Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 8749–8753, October 1991
Biochemistry

Expression of the rat growth hormone-releasing hormone gene in placenta is directed by an alternative promoter

(differential splicing/hypothalamus/regulatory peptides/gene expression)

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Communicated by Roger Guillemin, April 19, 1991 (received for review November 19, 1990)

ABSTRACT **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. GHRH has also been detected in other nonneural extrahypothalamic tissues, including rat placenta, although its role in the hormonal control of pregnancy and/or fetal development has not yet been defined. Here we present the isolation and characterization of cDNA clones corresponding to rat placental GHRH. The placental GHRH mRNA codes for a pre-pro-GHRH identical to that found in the hypothalamus, suggesting that the mature placental GHRH is identical to its hypothalamic counterpart. 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 kilobases upstream from the hypothalamic 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. Multiple transcription initiation sites have been found in the placental GHRH mRNA, which correlates to the lack of a consensus TATA box in the promoter region.**

Synthesis and secretion of anterior pituitary hormones are under the control of specific hypothalamic releasing and inhibiting hormones. Among them, the growth hormone (GH)-releasing hormone (GHRH) specifically interacts with the pituitary somatotrophs activating GH gene transcription (1, 2) and GH secretion (3, 4). GHRH also exerts a mitogenic effect on the somatotrophs *in vitro* (5) and is supposed to play an important role in the growth and differentiation of the GH-secreting cells (6). At present, little is known about the mechanism of GHRH action, although it apparently interacts with specific cell-surface receptors that trigger the synthesis of cAMP by stimulating adenylate cyclase activity (7, 8). Nevertheless, this final increase in cAMP is not yet clearly connected to the known GHRH-mediated effects.

Human GHRH is a C-terminal-amidated peptide of 44 amino acids initially isolated from pancreatic tumors (9, 10). Rat GHRH shares a homology of 67% with human GHRH, is 43 amino acids long, but is not amidated (11, 12). After cloning of the corresponding cDNAs, GHRH was established to originate from a precursor (pre-pro-GHRH) that is processed to mature GHRH by removal of the signal peptide and proteolytic cleavage at the N- and C-terminal regions (13–15). GHRH is encoded by a single-copy gene that has been cloned and characterized in humans and rat. In both species, the gene is organized into five small exons spanning over 10 kilobases (kb) on genomic DNA and encodes a mRNA of ≈700 nucleotides (nt) (15, 16).

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In the last few years several reports have indicated the presence of hypothalamic hormones, including GHRH, in nonneural extrahypothalamic tissues. Immunoreactivity against GHRH has been detected in a variety of sites, such as the gastrointestinal tract (17, 18), pancreas (19), testis (20), placenta (21, 22), and tumors (23, 24). Because of the importance of the placenta in the hormonal control of pregnancy in mammals, the study of the role of GHRH produced in the placenta is of special interest.

Recent results from our laboratory and others have shown that the GHRH gene is actively transcribed in rat (25, 26) and mouse (27) placenta. To further characterize GHRH expression in the placenta we present here the isolation of cDNA clones corresponding to rat placental GHRH mRNA.† We also report that placental and hypothalamic GHRH mRNAs differ in the region corresponding to the first exon as a consequence of the use in placenta of an alternative promoter located 10 kb upstream from the hypothalamic promoter.

MATERIALS AND METHODS

cDNA Cloning and Sequencing. A λgt11 cDNA library was constructed using poly(A)$^+$ RNA obtained from placentas of Sprague–Dawley rats on gestational days 18–20 following described protocols (28). Before amplification, the library was screened by using a cDNA corresponding to human GHRH as a probe (14). The inserts of the positive clones were sequenced by the dideoxynucleotide chain-termination method using T7 DNA polymerase (either from Pharmacia or United States Biochemical).

Southern Blot Analysis. Rat genomic DNA was digested with restriction enzymes, fractionated by electrophoresis on 1% agarose gels, transferred to nylon membranes (Nytran; Schleicher & Schuell), and hybridized to ^{32}P-radiolabeled probes according to standard procedures (29, 30).

RNA Blot Analysis. Total RNA from Sprague–Dawley rat placentas was obtained by successive phenol/chloroform and chloroform extractions of frozen disgregated tissue, followed by ethanol and lithium chloride precipitations (29, 30). Poly(A)$^+$ RNA fractions were obtained by affinity chromatography through oligo(dT)-cellulose (29). RNA samples were fractionated by electrophoresis on 1% agarose/formaldehyde gels, transferred to nylon membranes (Nytran; Schleicher & Schuell), and hybridized to ^{32}P-radiolabeled probes, according to standard procedures (29, 30). Northern (RNA) blot analysis was also done using as probes the synthetic oligonucleotides 5′-TGCAGTTGTGGGATCCAGGC-3′ (Oligo-1) and 5′-

Abbreviations: GH, growth hormone; GHRH, GH-releasing hormone; nt, nucleotide(s).

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†The sequences reported in this paper have been deposited in the EMBL/GenBank data base (accession nos. M73486 and M73487).

TTCTCCGTCCTGGGCCTGCATGTT-3' (Oligo-2) complementary to the placental GHRH mRNA (see Fig. 4*D*).

Isolation and Characterization of Genomic Clones. A rat genomic library constructed in λCharon 4A (Clontech) was screened with a DNA fragment corresponding to exon 1P as a probe (see *Results*). Positive clones were characterized by restriction mapping, Southern blot analysis, and DNA sequencing.

Determination of the Transcription Start Point. The 5' end of rat placental GHRH mRNA was determined by primer extension and RNase protection analyses. Primer-extension analysis was done by hybridizing ^{32}P-end-labeled Oligo-1 to 10 μg of rat placental poly(A)$^+$ RNA and extending the primer with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim), as described (29, 30). For RNase protection analysis, a genomic fragment extending 462 base pairs (bp) upstream from the *Bam*HI site at position −40 (see Fig. 4*D*) was subcloned into Bluescript to generate an antisense RNA probe that was hybridized to 10 μg of rat placental poly(A)$^+$ RNA and digested with RNase T1 (Bethesda Research Laboratory) and RNase A (Boehringer Mannheim), as described (29, 30). The primer-extended products and the RNase-resistant fragments were analyzed in polyacrylamide sequencing gels beside sequencing reaction fragments as molecular-size markers. Controls were done with calf thymus tRNA instead of rat placental poly(A)$^+$ RNA.

RESULTS

Previous data from our laboratory revealed the presence in rat placenta of a transcript of ≈700 nt hybridizing to a human GHRH cDNA clone (26). To characterize this mRNA we screened ≈200,000 recombinant clones of an unamplified cDNA library from rat placenta using as a probe the ^{32}P-labeled *Eco*RI–*Bam*HI fragment of human GHRH cDNA clone phGRF-54 (14). Five positive clones were purified after repeated plating. Their inserts were subcloned into *Eco*RI-digested Bluescript plasmids and sequenced. Fig. 1 shows the

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-195 GTACGTGCTCTGGAACATGCAGGCCCAGGACGGAGAAGGAGGCGTCCTGCTCCTGCCAGC

-135 CTTAAGATGGGAATTTTAGGGTCTGGACATCACTGGTGCTCCAGGTCAGCTTTCCTGGTT
                                                          E2
 -75 GCAGATCTCTCCTGGTCAAGGCTCCCAGCTCGCCTGGATCCCACAACTGCACAGTGTCCC
                 +1
 -15 GCCCAGGAGTGAAGGATGCCACTCTGGGTGTTCTTTGTGCTCCTCACCCTCACCAGTGGC
                   MetProLeuTrpValPhePheValLeuLeuThrLeuThrSerGly
                                          E3
  45 TCCCACTGCTCACTGCCCCCCTCACCTCCCTTCAGGGTGCGGCGGCATGCAGACGCCATC
     SerHisCysSerLeuProProSerProProPheArgValArgArgHisAlaAspAlaIle

 105 TTCACCAGCAGCTACCGGAGAATCCTGGGCCAATTATATGCCCGCAAACTGCTGCACGAA
     PheThrSerSerTyrArgArgIleLeuGlyGlnLeuTyrAlaArgLysLeuLeuHisGlu
                                      E4
 165 ATCATGAACAGGCAGCAAGGGGAGAGGAACCAGGAACAAAGATCCAGGTTCAACCGCCAT
     IleMetAsnArgGlnGlnGlyGluArgAsnGlnGluGlnArgSerArgPheAsnArgHis
                                                  E5
 225 TTGGACAGAGTGTGGGCAGAGGACAAGCAGATGGCCCTGGAGAGCATCTTGCAGGGATTC
     LeuAspArgValTrpAlaGluAspLysGlnMetAlaLeuGluSerIleLeuGlnGlyPhe

 285 CCAAGGATGAAGCTTTCAGCGGAGGCTTGAGCCCTCGGCCCCCAAACATAGCTGGACCCT
     ProArgMetLysLeuSerAlaGluAlaEnd

 345 GTTACTTCTACTTCAGTTCTGATCTTCTCCTTCCTCTGTGAATACAATAAAGACCCATCT

 405 CATCTGCAAAAAAAAA
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FIG. 1. Nucleotide sequence of the rat placenta GHRH cDNA clone λrpGHRH-1. Dots indicate position of the 5' end of cDNA clones λrpGHRH-2 to -5. Nucleotides are numbered by assigning position +1 to the ATG initiator codon. The deduced amino acid sequence of the pre-pro-GHRH is shown below the nucleotide sequence. The mature GHRH peptide is underlined. The distribution of exons is indicated by arrows on the basis of the hypothalamic mRNA (15). The placental-specific part of the untranslated leader is also underlined (positions −21 to −195) and contains the *Bam*HI restriction site (boxed) used to generate cDNA-derived probes. The putative polyadenylylation signal is boxed.

nucleotide sequence of the longest cDNA clone (λrpGHRH-1), which spans 607 bp [excluding the poly(A) tail]. The other cDNA clones (λrpGHRH-2 to -5) have their 5' ends clustered between positions −76 to −96 (Fig. 1). All the cDNA clones contain an open reading frame of 312 bp, encoding a polypeptide of 104 amino acids (Fig. 1), which is identical to rat hypothalamic pre-pro-GHRH (15). Nevertheless, the alignment of the nucleotide sequence of the placental and the hypothalamic cDNA clones revealed that they were completely different in the region upstream from the splice junction between hypothalamic exons 1 and 2, although they were identical downstream from this position (exons 2–5). Because in both cases the different region is part of the mRNA untranslated leader, this difference is not reflected in the encoded products.

To further characterize the placental GHRH mRNA untranslated leader, several approaches were undertaken. On the one hand, a computer search against a data bank showed no homology to any reported sequence. On the other hand, Southern blot analysis revealed that the GHRH mRNA untranslated leader hybridized to single genomic restriction fragments (Fig. 2*A*), thus indicating that it corresponds to a single-copy sequence in the rat genome. In addition, Northern blot analyses of rat placental RNA were done using as probes: (*i*) the human GHRH cDNA clone phGRF-54, (*ii*) a cDNA fragment containing most of the placental GHRH mRNA untranslated leader, and (*iii*) a cDNA fragment containing exons 2–5 (see legend to Fig. 2*B* for details). In all cases, a single transcript of ≈700 nt was detected (Fig. 2*B*).

To determine the genomic organization of the region encoding the placental untranslated leader, ≈200,000 genomic clones of a rat genomic library were screened with the *Eco*RI(linker)-*Bam*HI fragment from the cDNA clone λrpGHRH-1 as a probe. Two overlapping clones, λrGHRH-1 and λrGHRH-2 (Fig. 3*A*) spanning 22 kb of genomic DNA, were isolated and characterized by restriction enzyme mapping. Fig. 3*B* shows a composite restriction map of the newly cloned region overlapping that previously described (15). Southern blot analysis and sequencing data revealed that the placental untranslated leader is encoded by a single exon, hereafter referred to as exon 1P, located 10 kb upstream from the hypothalamic exon 1 (exon 1H) (Fig. 3*B*). That exon 1P is specific for the placental GHRH transcript was confirmed by Northern blot analysis of placental and hypothalamic RNA, with sequences corresponding to exon 1P and exon 1H as probes. When the *Eco*RI (linker)–*Bam*HI fragment from the cDNA clone λrpGHRH-1 was used as a probe, a tran-

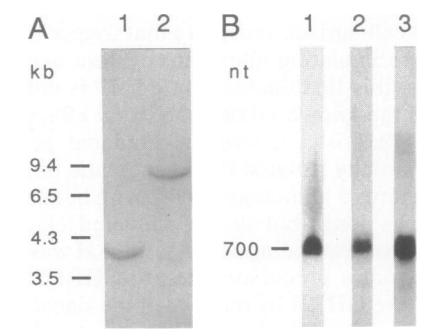

FIG. 2. Characterization of placental GHRH mRNA untranslated leader. (*A*) Southern blot analysis of *Eco*RI (lane 1) and *Hin*dIII (lane 2) digested genomic rat DNA with the *Eco*RI(linker)–*Bam*HI fragment of the cDNA clone λrpGHRH-1 as a probe. Size of molecular-weight markers is shown. (*B*) Northern blot analysis of placental poly(A)$^+$ RNA with probes *Eco*RI–*Bam*HI fragment of human GHRH cDNA phGRF-54 (lane 1), *Eco*RI(linker)–*Bam*HI fragment of the cDNA clone λrpGHRH-1 (lane 2), and *Bam*HI–*Eco*RI(linker) fragment of the same cDNA clone (lane 3). Approximate size of the hybridizing transcripts is shown.

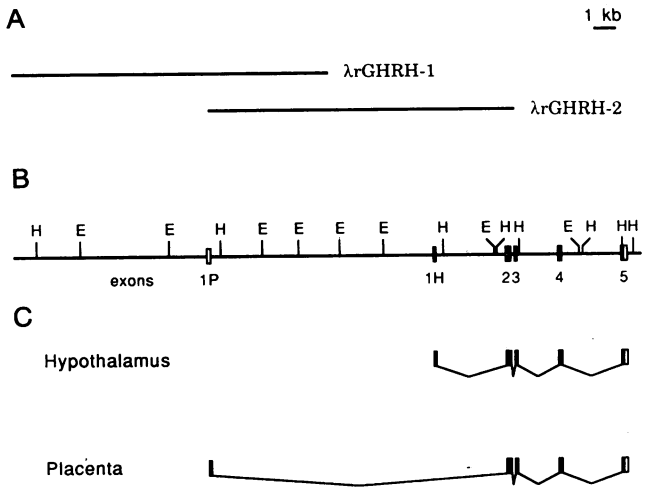

FIG. 3. Genomic organization of rat GHRH gene. (*A*) Region represented in the inserts of overlapping genomic clones λrGHRH-1 and λrGHRH-2 aligned to the restriction map of *B*. (*B*) Composite restriction map of our reported cloned region, together with that previously described (15). *Eco*RI (E) and *Hin*dIII (H) restriction sites are indicated. Exons are represented by boxes and named according to nomenclature indicated in text. Untranslated regions are represented by open boxes; translated regions are represented by dark boxes. (*C*) Structure of mature GHRH transcripts in hypothalamus and placenta.

script of ≈700 nt was detected in placental RNA but not in hypothalamic RNA. In an identical experiment with a genomic fragment containing exon 1H as a probe, a transcript of a similar size was identified only in hypothalamic RNA (data not shown). We conclude that in rat placenta the GHRH gene is expressed under the control of a different promoter from that reported for the hypothalamus. Thus, a combined mechanism based on the use of tissue-specific alternative promoters and differential splicing of exon 1 generates the mature GHRH transcripts in placenta and hypothalamus (Fig. 3*C*). The genomic nucleotide sequence corresponding to exon 1P and its 5′-flanking region is presented in Fig. 4*D*.

To define the 5′ end of the placental GHRH mRNA precisely, we performed primer-extension and RNase protection analyses. The results revealed the presence of multiple transcription start sites (Fig. 4 *A* and *B*) that are indicated in the nucleotide sequence of Fig. 4*D*. The relative intensity of the bands in the primer-extension and in the RNase protection analyses indicates that initiation of transcription at the three proximal sites (Fig. 4*D*) is more efficient than at the others. This observation is further supported by the results from Northern blot analysis of rat placental RNA with the oligonucleotide probes Oligo-1 and Oligo-2, which correspond to sequences located downstream and upstream from these major transcription start sites, respectively (Fig. 4*D*). Oligo-1 detected all the multiple transcripts present in placenta, whereas Oligo-2 only detected the longest less abundant transcripts (Fig. 4*C*). In addition, it is interesting to remark that four of the five placental GHRH cDNA clones we isolated have their 5′ end slightly downstream from the major transcription start sites (Fig. 1).

DISCUSSION

A current subject of investigation in endocrinology concerns the physiological significance of the expression of hypothalamic hormones in extrahypothalamic sites. Although the precise role of the extrahypothalamic synthesis of most of these hormones has not been fully assessed, their synthesis

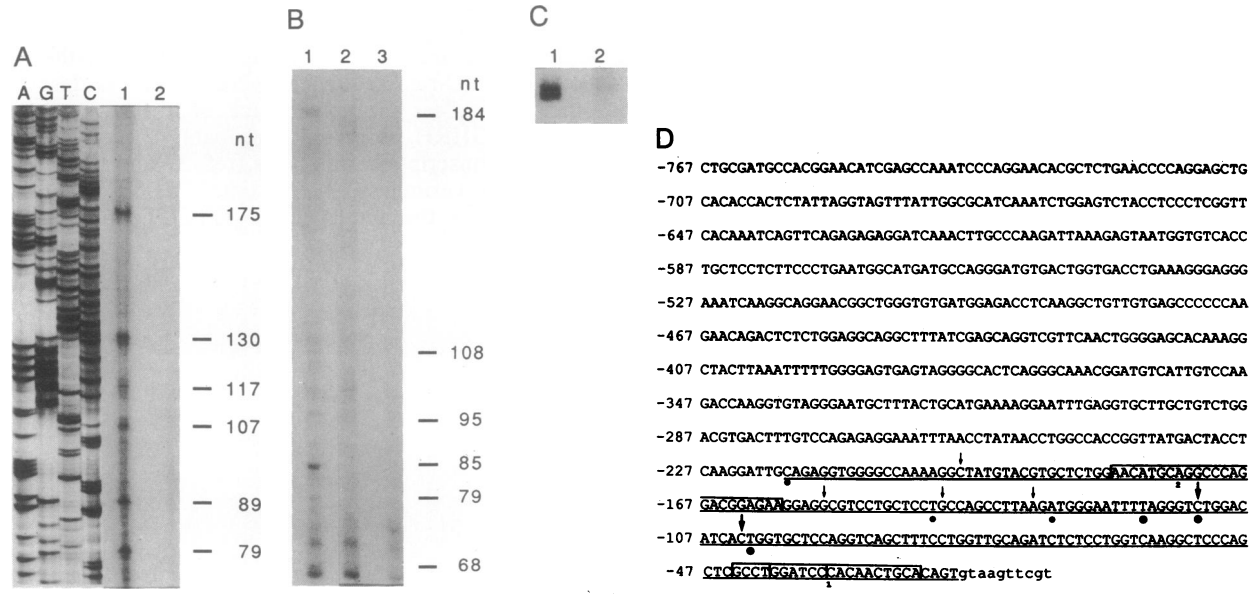

FIG. 4. Determination of the transcriptional start point of placental GHRH mRNA. (*A*) Primer-extension analysis. Synthetic oligonucleotide Oligo-1 (see *D*) was used to prime synthesis with reverse transcriptase from rat placental poly(A)$^+$ RNA (lane 1) or calf thymus tRNA (lane 2). The size of the primer-extended products is indicated. Because the sequencing ladder at left has been obtained by using Oligo-1 as sequencing primer on genomic subclones, it can be used to directly read the position of the mRNA 5′ ends on the sequence of *D*. (*B*) RNase protection analysis. RNA samples were hybridized to the described RNA probe, and the RNase-resistant products were analyzed. Lanes: 1, rat placental poly(A)$^+$ RNA: 2, calf thymus tRNA; 3, no RNA. The size of specific RNase-resistant products is indicated. (*C*) RNA blot analysis of rat placental RNA using as probes the synthetic oligonucleotides Oligo-1 (lane 1) and Oligo-2 (lane 2) (see *D*). The size of GHRH transcripts is ≈700 nt. (*D*) Nucleotide sequence of 572 bp upstream from the 5′ end of the cDNA clone λrpGHRH-1 derived from genomic clone λrGHRH-1. Exon 1P is underlined and contains the *Bam*HI restriction site (boxed at position −40) used to generate the RNA probe for RNase protection analysis. The first 10 nucleotides corresponding to the 5′ end of the placental first intron are in lowercase letters. The complementary sequences to Oligo-1 and Oligo-2 are boxed, and referred to as 1 and 2, respectively. The ends of primer-extended products and RNase-protected fragments are represented by vertical arrows and dots, respectively. The origins of transcription that appear more efficiently used are shown by thicker arrows and dots.

in placenta is of special interest because of the relevant role of this organ in the hormonal control of pregnancy in mammals.

To study the expression of GHRH in placenta, we have cloned and characterized the cDNA corresponding to rat placental pre-pro-GHRH. In this report we have shown that the rat placental GHRH mRNA contains an open reading frame that encodes a polypeptide identical to the hypothalamic pre-pro-GHRH. This observation strongly supports previous data (21, 22, 25), suggesting that the mature placental GHRH is identical to that found in the hypothalamus. Further, it also suggests that rat placenta must have a mechanism to process the GHRH precursor equivalent, if not identical, to that present in the hypothalamus. Nevertheless, the chromatographic detection in placenta, but not in the hypothalamus, of an additional peak of higher molecular weight showing GHRH immunoreactivity (22, 25) suggests that other GHRH-like polypeptides may occur in rat placenta.

In contrast to the identity of placental and hypothalamic GHRH at the protein level, their corresponding mRNAs clearly differ in the 5′-untranslated leader. An increased number of reports indicates that the 5′-untranslated leader is critical in the translatability of the mRNAs, thus representing an additional step in the regulation of gene expression in eukaryotic cells (for review, see ref. 31). In this respect, the formation of stem-loop structures in the untranslated leaders and the presence of AUG triplets preceding the translational initiator AUG are the best-documented mechanisms involved in the control of mRNA translation (31). For the GHRH gene, it could be speculated that the existence of two different untranslated leaders, one for each tissue-specific transcript, may confer different properties to the translatability of each mRNA. A detailed analysis of the rat GHRH mRNA untranslated leaders revealed several interesting features that could influence the translatability of the placental transcripts. On the one hand, the placental, but not the hypothalamic, GHRH untranslated leader contains two pairs of inverted repeats that can potentially generate the stem-loop structures shown in Fig. 5. Nevertheless, only the stem-loop structure located downstream from the most efficient transcriptional start points would affect the translation of the most abundant placental GHRH transcripts (Fig. 5). On the other hand, three AUG triplets preceding the initiator are located in the placental GHRH mRNA at positions −197, −179, and −129 (Fig. 5). Two short open reading frames of 26 and 41 amino acids initiate at the AUG located at position −197 and −129, respectively, and precede the pre-pro-GHRH peptide. The AUG at −179 is included in the open reading frame starting at the AUG at −197. Because these AUG triplets are located upstream from the most efficient transcriptional start points (Fig. 5), they would only influence the translation of the less abundant placental GHRH transcripts. In contrast, the hypothalamic GHRH mRNA contains only one AUG codon (out of frame) in its 5′-untranslated leader, and its proximity to the mRNA 5′ end (only 3 nt downstream) makes it very unlikely to be efficiently used (32). Another feature of the placental untranslated leader is the high number of CUG triplets (nine) preceding the AUG initiator codon. As recent reports have revealed that CUG codons can be used as initiator codons in eukaryotic cells (33–35) and that in all cases they are related with the synthesis of extended protein products (33–35), we examined this fact in relation to the presence of the high-molecular-weight forms of GHRH detected in rat placenta (22, 25). Nevertheless, the presence of an UGA stop codon in frame with the initiator AUG and located at position −6 discards the possibility that the higher-molecular-weight forms of placental GHRH arise from the use of any of these upstream CUG codons.

The different untranslated leaders in placental and hypothalamic GHRH transcripts result from alternative splicing of the first exon in combination with the use of an alternative promoter. Data obtained from the recently isolated mouse hypothalamic and placental GHRH cDNA clones (27, 36) suggest that mouse GHRH gene transcription can also be directed by two tissue-specific alternative promoters. Nevertheless, no experimental evidence for it has yet been provided.

The results from mapping the 5′ end of the placental GHRH mRNA revealed multiple transcriptional start sites scattered over 117 bp (Fig. 4*D*). When the nucleotide sequence of the region preceding these sites was examined, neither TATA box nor G+C-rich sequences were found. As described in other TATA-less promoters, lack of a TATA box in the placental GHRH promoter is probably the reason for the multiple transcription start sites (ref. 37 and the references therein). Nevertheless, the placental GHRH 5′-flanking region contains the sequence 5′-CGTCCTGCT-3′ (position

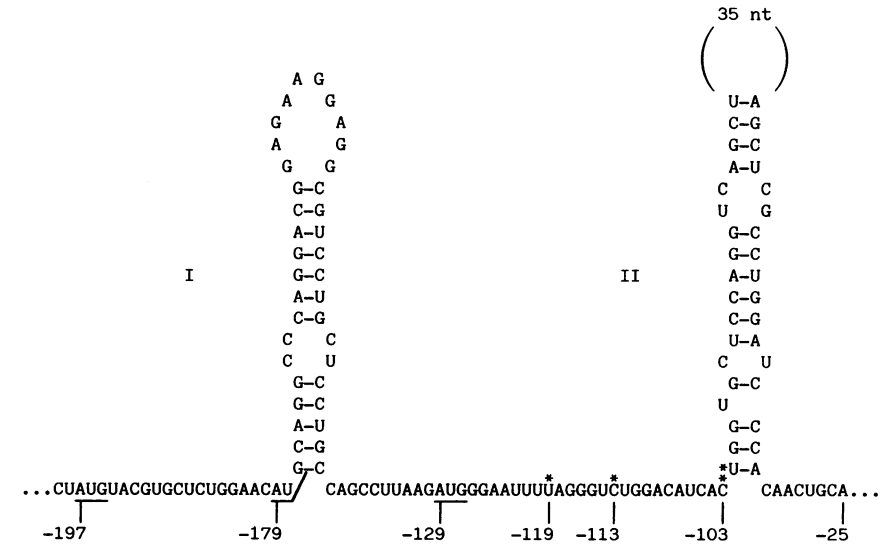

FIG. 5. Possible secondary structures in the untranslated leader of rat placental GHRH mRNAs. Numbering used is the same as for Figs. 1 and 4. Nucleotide pairings are indicated by dashes. The three AUG triplets preceding the initiator AUG codon are underlined. The three most efficient transcriptional start points are indicated by asterisks. The free energy of both structures was calculated with the MicroGenie computer program (Beckman) and resulted in −23.4 (structure I) and −23.2 kcal/mol (structure II).

−153) that is similar to the sequence 5′-CTCCCTGCT-3′ located at an equivalent position in the 5′-flanking region of the murine *Thy* gene, which also lacks TATA and G+C boxes and has multiple transcription start sites (38, 39). Similar sequences are also found in other genes with multiple transcription start sites but containing G+C boxes (38). In addition, a putative inverse CCAAT box, similar to that found also in the promoter region of the murine *Thy* gene (38) and in the human placental estradiol 17β-dehydrogenase gene (40), is located at position −223 (Fig. 4*D*). In spite of the existence of multiple transcriptional start points, the three proximal sites are the most efficient. This situation resembles that found in other placental genes, such as the human estradiol 17β-dehydrogenase (40) and the human corticotropin-releasing hormone (41) genes that, although they present a major transcription start site at the appropriate distance from a TATA box, also show other less efficient transcription start sites upstream.

The use in placenta of a promoter different from the hypothalamic promoter could be reflected in a different control of GHRH gene transcription in both tissues. At present, no data have yet been reported concerning the regulation of GHRH gene transcription, either in hypothalamus or in tumors. Nevertheless, some studies on transgenic mice expressing a GHRH minigene under control of a metallothionein promoter led to the suggestion that some tissue-specific regulatory elements may be located within the second intron, the 3′ end of the mRNA, or the 3′-flanking region (42). It is worthwhile pointing out that these three regions are depleted of repetitive DNA, which is abundant along the rest of the human GHRH gene (43). Thus, the differential and tissue-specific control of GHRH gene transcription would also involve elements located either into the gene or in its flanking regions. The transient existence of the placenta raises the question as to whether GHRH gene expression is developmentally regulated or, alternatively, triggered and/or modulated by other hormonal factors. Functional analysis of the GHRH placental promoter, either *in vitro* or *in vivo*, will give clues about the regulation of its expression.

We thank Dr. Ronald M. Evans for providing plasmid phGRF-54. We are grateful to Dr. Carme Caelles and Gerardo Jiménez for helpful suggestions and continuous encouragement, as well as to Isabel Calero for animal care guidance. S.G.-C. is a recipient of a predoctoral fellowship from the Fondo de Investigaciones Sanitarias de la Seguridad Social (88/0079), Spain. This work has been supported in part by Grants 87/1239 and 88/1462 from the Fondo de Investigaciones Sanitarias (to A.B.) and a Research Grant from the Comissió Interdepartamental de Recerca i Innovació Technològica (AR88), Generalitat de Catalunya (to S.G.-C.).

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