Identification and cDNA sequence of δ-preprotachykinin, a fourth splicing variant of the rat substance P precursor

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The neuropeptides substance P and neurokinin A are synthesised from a family of precursor polypeptides encoded by the preprotachykinin A (PPT) gene. In addition to a mRNA (β-PPT) containing all 7 exons of the gene, alternatively spliced mRNAs lacking either exon 4 (γ-PPT) or exon 6 (α-PPT) have been identified. We have determined the sequences of cDNA clones encoding four variants of PPT mRNA from rat dorsal root ganglion (DRG), including a novel mRNA species (δ-PPT) in which both exons 4 and 6 are absent. The sequence of δ-PPT predicts the existence of a novel tachykinin precursor polypeptide.

cDNA cloning; PCR; RNA splicing; Substance P; Tachykinin; Rat dorsal root ganglion

1. INTRODUCTION

The neuropeptides, substance P and neurokinin A, are synthesized from a family of precursor polypeptides encoded by the preprotachykinin A gene [1]. In rat and bovine tissues, the largest form of PPT mRNA (β-PPT) contains regions derived from all 7 exons of the corresponding gene, with sequences in exon 3 encoding substance P and sequences in exon 6 encoding neurokinin A.

Alternatively spliced forms lacking either exon 6 (α-PPT) or exon 4 (γ-PPT) are also found [2–5]; in all rat tissues so far examined, γ-PPT is the most abundant form of PPT mRNA [5–8]. We report here the characterisation of PPT splicing products in rat DRG, using the polymerase chain reaction (PCR). We have identified a novel and relatively abundant splicing variant of PPT mRNA lacking both exons 4 and 6 (δ-PPT), which encodes a predicted polypeptide containing the sequence of substance P but not of neurokinin A. The expected processing products of rat δ-PPT include a C-terminal peptide of 22 amino acids unique to the δ-PPT precursor.

2. EXPERIMENTAL

2.1. *cDNA synthesis*

DRG were dissected from adult male rats (Ham Wistar; 200–250 g) and RNA was isolated by the guanidinium thiocyanate/caesium chloride method [9]. Poly(A)$^+$ RNA was isolated by chromatography on oligo(dT) cellulose (kit obtained from Pharmacia). Single-stranded cDNA was reverse transcribed from 10 μg total RNA or 5 μg poly(A)$^+$ RNA using a commercially available kit (Amersham International) with oligo(dT) as the primer.

2.2. *Amplification of PPT cDNAs by PCR*

The oligonucleotides (synthesised by Oswel DNA service, University of Edinburgh) used for PCR were 5′-AGAATTCAACATGAAA-ATCCTCGTG-3′ (Oligo 1: corresponding to a region in exon 2 which includes the initiator codon ATG of rat PPT mRNA, with an *Eco*RI restriction site introduced at bases 2–7) and 5′-TGGATCCTCCGCG-GACACAGATGGAGAT-3′ (Oligo 2: complementary to a region in exon 7 of rat PPT mRNA immediately 3′ to the termination codon, with a *Bam*HI restriction site introduced at bases 2–7). Reactions contained 10 ng single-stranded cDNA, 200 pmol of each oligonucleotide, 200 μM dATP, dCTP, dGTP and dTTP and 2.5 units Amplitaq DNA polymerase in 100 μl PCR buffer (Perkin-Elmer Cetus). 40 cycles of PCR (45 s at 94°C, 45 s at 50°C, 2 min at 72°C) were performed and polymerisation was continued for a further 5 min at 72°C at the end of the last cycle. PCR products were analysed by electrophoresis on 2% agarose or 5% polyacrylamide gels.

2.3. *Cloning and sequence analysis*

PCR products were cleaved with *Bam*HI and *Eco*RI and inserted between the corresponding restriction sites of the plasmid pGem3 (Promega). Clones were sequenced on both strands by the method of Sanger et al. [10] after subcloning into bacteriophages M13 mp18 and M13 mp19.

3. RESULTS

3.1. *Characterisation of PPT splicing products in rat DRG*

To investigate the forms of PPT mRNA in rat DRG, cDNA synthesised from DRG poly(A)$^+$ RNA was amplified by PCR and the products analysed by polyacrylamide gel electrophoresis (Fig. 1). The PCR products were resolved into three bands of approximately 450, 400 and 350 bp. The size of the largest cDNA was close to that predicted for the PCR product of β-PPT (454 bp) whereas the band at ~400 bp was

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Abbreviations: cDNA, complementary DNA; mRNA, messenger RNA; PCR, polymerase chain reaction

The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession number no. X56306

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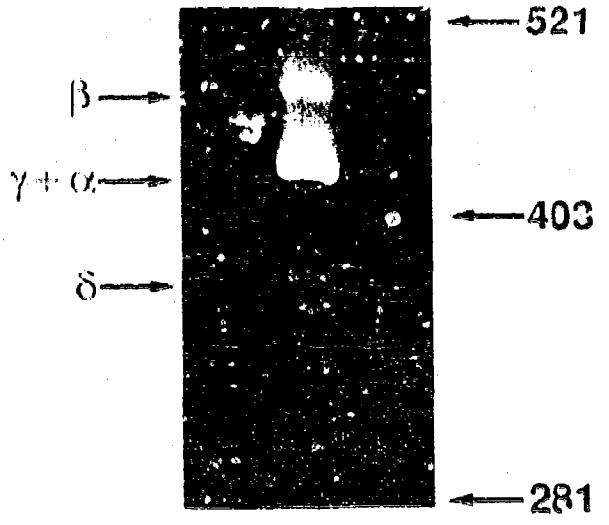

Fig. 1. Polyacrylamide gel electrophoresis of products obtained by PCR amplification of PPT cDNA. cDNA was synthesised from 5 μg poly(A)$^+$ RNA and amplified by PCR as described in the text. Arrows indicate the positions of α-, β-, γ- and δ-PPT and the position and size (in bp) of DNA standards (*Alu*I digested pBR322) run on the same gel.

consistent with the presence of γ- and α-PPT (predicted PCR products of 409 bp and 400 bp, respectively). The size of the smallest PCR product (350 bp) was consistent with a novel splicing variant lacking both exons 4 and 6 (δ-PPT: predicted size 355 bp). Densitometry indicated α- and γ-PPT together constituted some 70% of total PPT mRNA in DRG; the remaining 30% consisted of δ-PPT and β-PPT in approximately equal amounts.

3.2. *Identification and sequence of a novel PPT cDNA*

To establish whether the PCR product of ~350 bp represented a novel splicing variant, PCR amplified cDNA was cloned into the vector pGem3 and clones were characterised by restriction analysis. Clones were classified by insert size and by the presence or absence of a restriction site for *Dra*I, which restricts PPT cDNA once in exon 6 and therefore cleaves β- and γ-PPT but not α-PPT. Clones containing inserts of 4 size classes were obtained. The majority of clones contained inserts with the properties predicted for γ-PPT (inserts of ~410 bp with an internal *Dra*I site). In addition, clones with the properties expected of β-PPT (insert ~450 bp, internal *Dra*I site), α-PPT (insert ~400 bp, no *Dra*I site) and the proposed δ-PPT variant (insert ~350 bp, no *Dra*I site) were found at a lower frequency.

Representative clones of each of the four size classes were sequenced (Fig. 2): clones corresponding to α-, β- and γ-PPT were identified, together with clones with the structure predicted for δ-PPT. The sequences of α-, β- and γ-PPT were identical to those reported previously [4,5], whilst the δ-PPT clones contained sequences corresponding to exons 2, 3, 5 and 7. The sequence of δ-PPT encodes a novel PPT polypeptide of 115 amino acids (M_r 11 375).

4. DISCUSSION

We have shown that in rat DRG, as in other rat tissues [6–8], γ-PPT is the predominant splicing variant of PPT mRNA, but β-PPT and a novel splicing variant, δ-PPT, are also present in significant amounts. The order of abundance of the four splicing variants of PPT mRNA in DRG, estimated by polyacrylamide gel electrophoresis of PCR-amplified cDNA and from the observed frequency of the four size classes of cloned cDNA, was γ-PPT ≥ β-PPT ≅ δ-PPT ≥ α-PPT. In previous studies [4–5] PPT clones lacking exon 6 and presumed to encode α-PPT were identified in rat brain cDNA libraries either by restriction enzyme analysis [4] or by sequencing of partial cDNA clones [11]. Our results suggest that these clones may have encoded δ-PPT. Although the presence of α-PPT mRNA at low levels in rat tissues has been confirmed by nuclease protection assays using a synthetic construct with the structure proposed for rat α-PPT [12], we have obtained the first direct sequence data for rat αPPT cDNA.

In bovine tissues, the splicing of PPT mRNA has been reported to differ between tissues, with β-PPT predominating in nervous tissue and α-PPT predominating in thyroid and gut [3]. In contrast, γ-PPT is the most abundant form of PPT mRNA in the rat, and the splicing pattern of PPT gene transcripts has been reported to be relatively constant in all tissues studied [5–8]. These data were obtained from nuclease protection assays [12] using either end-labelled cDNA probes (which would not detect δ-PPT mRNA) or uniformly-labelled cRNA probes (where the only indication of the presence of δ-PPT would be a 45 bp RNA fragment that could easily be overlooked). In the light of our findings, the tissue distribution of the splicing variants of PPT mRNA and the possibility that splicing can be regulated by physiological stimuli should be re-examined.

The products of post-translational processing of the δ-PPT polypeptide may be predicted on the basis of previous studies [13,14]. Two polypeptides are likely to be produced in addition to substance P; a N-flanking peptide of 37 amino acids encoded by all four splicing variants of PPT mRNA [14] and a C-flanking peptide of 22 amino acids (amino acids 72–93 of the δ-PPT polypeptide) which is encoded uniquely by δ-PPT mRNA. The findings of McGregor et al. [13] may provide some evidence for the expression of the δ-PPT polypeptide in rat tissues. In chromatographic studies of peptides immunoreactive with antisera to the C-terminus of the PPT polypeptide they found evidence for the presence of the predicted C-flanking peptide of β-PPT but not for the corresponding processing product of α-PPT. An additional peptide detected in these

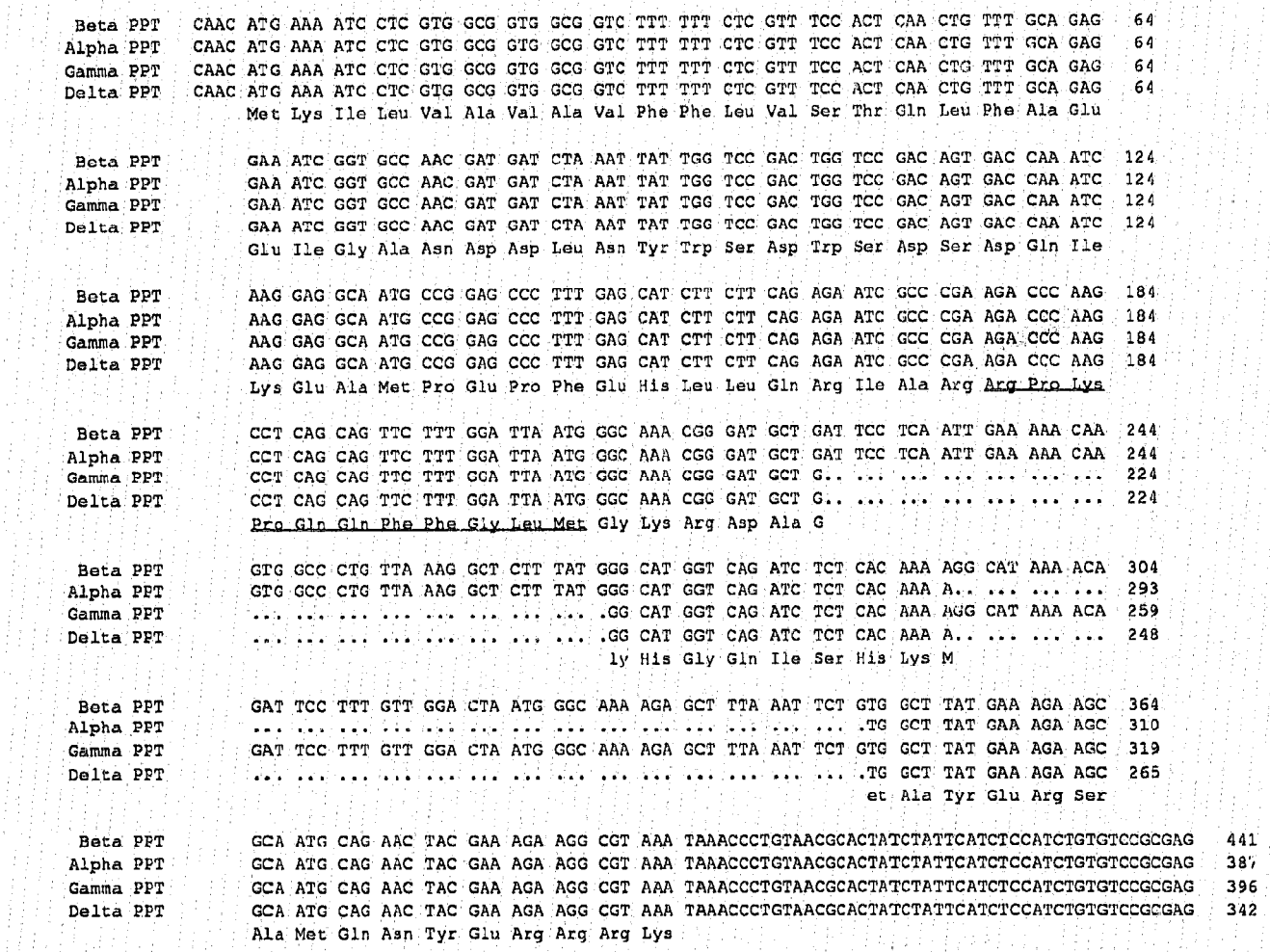

Fig. 2. Nucleotide sequences of cDNA inserts coding for rat α-, β-, γ- and δ-PPT. The predicted amino acid sequence of rat δ-PPT is indicated below the aligned cDNA sequences: the sequence of substance P is underlined.

studies but not fully characterised may correspond to the C-flanking peptide of δ-PPT. The existence and possible biological activity of this peptide remain to be confirmed.

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