communicated by Gerald D. Fischbach, September 17, 1986

**ABSTRACT** Synthetic oligonucleotides were used to screen a rat striatal cDNA library for sequences corresponding to the tachykinin peptides substance P and neurokinin A. The cDNA library was constructed from RNA isolated from the rostral portion of the rat corpus striatum, the site of striatonigral cell bodies. Two types of cDNAs were isolated and defined by restriction enzyme analysis and DNA sequencing to encode both substance P and neurokinin A. The two predicted preprotachykinin protein precursors (130 and 115 amino acids in length) differ from each other by a pentadecapeptide sequence between the two tachykinin sequences, and both precursors possess appropriate processing signals for substance P and neurokinin A production. The presence of a third preprotachykinin mRNA of minor abundance in rat striatum was established by S1 nuclease protection experiments. This mRNA encodes a preprotachykinin of 112 amino acids containing substance P but not neurokinin A. These three mRNAs are derived from one rat gene as a result of differential RNA processing; thus, this RNA processing pattern further increases the diversity of products that can be generated from the preprotachykinin gene.

Of all the naturally occurring neuropeptides, the undecapeptide substance P (SP) is perhaps the peptide best documented as a neurotransmitter and/or neuromodulator substance in both central and peripheral nervous systems (1, 2). SP belongs to a family of structurally related peptides, the tachykinins. Peptides in this family include neurokinin A (NKA, also known as substance K) and neurokinin B (see ref. 3 for discussion); they all exhibit similar biological activities—including contraction of smooth muscle and hypotension—and share a common COOH-terminal sequence, Phe-Xaa-Gly-Leu-Met-NH<sub>2</sub> but possess distinct NH<sub>2</sub>-terminal sequences that convey receptor specificities.

Little is known about the biosynthesis of the tachykinins and the regulation of preprotachykinin (PPT) gene expression (4). Nawa *et al.* (5) have recently cloned cDNAs that correspond to two types of bovine PPT mRNAs ( $\alpha$  and  $\beta$ ) that are derived from one gene (5, 6). Because most information about the localization and functions of the tachykinins and their receptors has involved the rat nervous system, it is the system of choice for an analysis of tachykinin gene expression. It is important to identify the primary structure of rat PPT mRNAs, the precursor proteins encoded by these mRNAs, and the specific gene(s) encoding the PPT mRNAs as well as to understand its regulation. The development of a specific and sensitive assay for individual PPT mRNAs is also necessary. We report here progress made toward these goals, describing three rat PPT mRNAs that encode SP and NKA derived from a single gene; differential RNA processing

yields the structural differences of these peptide precursors. A preliminary account of this work has appeared (7).

### EXPERIMENTAL PROCEDURES

**Materials.** Avian myeloblastosis reverse transcriptase was obtained from J. Beard (Life Sciences, St. Petersburg, FL). Restriction endonucleases, terminal nucleotidyltransferase, DNA polymerase I and Klenow fragment of DNA polymerase I, T4 polynucleotide kinase, and SP6 polymerase were purchased from Bethesda Research Laboratories, New England Biolabs, or Promega Biotec (Madison, WI). S1 nuclease was from Sigma, and oligo(dC)-tailed *Sst* I-digested pUC19 was a gift of P. Rotwein (Washington University). Oligonucleotides 1 and 2 (Fig. 1) were from J. McKelvy (State University of New York, Stony Brook) and M. Zoller (Cold Spring Harbor, NY), respectively, whereas other oligonucleotides (Figs. 1 and 3) were synthesized by the Washington University Protein Chemistry Facility. Other chemicals were of the highest purity available. [ $\gamma$ -<sup>32</sup>P]ATP (>6000 Ci/mmol; 1 Ci = 37 GBq), [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol), [ $\alpha$ -<sup>32</sup>P]CTP (3000 Ci/mmol), and dATP[ $\alpha$ -<sup>35</sup>S] (1200 Ci/mmol) were obtained from Amersham.

**RNA Preparation and Construction of Rat Striatal cDNA Library.** The rostral portion of the caudate putamen complex (20–30 mg of tissue per animal; ref. 8) from 100 Sprague-Dawley rats (Holtzmann, Madison, WI) was used for RNA isolation by the guanidine isothiocyanate method (9). Poly(A)<sup>+</sup> RNA was isolated using oligo(dT)-cellulose chromatography (10). Double-stranded cDNA was synthesized from 2  $\mu$ g of poly(A)<sup>+</sup> RNA by reverse transcriptase and DNA polymerase I for first- and second-strand synthesis, respectively (11). The cDNA was size-fractionated [ $>550$  base pairs (bp)] and isolated, and homopolymer poly(dG) tails were added with terminal nucleotidyltransferase. Poly(dC) tails were similarly added to *Sst* I-digested pUC19. Hybridized cDNA/vector (12) was used to transform *Escherichia coli* DH-5 cells provided by D. Hanahan (Cold Spring Harbor, NY) (13). The recombinant plasmids resulted in 120,000–150,000 ampicillin-resistant transformants.

**Identification of PPT-Encoding cDNA Clones and Nucleotide Sequence Analysis.** The cDNA library was screened with an oligonucleotide (oligo 1, CCAAAGAAGCTGCTG) corresponding to the Gln-Gln-Phe-Phe-Gly region of rat SP and bovine PPT mRNAs (5), excluding the third nucleotide of the glycine codon. Oligonucleotides were 5' end-labeled and used for colony and blot hybridization (14). Melting temperatures (15) were calculated [ $T = 4(G+C) + 2(A+T)$ ]. Plasmid DNA was prepared (16), and inserts were excised at *Eco*RI/*Bam*HI sites. Sequences were analyzed using the Sanger method (17)

Abbreviations: SP, substance P; NKA, neurokinin A; PPT, preprotachykinin.

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with M13mp18 and M13mp19 templates, dATP[ $\alpha$ - $^{35}$ S] and buffer gradient acrylamide gels (18).

**RNA Blot Hybridization.** Poly(A)<sup>+</sup> RNA (4  $\mu$ g) was denatured in 6% (vol/vol) formaldehyde and electrophoresed on 1.5% agarose gels containing 6% formaldehyde (19). After electrophoresis the gel was soaked in 10 $\times$  standard saline citrate (1 $\times$  SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) for 30 min, and the RNA was transferred to nitrocellulose (20). Hybridizations were done with nick-translated cDNA, antisense cRNA, or a 45-mer oligonucleotide. Briefly, blots were preincubated at 55°C for 2 hr in hybridization buffer [50% formamide/0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.05 M Tris-HCl, pH 7.5/1 M NaCl/3.75 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>/1% (wt/vol) sarcosyl/10% (wt/vol) dextran sulfate containing 100  $\mu$ g of salmon sperm DNA per ml]. RNA-cDNA and RNA-cRNA blots were hybridized at 55°C for 18 hr in buffer containing 1  $\times$  10<sup>6</sup> cpm/ml [<sup>32</sup>P]cDNA (prepared by nick-translation, ref. 21) or [<sup>32</sup>P]cRNA probe (prepared by SP6 polymerase transcription from pG2SP31-1; specific activity, 4.8  $\times$  10<sup>8</sup> cpm/ $\mu$ g). The plasmid pG2SP31-1 was prepared by isolating the cDNA insert from pSP31-1 at the *Bam*HI/*Eco*RI sites of the polylinker and inserted it into pGEM2. After hybridization, membranes were washed four times with 250 ml of 0.1 $\times$  standard saline citrate containing 0.1% sarcosyl for 30 min at 65°C or 68°C for cDNA and cRNA probes, respectively. Oligonucleotide-RNA hybridization (exon 4 probe, residues 221–226 of  $\beta$ -PPT; Fig. 3) was done at 55°C for 18 hr. Membranes were washed four times with 250 ml 4 $\times$  standard saline citrate containing 0.1% NaDodSO<sub>4</sub> for 30 min at 65°C. Membranes were autoradiographed with Kodak XAR-5 film at –80°C with an intensifying screen.

**S1 Nuclease Protection Experiments.** Protection experiments for rat PPT mRNAs were done using the general methods of Favalaro *et al.* (22). Briefly, two cDNA fragments were isolated from pSP27-4 and were used as probes to protect the 5' and 3' ends of the  $\alpha$ - and  $\gamma$ -PPT mRNAs. An *Rsa* I fragment encompassing part of the pUC19 vector and 738 bp of the 5' end of the pSP27-4 insert was isolated, dephosphorylated with calf intestinal phosphatase and end-labeled with T4 kinase. A 661-bp *Nci* I/*Rsa* I fragment from pSP27-4 (nucleotides from –22 to 639) was isolated and end-labeled with T4 polymerase. The probes were hybridized with total RNA in 80% (vol/vol) formamide for 3 hr at 45°C or 46°C, respectively, and digested with 100 units of S1 nuclease at 37°C for 30 min.

**Southern Blot Hybridizations.** DNA isolated from Sprague-Dawley rat liver was used for genomic blot hybridizations (16). Hybridization was done with nick-translated [<sup>32</sup>P]cDNA insert from pSP27-4 (specific activity, 4.2  $\times$  10<sup>8</sup> cpm/ $\mu$ g). The filter was washed at 55°C using solutions described for RNA blots.

**Data Analysis.** Nucleotide sequences were analyzed with an IBM AT computer and Microgenie software (23); GenBank<sup>‡</sup> was used for nucleotide sequence homology searches.

## RESULTS

**Isolation and Identification of Rat PPT-Encoding cDNA Clones.** A cDNA library of 120,000–150,000 recombinants was constructed with striatal poly(A)<sup>+</sup> RNA and was screened for PPT-encoding cDNAs with oligonucleotides corresponding to SP and NKA sequences. Positive hybridization by SP-corresponding oligodeoxynucleotide CCAAA-GAAGTCTG was seen with 18 colonies. The colonies were isolated, and cDNA inserts were excised from the plasmid DNA. Partial nucleotide sequence analysis of plasmids pSP 15-1, 28-1, and 35-1 revealed homology to the 5' end of the

Table 1. Classification of rat tachykinin-encoding cDNA clones

cDNA	Insert, bases	SP probe	NKA probe	5'-UT probe	Hae III
pSP4-1	960	+	+	–	+
pSP10-1	860	+	+	–	–
pSP10-2	1020	+	+	+	–
pSP15-1	450	+	–	+	–
pSP17-1	960	+	+	–	+
pSP21-1	980	+	+	–	+
pSP21-2	1120	+	+	+	–
pSP27-1	1050	+	+	+	–
pSP27-2	740	+	+	–	–
pSP27-3	1050	+	+	+	–
pSP27-4	1130	+	+	+	+
pSP27-5	860	+	+	–	–
pSP28-1	980	+	+	–	+
pSP29-2	740	+	+	–	+
pSP30-1	850	+	+	–	+
pSP31-1	1100	+	+	+	–
pSP35-1	1020	+	+	+	–
pSP35-5	960	+	+	–	–

The SP and NKA probes are oligonucleotides 1 and 2, and the 5'-UT probe corresponds to residues –27 to –10 described in Fig. 2. UT, Untranslated region. +, Positive hybridization.

coding region of bovine PPTs (5). Southern blot hybridization analysis of 18 cDNA inserts, using the insert of pSP 28-1 labeled by nick-translation (21), demonstrated that the 18 cDNA inserts were homologous. Table 1 summarizes hybridization data of these cDNAs. Seventeen of 18 inserts hybridized to the oligonucleotide GCCATTAGTCCAACAAA-

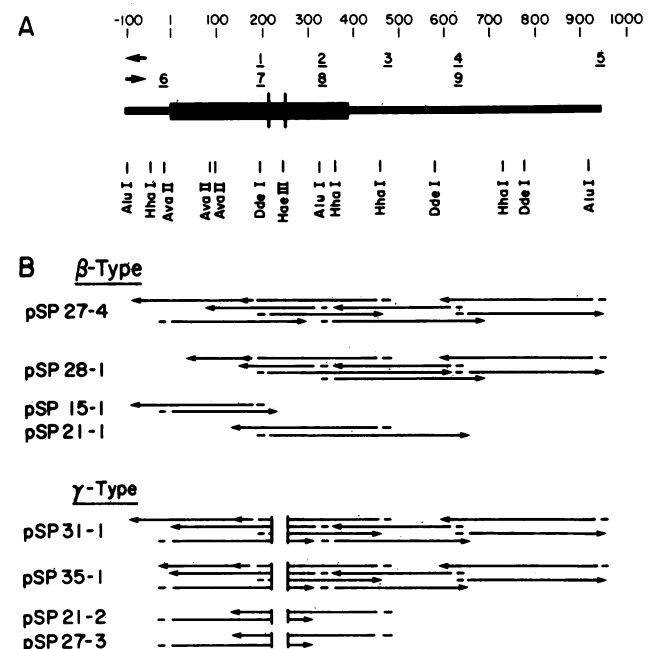


FIG. 1. Restriction map and DNA sequencing strategy for various cDNAs corresponding to  $\beta$ - and  $\gamma$ -PPT mRNAs. (A) The nucleotide length is numbered from the A of the AUG initiation codon. Arrows show the direction of nucleotide sequence analysis of M13 subclones using internal oligodeoxynucleotide primers, indicated by numbers 1–9. Oligonucleotides 1–5 were derived corresponding to residues 184–197, 305–324, 458–472, 619–633, and 924–936; and oligonucleotides 6–9 were derived corresponding to residues –21 to –7, 185–198, 310–324, and 619–633. The thick bar denotes the coding region, and the bracketed region within the coding region depicts a segment of 45 nucleotides expressed in the  $\beta$ -PPT mRNA. Selected restriction sites are indicated. (B) The extent of DNA sequencing for various cDNAs.

<sup>‡</sup>National Institutes of Health (1986) Genetic Sequence Databank: GenBank (Research Systems Div., Bolt, Beranek, and Newman, Inc., 10 Moulton St., Cambridge, MA 02238), Tape Release No. 40.0.



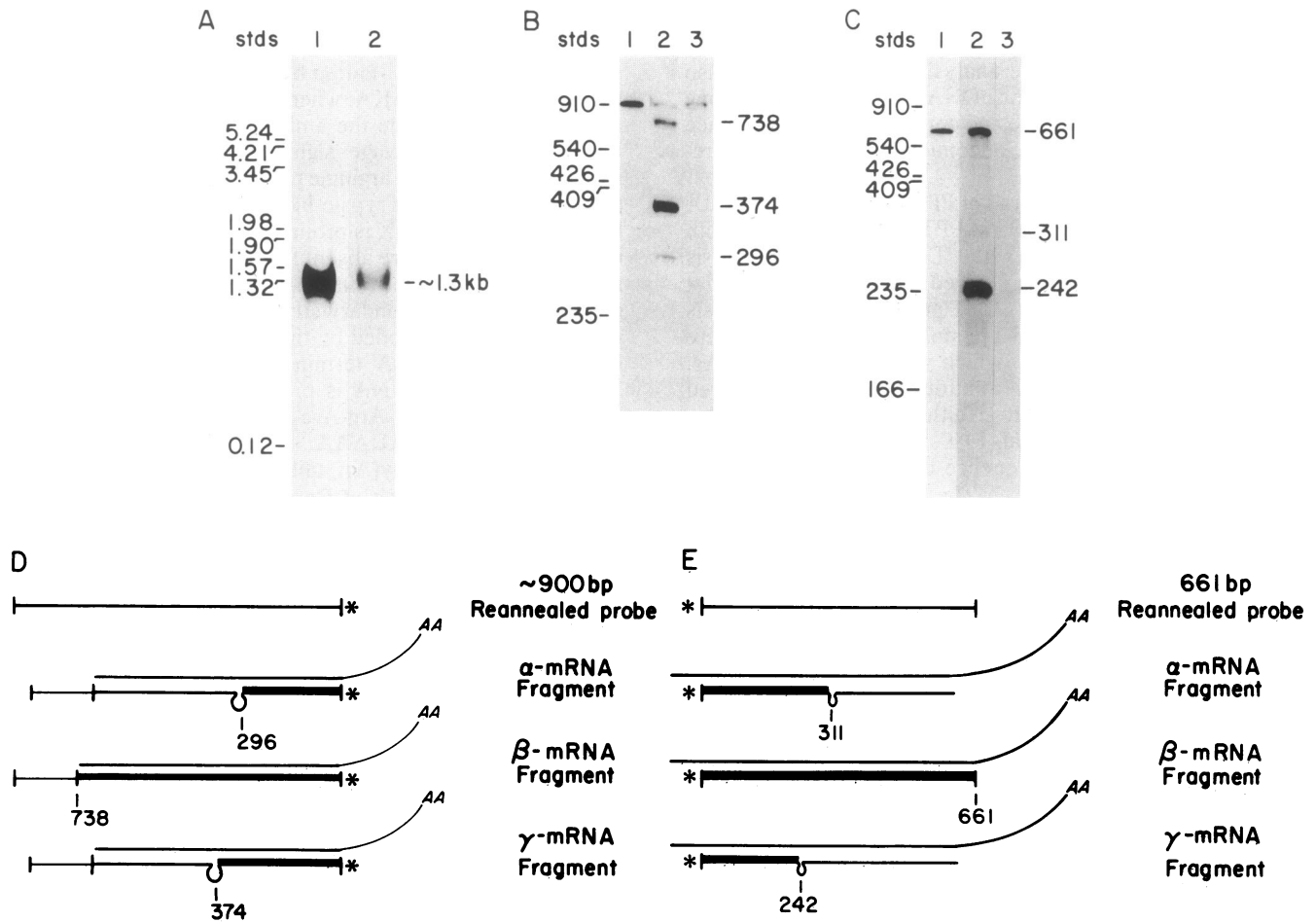


FIG. 3. Hybridization analysis of rat PPT mRNAs. (A) Striatal poly(A)<sup>+</sup> RNA (4 μg) was electrophoresed, blotted, and hybridized to a cRNA probe corresponding to γ-PPT mRNA (4.8 × 10<sup>8</sup> cpm/μg) (lane 1), or hybridized to an end-labeled oligodeoxynucleotide corresponding to position 221–266 of β-PPT (1.95 × 10<sup>8</sup> cpm/μg) (lane 2). Standards (stds) used were *Hind*III/*Eco*RI-restricted λ DNA, rat ribosomal RNA, and wheat germ tRNA. (B and C) S1 nuclease protection experiments. RNA from striatum or liver was annealed to a <sup>32</sup>P-labeled *Rsa* I (B) or to a <sup>32</sup>P-end-labeled *Nci* I/*Rsa* I (C) fragment isolated from pSP27-4, digested with S1 nuclease, and electrophoresed on a 6% polyacrylamide/7 M urea gel. Lanes: 1, <sup>32</sup>P-labeled probe; 2 and 3, protected species from the striatum and liver, respectively. Standards: B, <sup>32</sup>P-labeled *Dde* I-digested pUC19; C, <sup>32</sup>P-end-labeled *Nci* I/*Rsa* I-digested pSP27-4. (D and E) Diagrams of striatal PPT mRNAs protected in the S1 nuclease experiments shown in B and C.

2, striatal RNA with lane 3, liver RNA). In addition, another fragment of 296 bases is protected, apparently corresponding to the 3' end of a putative α-PPT mRNA. The presence of α-PPT mRNA was further established in another assay in which the end-labeled *Nci* I/*Rsa* I fragment protects a species of 311 bases (Fig. 3C, lane 2) that corresponds to the 5' end of α-PPT mRNA. Also protected are species of 661 and 242 bases that correspond to β-PPT mRNA and the 5' end of γ-PPT mRNA. Fig. 3 D and E shows a diagram of the protected RNA species.

**One Rat PPT Gene Encodes Three PPT mRNAs.** To determine the number of genes encoding SP and NKA, rat genomic DNA was digested with various restriction enzymes, electrophoresed, blotted, and hybridized with 5'- and 3'-specific cDNA probes derived from pSP27-4 (*Bgl* II/*Eco*RI and *Bgl* II/*Bam*HI fragments). These hybridization results demonstrate the existence of one PPT gene of less than 10 kilobases encoding α-, β-, and γ-PPT mRNAs. We have also isolated two Charon 4A phages containing this rat PPT gene (M.S.C. and J.E.K., unpublished results) from a rat genomic library, demonstrating that the rat haploid genome contains a single gene encoding SP and NKA.

The three rat PPTs, translated from mRNAs derived from one rat gene and differentiated through precursor (mRNA) splicing, are illustrated in Fig. 4.

### DISCUSSION

These investigations describe the primary structure of three rat PPT mRNAs. The cDNA sequences and the S1 nuclease

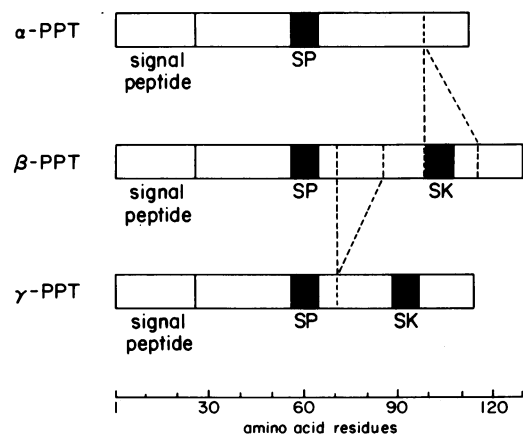


FIG. 4. Schematic of three rat PPTs encoding the neuropeptides SP and NKA or only SP. The regions encoding SP and NKA (SK, also known as substance K) are shown by solid boxes, and the signal peptide region is shown by open boxes. Dashed lines indicate the differentially spliced PPT coding regions.

protection experiments define  $\alpha$ -,  $\beta$ -, and  $\gamma$ -PPT mRNAs that encode PPTs of 112, 130, and 115 amino acid residues, respectively. The  $\gamma$ -PPT mRNA is the most abundant in rat brain. The  $\alpha$ - and  $\gamma$ -PPT precursors differ from  $\beta$ -PPT by the absence of either an 18-amino acid peptide that produces NKA or by a pentadecapeptide sequence COOH-terminal to the SP sequence processing sites. These three PPT mRNAs are derived from a single rat PPT gene encoding SP and NKA. The size of the PPT mRNAs is  $\approx$ 1.3 kb, and the mRNAs are not completely resolved by denaturing vertical agarose gels. Quantitative S1 nuclease protection experiments demonstrate that  $\beta$ -PPT and  $\gamma$ -PPT mRNAs comprise 95% of the total PPT mRNA in rat striatum. In addition, a minor PPT mRNA species was detected that corresponds to  $\alpha$ -PPT mRNA isolated from bovine brain (5). As yet we have not isolated a cDNA corresponding to the rat  $\alpha$ -PPT mRNA, due to its low abundance. Thus, all three PPT mRNAs in rat striatum encode SP, whereas  $\beta$ - and  $\gamma$ -PPT mRNA encode SP and NKA. It is clear that rigorous analysis of PPT gene expression necessitates methods able to discriminate among the various PPT mRNAs.

The rat PPT mRNAs and gene structurally resemble the bovine PPT mRNAs and gene (6). From a computer-derived alignment of rat  $\beta$ -PPT mRNA and bovine  $\beta$ -PPT mRNA, homologies of 91.1%, 50.4%, and 64.4% were observed for the coding, 5'-untranslated, and 3'-untranslated regions, respectively. At the protein level, a 93.1% homology was found. Rat PPT mRNA sequences showed no homology to other sequences in GenBank.

The three rat PPT mRNAs are derived from one gene that contains seven exons responsible for PPT mRNA. It is remarkable that the nucleotide regions absent in  $\alpha$ - and  $\gamma$ -PPT mRNA are encoded by exon 6 (54 nucleotides) and exon 4 (45 nucleotides) of the rat PPT I gene (M.S.C. and J.E.K., unpublished results). The expression of three PPT mRNAs from the rat PPT I gene must result from alternative RNA processing. A third mammalian tachykinin, neurokinin B (3), is produced by the expression of a different PPT gene.

The existence of mRNAs encoding three different PPTs suggests regulation of tachykinin gene expression at the level of precursor processing. Tachykinin peptides SP and NKA can be produced from both  $\beta$ - and  $\gamma$ -PPT, whereas only SP can be produced from  $\alpha$ -PPT mRNA. These tachykinins would be liberated from the PPTs after endoproteolysis and appropriate post-translational processing. Recently, Tatamoto *et al.* (30) have isolated a peptide from porcine brain that corresponds to an NH<sub>2</sub>-terminally extended derivative of NKA, termed neuropeptide K. This peptide, which possesses partially different biological activities with different time courses compared with NKA (30), can only be derived from  $\beta$ -PPT, because it contains amino acid residues 72–107 from  $\beta$ -PPT and is not present in either  $\alpha$ - or  $\gamma$ -PPT. The secondary structure predicted for rat  $\beta$ -PPT is that amino acid residues 72–88, the region present in  $\alpha$ - and  $\beta$ -PPT but absent in  $\gamma$ -PPT, display a completely  $\alpha$ -helical structure. This region may allow for different patterns of post-translational processing due to precursor folding to produce neuropeptide K, or the region may harbor a biologically active peptide *per se*. In addition, this region contains paired leucine residues at positions 83–84 in  $\beta$ -PPT that may represent a processing site, because in the glycopeptide region of the vasopressin/neurophysin common precursor, protein cleavage occurs beyond the carboxyl terminal of paired leucine residues (31). Paired leucine residues also occur at residues 51–52 of  $\beta$ -PPT. Furthermore, other so-called spacer peptide regions of the PPTs may have biological functions themselves, and the different PPTs, like proopiomelanocortin (32), may be polyproteins in which the biosynthesis and/or processing of each

peptide region is regulated in a tissue-specific manner. The function of these other peptide regions in the PPTs represents an important area for further investigation.

These experiments demonstrate the existence of three mRNA species encoding the rat PPTs. The deduced structures of the precursors should provide testable hypotheses for post-translational processing studies, and the cDNAs should be useful tools for the examination of PPT gene expression and its regulation.

This work was supported in part by National Institutes of Health Grant NS 21937 and the Pew Memorial Trust. J.E.K. is a Pew Scholar in the biomedical sciences.

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