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Differential splicing of the growth hormone-releasing hormone gene in rat placenta generates a novel pre-proGHRH mRNA that encodes a different C-terminal flanking peptide

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Abstract We have isolated and characterized a novel rat placental pre-proGHRH mRNA (pre-proGHRH-2 mRNA). This mRNA is generated by an alternative splicing process which results in the presence of an additional exon of 156 bp (designated exon 4.5) located between exons 4 and 5 of the previously reported hypothalamic and placental pre-proGHRH mRNA (pre-proGHRH-1 mRNA). Since the sequences encoding mature GHRH are included within exons 3 and 4, the processing of pre-proGHRH-2 would not affect the synthesis of mature GHRH but would generate a C-terminal peptide (designated GCTP-2) different from that previously reported in the hypothalamus and placenta (GCTP-1). The putative GCTP-2 has 64 amino acids, and the first 18 N-terminal residues are identical to those present in GCTP-1 (30 amino acids long). Pre-proGHRH-2 mRNA has not been detected in the hypothalamus.

Key words: Gestation; Gene expression; Hypothalamus; Regulatory peptide

1. Introduction

Growth hormone-releasing hormone (GHRH) is a hypothalamic peptide that plays a critical role in controlling the synthesis and secretion of growth hormone by the anterior pituitary. Human GHRH is a C-terminal amidated peptide of 44 amino acids, which was initially isolated from pancreatic tumors [1,2]. The primary sequence of hypothalamic GHRH has been established in other species, most of which show great similarity [3]. In contrast, rat GHRH is a 43 amino acid long, non-amidated peptide, which is only 67% homologous with human GHRH [4,5]. After cloning of the corresponding human cDNA, it was established that GHRH originates from a precursor (pre-proGHRH) that is processed to mature GHRH by removal of the signal peptide and proteolytic cleavage at the C-terminal region [6–8]. A model for the processing of human pre-proGHRH in transgenic mice has been proposed [9].

GHRH has also been detected in non-neural extrahypothalamic tissues of different species. In particular, GHRH expression in placenta has been reported in rat [10–14], mouse [12,15] and human [16]. Rat placenta contains a pre-proGHRH mRNA which encodes a pre-proGHRH identical to that found in the hypothalamus [14]. Nevertheless, the placental and the hypothalamic GHRH mRNAs differ in the region corresponding to the untranslated exon 1 because of the use of an alternative promoter in the placenta, located 10 kb upstream from the hypothalamic promoter [14]. In the rat, GHRH is encoded by a single copy gene organized into six small exons spanning over 20 kb of genomic DNA [14,17]. Exons 2–5 are contained in both hypothalamic and placental GHRH mRNA, while exon 1 is specific to placenta or hypothalamus (exon 1P and 1H, respectively).

The processing of pre-proGHRH is mediated by endoproteolytic cleavages directed by arginines flanking the mature GHRH [6,9]. In the hypothalamus, and also probably in placenta, this processing yields mature GHRH and a C-terminal flanking peptide (designated GCTP) of uncertain function. Human GCTP has been co-localized with GHRH in hypothalamic neurons [18] and has been suggested to be involved in the control of feeding behavior [19].

Here we present evidence for the expression in rat placenta of a novel form of pre-proGHRH mRNA generated by a differential splicing process. This form of pre-proGHRH mRNA encodes a pre-proGHRH with a different C-terminal flanking peptide.

2. Materials and methods

2.1. Preparation of samples

Female Sprague-Dawley rats weighing 200–230 g were caged overnight with males and the presence of a copulation plug was verified the following morning, which was considered day 0 of pregnancy.

Fetuses from days 13 to 20 of gestation were exteriorized by caesarean section and killed by decapitation. Tissues were removed, frozen on liquid nitrogen and stored at −80°C until used.

2.2. cDNA cloning from a rat placenta cDNA library

A rat long-term placenta cDNA library constructed in the vector λgt10 [20] was screened under high stringency hybridization conditions using as a probe the insert of the cDNA clone λrpGHRH-1 corresponding to the rat placental GHRH mRNA previously described [14]. Positive clones were purified and their inserts were subcloned into Bluescript plasmids (Stratagene).

2.3. Reverse transcription of total RNA, PCR amplification of cDNA and cloning of PCR products

Total RNA was isolated from rat placenta by the guanidinium thiocyanate method [21]. For the reverse transcription reaction, 2 μg of rat hypothalamic or placental total RNA in 3.75 μl of water was incubated at 65°C for 2 min, quenched on ice and adjusted to a final volume of 10 μl containing 1×PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.02% Triton X-100), 2.5 mM $MgCl_2$, 1 μg of bovine

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Abbreviations: GHRH, growth hormone-releasing hormone; nt, nucleotide(s); GCTP, GHRH C-terminal peptide

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serum albumin (Promega), 0.5 μg oligo(dT) (Promega), 10 units of RNasin (Promega), 200 μM each dNTP (Boehringer Mannheim) and 200 units of M-MLTV reverse transcriptase (Boehringer Mannheim). After incubation for 60 min at 42°C, the enzyme was inactivated at 95°C for 15 min, and samples were stored at 4°C until use.

Two oligonucleotides were used for PCR amplification of cDNA: an upstream primer, corresponding to sequences located in exon 2 (5′-GAGTGAAGGATGCCACTCTGGG-3′) and a downstream primer, complementary to sequences within exon 5 (5′-CCGAGGGCTCAA-GCCTCCGC-3′) (Fig. 1). 3 μl of the previously obtained cDNA reaction was diluted to a final volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.02% Triton X-100, 2 mM $MgCl_2$, 50 μM each dNTP, and 0.4 μM of each primer. Samples were overlaid with half a volume of mineral oil. After 2 min at 94°C, the temperature was lowered to 80°C and 1 μl of 1×PCR buffer containing 1 U of Taq polymerase (Promega) was added. Thirty cycles of amplification were carried out in a Programmable Thermal Controller (MJ Research, Inc.) using a cycling program of 94°C for 1 min, 68°C for 1 min, and 72°C for 1 min, followed by a 5 min final extension at 72°C. PCR products were fractionated by agarose gel electrophoresis, purified and cloned into the pGEM-T plasmid (Promega) according the manufacturer's instructions. Densitometric analysis of the ethidium bromide stained DNA fragments following agarose gel electrophoresis was performed in a CUE-2 Image Analyzer (Olympus).

For mapping exon 4.5, we performed long PCR amplification (Expand Long Template PCR System from Boehringer Mannheim) using the exon 2 and exon 5 specific primers previously described (Fig. 1). The PCR amplified product was further amplified for exon 4 to 4.5, exon 4.5 to 5 and exon 4 to 5 using specific primers (5′-GCAGAG-GACAAGCAGATGG-3′, exon 4 upstream; 5′-TACAGCGGCAG-GAGACACT-3′, exon 4.5 downstream; 5′-CCAGCACATAAAA-GAACACA-3′, exon 4.5 upstream).

2.4. DNA sequencing

The cDNA subclones and the PCR cloned products were sequenced by the dideoxynucleotide chain termination method using T7 DNA polymerase [22,23] (United States Biochemical Corporation).

2.5. Northern blot analysis

Total hypothalamic and placental RNA was obtained from Sprague-Dawley rats on gestational days 18–20 by successive phenol/chloroform and chloroform extraction of the frozen ground tissues, followed by ethanol and lithium chloride precipitation [22,23]. RNA samples (10 μg) were fractionated by electrophoresis on 1% agarose/formaldehyde gels, transferred to nylon membranes (Nytran, Schleicher and Schuell) and hybridized with the ^{32}P-radiolabeled *Ava*II-*Hin*fI fragment of the novel placental GHRH cDNA probe (Fig. 1) according to standard procedures [22,23].

2.6. Southern blot analysis

PCR amplified products were fractionated by electrophoresis on 1% agarose/formaldehyde gels, transferred to nylon membranes (Nytran, Schleicher and Schuell) and hybridized with the ^{32}P-radiolabeled rat placental pre-proGHRH-1 cDNA [14] according to standard procedures [22,23].

3. Results

A cDNA clone (λrpGHRH-2) corresponding to a novel form of pre-proGHRH mRNA (referred to as pre-proGHRH-2 mRNA) was isolated after screening a λgt10 rat placenta cDNA library. Fig. 1 shows the nucleotide sequence of the 495 bp *Eco*RI insert present in λrpGHRH-2. Pre-proGHRH-2 mRNA has an open reading frame of 414 bp, which encodes a pre-proGHRH of 138 amino acids that is different from that previously reported in the hypothalamus and placenta [14,17]. This novel form of pre-proGHRH mRNA is generated by an alternative splicing process which results in the presence of an additional exon of 156 nt (referred to as exon 4.5) located between exons 4 and 5 in the GHRH mRNAs described previously [14,17]. Exon 4.5 has an open reading frame, following that of exon 4, that encodes 46

Fig. 1. Nucleotide sequence of the rat placenta pre-proGHRH-2 cDNA clone. Nucleotides are numbered by assigning position +1 to the ATG initiator codon. The deduced amino acid sequence of the pre-proGHRH-2 is shown below the nucleotide sequence. The mature GHRH peptide is represented in italics. The amino acids encoded by exon 4.5 are underlined. The distribution of exons is indicated by arrows on the basis of the previously characterized hypothalamic and placental pre-proGHRH mRNA [14,17]. *Ava*II and *Hin*fI restriction enzyme sites are boxed. The positions corresponding to the oligonucleotide primers used for PCR amplification are dash underlined.

amino acids (Fig. 1). The presence of a stop codon near the end of exon 4.5 avoids translation of exon 5.

The occurrence of this novel pre-proGHRH mRNA was confirmed by reverse transcription of rat placental total RNA followed by PCR amplification of the cDNA using specific primers for exons 2 and 5 of pre-proGHRH mRNA (see Fig. 1 and Section 2). Electrophoretic analysis of the PCR amplified products showed two DNA bands of about 330 and 490 bp (Fig. 2). The amplified fragments were purified and cloned into a plasmid vector. Sequence analysis revealed cDNA inserts of 488 bp and 332 bp corresponding respectively to the pre-proGHRH-2 mRNA and the placental pre-proGHRH-1 mRNA previously reported [14]. The densitometric analysis of the PCR products obtained at different cycles of amplification (10–30 cycles) indicated that the relative abundance of the 488 bp fragment is about 10% the amount of the 332 bp fragment (data not shown).

Northern blot analysis of hypothalamic and placental total RNA using an exon 4.5 specific probe (*Ava*II-*Hin*fI fragment, Fig. 1) revealed that the pre-proGHRH-2 transcript was detected in placenta but not in the hypothalamus (Fig. 2A). These results were further confirmed by RT-PCR analysis of hypothalamic RNA, where a single 332 bp fragment was amplified (Fig. 2B,C). The 488 bp band detected in the placenta was never detected in the hypothalamus, even after increasing the number of cycles of the PCR or by longer exposures of the corresponding Southern blots (data not shown). Pre-

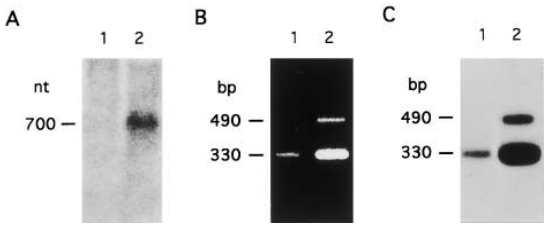

Fig. 2. Expression of rat pre-proGHRH mRNAs. A: Northern blot analysis of rat hypothalamic (lane 1) and placental (lane 2) total RNA (10 μg) using as a probe the *Ava*II-*Hin*fI fragment corresponding to exon 4.5 (see Fig. 1). The approximate size of the hybridizing transcript is shown. B: Ethidium bromide staining after agarose gel electrophoresis of RT-PCR amplification products of rat hypothalamic (lane 1) and placental (lane 2) pre-proGHRH mRNAs. The approximate size of RT-PCR amplified cDNA fragments is indicated. C: Southern blot analysis of the agarose gel containing the PCR amplification products of rat hypothalamic (lane 1) and placental (lane 2) pre-proGHRH mRNAs shown in B. Time exposure was 3 days.

proGHRH-2 mRNA was detected in the placenta at 14, 16, 18, 20 and 21 days of gestation (data not shown).

The relative position of exon 4.5 in respect to exons 4 and 5 has been mapped by PCR using exon specific primers (for details, see Section 2). Exon 4.5 is located 1.7 kb downstream of exon 4 and 800 bp upstream of exon 5 (Fig. 3).

The biological significance of the pre-proGHRH-2 is currently unknown, although its processing to yield mature GHRH according to the model proposed by Brar et al. [9] would result in the synthesis of a C-terminal peptide (here referred to as GCTP-2) different from that generated in the hypothalamus [18], and also suggested to occur in the placenta [14]. The putative placental GCTP-2 has 64 amino acids, the first 18 N-terminal residues being identical in GCTP-1 and GCTP-2 (Fig. 4).

A computer search against the GenEMBL databank revealed that the amino acid sequence encoded by exon 4.5 has no significant similarity to any previously reported protein. Nevertheless, high levels of similarity were detected at the DNA level with two particular sequences corresponding to the non-coding regions of UDP glucuronosyltransferase [24], lysozyme gene [25] and the kallikrein-like serine protease [26]. In addition, the nucleotide sequence of exon 4.5 also shows significant similarity with the rat 1B12 repetitive element [27].

4. Discussion

It is well documented that the placenta synthesizes a variety of peptide and steroid hormones having essential roles during pregnancy [28]. In addition, the placenta produces hormones, growth factors and cytokines that are also synthesized in other tissues [29,30], among them a number of hypothalamic peptides, including GHRH. Immunodetection of GHRH was first shown in rat placenta by Meigan et al. [11]. Expression of the GHRH gene in placenta was further demonstrated in rat [10,12–14], mouse [12,15] and human [16]. The rat placental pre-proGHRH mRNA contains an open reading frame that encodes a polypeptide identical to the hypothalamic pre-proGHRH [14]. Nevertheless, the placental and the hypothalamic GHRH mRNAs differ in the region corresponding to the untranslated exon 1 because of the use in the placenta of an upstream alternative promoter. A combined mechanism involving the use of tissue-specific alternative promoters and the differential splicing of exon 1 generates the mature GHRH transcript in placenta and hypothalamus [14].

Here we present evidence for the presence in rat placenta of a pre-proGHRH mRNA different from that previously characterized [14]. This novel pre-proGHRH mRNA (pre-proGHRH-2 mRNA) has an additional exon (designated exon 4.5) of 156 bp, located between exons 4 and 5, and contains an open reading frame encoding a pre-proGHRH form of 138 amino acids (Fig. 1). Since the sequences encoding mature GHRH are included within exons 3 and 4, the expected processing of the pre-proGHRH-2 would not affect the synthesis of mature GHRH but would generate a novel C-terminal peptide (designated GCTP-2) (Fig. 4) different from that previously reported in hypothalamus and placenta (GCTP-1).

This novel pre-proGHRH mRNA found in placenta was not detected in the hypothalamus (Fig. 2). The relatively high level of pre-proGHRH-2 mRNA during the second half of gestation suggests that it may significantly contribute to the synthesis in placenta of both mature GHRH and GCTP-2.

It is well established that many peptide hormones and neuropeptides are produced from large inactive precursors through endoproteolytic cleavage at sites usually marked by paired basic residues (primarily Lys-Arg and Arg-Arg) or occasionally by monobasic residues (primarily Arg) [31,32]. A detailed analysis of the primary sequence of pre-proGHRH-1 and pre-proGHRH-2 reveals that they contain a high proportion of dibasic and monobasic sites, thus providing the possibility of a variety of alternative processing events. In this respect, the chromatographic detection in placenta, but not in the hypothalamus, of an additional peak of high molecular weight (about 10 kDa) showing GHRH immunoreactivity [11] suggested that other GHRH-like polypeptides may be present in rat placenta. To date, the molecular nature of this high molecular weight GHRH immunoreactive material has not been established. ProGHRH-1, which contains 74 amino acids

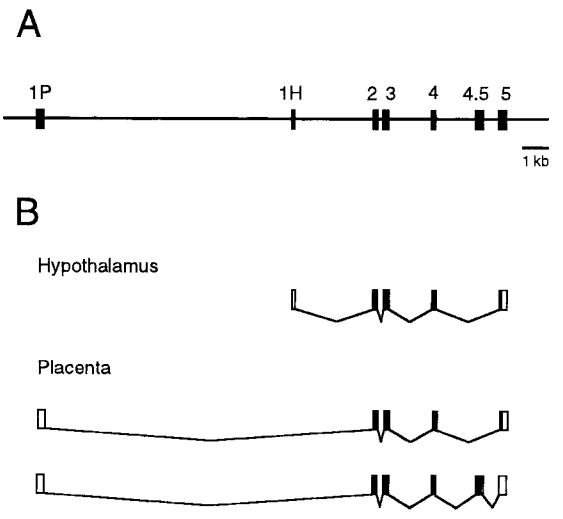

Fig. 3. Genomic organization of rat GHRH gene. A: Schematic representation of the rat GHRH gene. Exons are represented by boxes and named according to nomenclature indicated in text. Exon sizes are not drawn to scale. B: Structure of mature GHRH transcripts in hypothalamus and placenta. Untranslated regions are represented by open boxes; translated regions are represented by dark boxes.

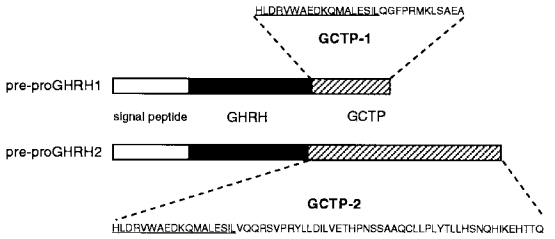

Fig. 4. Schematic representation of rat placental pre-proGHRH-1 and pre-proGHRH-2. The amino acid sequences of GCTP-1 and GCTP-2 are shown. Identical residues in GCTP-1 and GCTP-2 are underlined.

and has an estimated molecular size of 9 kDa, would represent a good candidate. Nevertheless, the involvement of the novel form of pre-proGHRH cannot be ruled out. In this respect, the analysis of the different processing possibilities of pre-proGHRH-2 directed by dibasic amino acids predicts a peptide of 87 residues with an estimated molecular size of 10.3 kDa. This peptide, extending from residue 22 of mature GHRH (flanked by the dibasic site Arg-Lys) to the end of GCTP-2, should display both immunoreactivity against GHRH and the appropriate molecular weight.

The physiological significance of the hypothalamic and placental GCTP-1 has not been assessed, although it has been reported that human hypothalamic GCTP influences the feeding behavior of rats when injected into the brain [19]. Furthermore it has been shown that GCTP-1 is co-secreted with GHRH in rat hypothalamic neurons [18]. The data presently available on placental GCTP-2 do not indicate whether it might have a functional role during pregnancy as a processed peptide or, alternatively, promote a differential processing and/or stability of the corresponding pre-proGHRH, thus regulating the synthesis of either mature GHRH or GHRH-like peptides. Additional experiments are needed to assign the physiological role of the novel placental pre-proGHRH reported here.

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