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Isolation and Characterization of the Rat Prolactin-Releasing Peptide Gene: Multiple TATA Boxes in the Promoter Region

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The prolactin-releasing peptide (PrRP) gene is a novel bioactive peptide expressed in very restricted regions in the brain. To explore the molecular mechanism of PrRP gene expression, we cloned and characterized the gene and its promoter region. The gene spans approximately 2.4 kb and contains three exons and two introns. 3′RACE analysis showed that a polyadenylation signal 103 bp downstream from the stop codon was functional. Primer extension analysis indicated three transcriptional start sites (TSSs) 92, 199, and 325 bp upstream from the translational start site. Interestingly, in addition to the putative binding sites for SP1-1, AP-2, and Oct-2A, three characteristic TATA boxes were identified close to these TSSs. Transient transfection study using a series of deletion mutants revealed that the middle TATA box is important for the promoter activity. Furthermore, the cloned 1.6 kb promoter region was active only in neuron- and pituitary-derived cell lines, and the promoter region −1600∼−800 bp worked as a negative regulatory element. We demonstrated for the first time, the genomic organization and promoter function of the PrRP gene, and this knowledge will facilitate elucidation of transcriptional control of the PrRP gene.

Key Words: **PrRP; gene; promoter.**

Prolactin-releasing peptide (PrRP) is a newly identified peptide, which is isolated from bovine hypothalamic tissues as a ligand of an orphan seven transmembrane domain receptor (hGR3) expressed extensively in the anterior pituitary (1–3). From the analysis of the rat PrRP cDNA, it was expected that two isoforms PrRP31 and PrRP20 would be generated, and PrRP20 was corresponding to the C-terminal 20 residues of PrRP31. Although both have been demonstrated to have similar abilities to cause the secretion of prolactin from the rat anterior pituitary, the function of PrRP as a physiological regulator of prolactin secretion remains controversial (4–6). Furthermore, alternative roles for PrRP such as acute effect on food intake and body weight, stimulation of oxytocin release, and stimulation of ACTH secretion from the pituitary have been recently reported (7–9).

Northern blot analysis of PrRP mRNA showed its expression specifically in the human medulla oblongata, and recent *in situ* hybridization studies indicated its expression in more restricted brain regions including the caudal part of the dorsomedial nucleus of the hypothalamus, the caudal part of the solitary tract nucleus, and in the caudal ventrolateral medulla (10–12).

As an initial step towards elucidation of the mechanism of expression of the PrRP gene, we cloned its gene and characterized its promoter region.

MATERIALS AND METHODS

Isolation of rat prolactin-releasing peptide genomic clones. A rat spleen genomic DNA library (Lambda FIX II; Stratagene, USA) was screened using a ^{32}P-labeled rat PrRP cDNA (PrRP1). The PrRP1 cDNA was generated by PCR using the primers, 5′-atggccctgaagacgtggcttct-3′, and 5′-ttatccacgctgagagaacttggtg-3′, and rat medullar oblongata cDNA as a template. Filter hybridization and restriction endonuclease mapping were performed using the previously described method (13). All hybridized genomic fragments were sequenced using a PRISM model 310 auto sequencer (Applied Biosystems, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR) and 5′ rapid amplification of cDNA end. Primers were designed to span each exon/exon boundary. Amplification products were subcloned into the pGEMT-Easy plasmid (Promega, USA) and sequenced as above.

The nucleotides sequences reported in this paper will appear in the DDBJ, and GenBank/EMBL Data Bank with Accession Nos. AB040611, AB040612, and AB040613.

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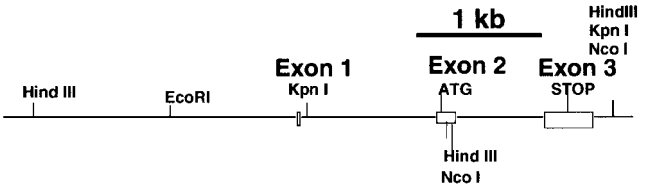

FIG. 1. Schematic representation of the rat PrRP gene. Boxes denote exons, while thin lines denote introns and flanking regions. ATG represents the translational initiation site; STOP, stop codon.

To obtain the 5′ portion of the rat PrRP cDNA, 5′ RACE (rapid amplification of cDNA ends) was performed using rat hypothalamus Marathon Ready cDNA (Clontech, USA).

Primer extension analysis. To determine the transcriptional start site of the PrRP gene, primer extension was carried out using the synthetic oligonucleotide PE1 (+93∼+117, the most downstream TSS was numbered as +1), 5′-acagaagccacgtcttcagggccat-3′, PE2 (−141∼−117), 5′-ctcttctctcatgaag acactcgtc-3′ and PE3 (+1∼+25), 5′-tgagtggtgataaggctgcctgggc-3′. The oligonucleotides were end-labeled with [γ-^{32}P]ATP, hybridized to 5 μg of poly(A)$^+$RNA extracted from the rat medulla and extended using AMV reverse transcriptase. Forty micrograms of yeast tRNA was used as a negative control. The primer-extended products were separated on an 8 M urea 6% polyacrylamide gel which was then dried and exposed to Kodak XAR-5 film. The sizes of the resulting labeled primer-extended products were inferred from their positions relative to ϕx174 RF DNA digested with *Hae*III and a sequencing ladder which was obtained using the same primer (PE1) and 5′RACE product as a template. For primer extension with primers PE2 and PE3, a sequencing ladder was obtained using a primer, PE4 (−8∼−32), 5′-gaggcctgcctggctgtgcaccttt-3′ and a cloned genomic DNA as template.

Analysis of the 3′ untranslated region by 3′ rapid amplification of cDNA ends (3′RACE). To elucidate functional polyadenylation signals, 3′ RACE was performed with total RNA from the medulla oblongata and primers below; an oligo(dT)$_{17}$ + adapter primer (5′-gactcctgcagacatcg attttttttttttttttt-3′), adapter primer (5′-gactcctgcagacatcga-3′), and a sequence-specific primers RE1, 5′-atggcccctgaagacgtggcttct-3′ and RE2, 5′-ggcttccagccgagcccaccag-3′ (13).

Cell culture and transfection. HTB-185, HTB-10, CV-1, C6, and GH_4C_1 cells were cultured in Dulbecco's modified Eagle medium or RPMI 1640 supplemented with 10% (v/v) fetal bovine serum. These cell lines were derived from human cerebellum-medulloblastoma, human neuroblastoma, monkey kidney, rat glioma, and rat pituitary tumor, respectively. Transient transfection was performed by the calcium phosphate precipitation method with 3 μg of reporter construct. The cells were then harvested after a further 48 h. Luciferase assay was performed as described previously (14). For neuron-derived HTB-185 and HTB-10 cells, pfx-6 (Invitrogen, USA) was used for transfection.

Plasmid construction. The pGL3 basic vector (Promega, USA) is a promoterless luciferase expression vector. The pGLTK contains a thymidine kinase (TK) promoter sequence linked to pGL3 basic and was used to monitor transfection efficiency in each cell line and as an internal standard between different experiments. The rat PrRP gene *Hin*dIII-*Kpn*I fragment containing approximately 1.6 kb of the promoter region, exon 1, approximately 250 bp first intron, was subcloned into the pGL3 basic vector and named pHK1.6Luc. In the pEK0.8Luc, the *Hin*dIII-*Eco*RI 0.8 kb fragment was deleted from the pHKL1.6Luc. To generate a series of deletion mutants of TATA boxes, a sense primer 5′-atcgataagcttttctacaggaat-3′ and antisense primers, TA1 5′-tgaggtggtgataagcttgcctggc-3′, TA2 5′-gccaacaagaagcttaagacgccccc-3′, or TA3 5′-actgcgaagcttctctcatgaagaca-3′, corresponding to downstream of the TATA box at -32, at -92 and at -242, respectively, were used for PCR amplification and the plasmid pHKL1.6 as the template. These plasmids were named pTA1, PTA2, and pTA3, respectively.

Statistical analysis. Statistical analysis was performed by ANOVA and Duncan's multiple range test.

RESULTS

Comparison of the genomic sequence with the cDNAs obtained by RT-PCR and 5′ RACE established the organization of the rat PrRP gene. The gene consisted of three exons and two introns that were flanked by typical splice donor and acceptor sequences (Fig. 1). A short exon 1 encoded only the 5-untranslated region and was located approximately 1.0 kb upstream from exon 2. Exon 2 started 56 bp upstream of the ATG translation initiation codon and encoded 97 base pairs of the coding region. Following intron 2, approximately 650 base pairs in length, exon 3 encoded the rest of the coding sequence and the entire 3′ untranslated region (Fig. 2).

Sequence analysis of the 3′ RACE product indicated a single functional polyadenylation signal (AATAAA) 103 bp downstream of the stop codon in the rat medulla oblongata, and the adenine residue 22 bp downstream of the signal was the polyadenylation site (Fig. 3A).

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-300 gcgtgaagaggggaggttaatgtgttttaaagcacttggcccaaatgaa
                                          CREB
-250 attatataattcaggaag*gaacaaatcacatcactgacgagaggggtttt

-200 tgttgggtgtttttttttttttttaaggtaaaaagaaaggaaaagaagaag
                        oct2A                  *
-150 aaaaaaaaagacgagtgtcttcatgagagaagaggcgcagtgggaactgc
                                              SP1
-100 aggctataagcaaagacgccatctcaaggaggggaccgggggcgtctttt
                                                   SP1
-50  gctccttgttggcctataaaggtgcacagccaggcaggcctcacctcac
      * 1st exon
+1   GCCCAGGCAGCCTTATCACCACTCAAGGGACAGAGGgtgagtaacttggc
     agtgctggagaggggtgggtgttgctgtgacttctgcccggcttactggg
     tagccaggtgccggccccaaacaggacaagcagagagagctgcctgtggt
     ctgtg--------(1st Intron, approx. 1.0 kb)--------
     aaaccacctagggtccctcctggctttgtgtatacagctcacccttgcac
               2nd exon
     ctctacccccagGCTGCACCATCCCCTGTGGGATCCGAACTGCCGGGCAT
+75  CATCCCGGAAGACGGAGCATGGCCCTGAAGACGTGGCTTCTGTGCTTGCT
                    M  A  L  K  T  W  L  L  C  L  L
+125 GCTGCTAAGCTTGGTCCTCCCAGGGGCTTCCAGCCGAGCCCACCAGCACT
       L  L  S  L  V  L  P  G  A  S  S  R  A  H  Q  H
+175 CCATGGAGACAAGAAgtgagtgcctgacttatggaaacagcctctgacat
     S  M  E  T  R
     ccttccatccattcacttcccagatgccgtgagtgtccaaggcatggggc
     ttggccatctcctaagccagttgttccc-----(2nd intron,
     approx. 650bp)---gcttaggggctctgtgtggggaacactgcaccc
                                                3rd exon
     gtgtgtcaggcgtcccatacgctgatctggttctttcatttccagCCCCT
                                                  T  P
+195 GATATCAATCCTGCCTGGTACACGGGCCGCGGGATCAGGCCTGTGGGCCG
      D  I  N  P  A  W  Y  T  G  R  G  I  R  P  V  G  R
+245 CTTCGGCAGGAGAAGGGCAACCCCGAGGGATGTCACTGGACTTGGCCAAC
       F  G  R  R  R  A  T  P  R  D  V  T  G  L  G  Q
+295 TCAGCTGCCTCCCACTGGATGGACGCACCAAGTTCTCTCAGCGTGGATAA
     L  S  C  L  P  L  D  G  R  T  K  F  S  Q  R  G
+345 caccccagctcgagaagacagtgctgctgagcccaagcccacactccctg
+395 tcccctgcagaccctcctctaccctccctctcctctgctttctctctaat

+445 ctaataaaagtgttggctttgtttactct*acacctgtaactgcgcggtaa
     caactggaagatgttgtctctccc
```

FIG. 2. Nucleotide sequence of the rat PrRP gene. Exons are shown in upper case letters. Introns and 3′ untranslated region are indicated in lower case letters. Proposed transcriptional initiation sites are shown with an asterisk. The TATA boxes and polyadenylation signal are underlined.

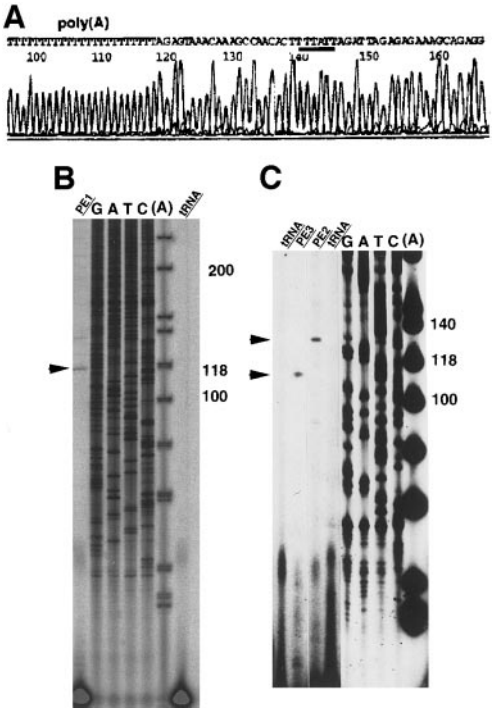

FIG. 3. 3′RACE and primer extension study of the rat PrRP gene. (A) Representative sequence data from the 3′RACE study. (B) Primer extension using the oligonucleotide PE1. (C) Primer extension study with PE2 (lane PE2) and PE3 (lane PE3) primers.

Although electrophoresis analysis of 5′RACE products showed three major amplified fragments, sequence analysis of these fragments indicated several 5′ ends of PrRP cDNAs within approximately 200 bp (data not shown). Therefore, to determine the exact site of transcription of the rat PrRP gene, we used three different primers, PE1, PE2, and PE3 covering this region in the primer extension analysis. Each primer extension with 5 μg of poly(A)$^+$RNA from rat medulla oblongata gave three different strong signals at 92, 199, and 325 bp from the translational initiation site, respectively, while no significant signals were found in yeast transfer RNA (Fig. 3B). We numbered the most downstream TSS as +1. Inspection of the sequence of the promoter region indicated three typical TATA boxes within 250 bp (at −32, at −92, and at −242) (Fig. 2). Characteristic poly(dA) sequences around −150 and poly (dT) sequences around −180 were observed, and several possible regulatory elements were identified including complete sequence matches for Sp1, AP-2, and OCT-2A binding sites (Fig. 2).

To determine whether the putative promoter region is functional, a fragment containing approximately 1600 bp of the promoter region was subcloned into a luciferase reporter plasmid and transfected into five different cell lines. As shown in Fig. 4, the strongest promoter activity of the PrRP gene with approximately 17% of TK promoter was observed in HTB-185 cells. The activity in GH4C1 cells was 12%. Lower activity (less than 10% of TK) was observed in CV-1, HTB-10 and C6 cells. In HTB-185 cells, deletion of the region between approximately −1600 and −800 -bp resulted in a significant increase in promoter activity (pEK0.8Luc) (Fig. 4B).

To determine which TATA box among the three of them was important for the promoter activity, we used three different plasmids deleting TATA boxes in series. Although promoter activity of the pTA2 excluding the TATA box at −32 was similar to those of pHK1.6Luc and pTA1 that included all TATA boxes, the pTA3 deleting both TATA boxes at −32 and −92 showed only 19% of that of pHK1.6Luc (Fig. 4B)

DISCUSSION

In the present study, we established the complete structure of the rat PrRP gene which contains three exons and two introns and spans a region of approximately 2.4 kb. Analysis of the 5′RACE study showed a short exon in the untranslated region. On the basis of the analyses for rat PrRP cDNA, it was expected that two isoforms PrRP31 and PrRP20 would be generated, and PrRP20 would correspond to the C-terminal 20 residues of PrRP31. PrRP31 and 20 have both been demonstrated to have similar abilities to induce ara-

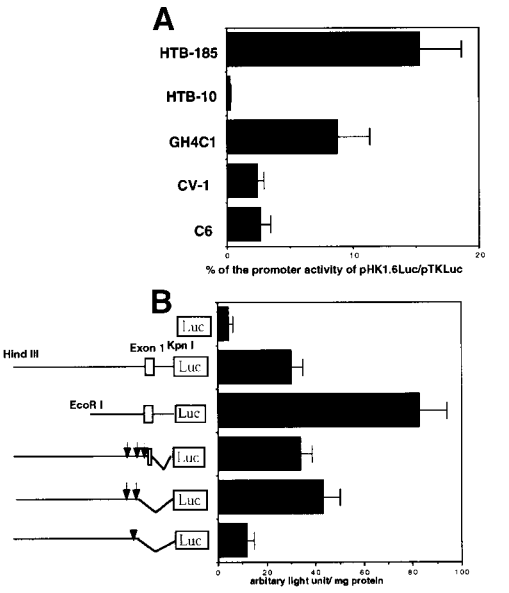

FIG. 4. Analysis of the rat PrRP gene promoter. (A) pHK1.6Luc was transiently transfected into HTB-185, HTB-10, GH4C1, C6, and CV-1 cells. Values represent means ± SE. of triplicate determinations. At least three independent experiments were performed. (B) HTB-185 cells were transiently transfected with the indicated plasmids. Schematic representation of the plasmid are shown on the left. The arrows indicate TATA box, Luc for luciferase cDNA, and the box for exon 1.

chidonic acid metabolite release from the rat anterior pituitary cells. It is of interest to notice that the second intron, approximately 650 bp in length, was located at the cleavage site of PrRP30 and 20.

Sequence analysis of the PrRP promoter region revealed three characteristic TATA boxes within 200 base pairs, and the primer extension study using the rat medulla oblongata mRNA showed three major transcriptional start sites close to these TATA boxes. However, sequence analysis of the 5′RACE study showed several other 5′ends of PrRP cDNAs within this region. These findings of 5′RACE may be due to the premature extension of cDNA or hairpin loop formation of the priming of the second strand of cDNA. However, considering the high sensitivity of the PCR technique, there is still the possibility of other minor transcriptional start sites of the PrRP gene.

To explore how complexes of these TATA boxes worked for the promoter activity, the transient transfection study was performed using a series of deletion mutants of the TATA boxes. The findings showed the TATA box at −32 was not required for the initial promoter activity of the rat PrRP gene. In contrast, additional deletion of the TATA box at −92 almost abolished its promoter activity, indicating that TATA at −92 is essential for the expression of the PrRP gene. Furthermore, the PrRP gene showed significant promoter activity only in the HTB-185 and GH4C1 cells, and lower activity was observed in other cell lines examined. Interestingly, Zhang *et al.* recently reported expression of PrRP mRNA in the normal human pituitary and pituitary adenomas (15). Therefore, the cloned promoter region of the PrRP gene in the present study may be responsible for tissue-specific expression of the PrRP gene. In addition, deletion of −1600∼−800 bp of the PrRP gene promoter region significantly increased the promoter activity indicating a negative regulatory element such as a repressor in this region.

Although the exact mechanism involving strict expression of PrRP gene remains to be determined, these characteristic futures of the rat PrRP gene may contribute to expression of PrRP gene in the brain.

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