Cloning and Characterization of the 5'-Flanking Region of the Human Growth Hormone-releasing Hormone Receptor Gene*

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We cloned the 5'-flanking region of the human growth hormone-releasing hormone receptor (GHRH-R) gene and determined the nucleotide sequence of 2.7 kilobases upstream from the translation start site. RNAase protection analysis showed the major transcription start site is 122 base pairs upstream from the translation start site. The 5'-end of the longest product of 5'rapid amplification of cDNA ends was close to the site. There were no typical TATA homologies but several putative regulatory elements including Pit-1-binding site-like element. Transient transfection studies using a luciferase reporter gene demonstrated that 5'-flanking region had promoter activity in GH3 cells (derived from rat pituitary tumor) but not in nonpituitary cells, BeWo and HeLa cells. However, co-transfection of Pit-1 expression vector increased luciferase activity in BeWo cells. Deletion study showed that the regions from –310 to –130 and from –130 to –120 were important for the GHRH-R gene expression in GH3 cells, although the latter contributed less to the gene expression. In BeWo cells co-transfected with Pit-1 expression vector, the region from –310 to –130 was essential for the Pit-1-dependent expression of GHRH-R gene. The region from –310 to –120 has two putative Pit-1-binding sites, P1 and P2, located from –120 to –123 and from –171 to –160, respectively. Both mobility shift assay and DNase-I footprint analysis showed that P2 had much higher Pit-1 binding affinity than P1. Mutation of P2 decreased GHRH-R gene expression in GH3 cells. These findings were consistent with the results that the region from –310 to –130 is an important element for Pit-1-dependent expression of GHRH-R gene.

Growth hormone-releasing hormone (GHRH) plays a major role in stimulating both synthesis and release of growth hormone (GH) in the anterior pituitary through its specific receptor. So far, the GHRH receptor (GHRH-R) has been cloned in human (1, 2), mouse (3), rat (1), and pig (4). The GHRH-R is a member of G protein-coupled receptor family and transduces GHRH-dependent increase in intracellular cAMP via G activation for stimulating somatotroph proliferation and GH gene expression (1–4). Lin et al. (5) demonstrated that one amino acid substitution of the GHRH-R in the little mouse, showing genetically transmitted dwarfism, caused GH deficiency and somatotroph hypoplasia. In addition, an amber-type mutation (Glu72Stop) of the GHRH-R in humans was demonstrated to cause profound GH deficiency (6). These genetic disorders suggest the physiological significance of GHRH-R in hypothalamic-pituitary GH axis.

The relationship between the dynamics of GHRH-R expression and GH secretion remains to be clarified. Not only the functional defects of GHRH-R but also the amount of GHRH-R should affect GH synthesis and secretion in the pituitary. Hypothalamic hormone, neurotransmitters, various hormonal states, and nutrition could all modulate the activity of the GHRH-GH axis. For instance, glucocorticoids potentiate GHRH action and enhance GH secretion in rats (7–9), whereas they have a biphasic effect in humans (10, 11). A recent study showed that GHRH-R gene expression was increased by glucocorticoids in rats (12). The result suggests that glucocorticoids stimulate GH synthesis and secretion, at least in part, via up-regulation of GHRH-R gene expression. Interestingly, Horikawa et al. (13) observed a marked reduction of GHRH-R mRNA in GHRH-deprived neonatal rats. This observation suggests that GHRH up-regulates GHRH-R expression in addition to direct action to stimulate GH synthesis and secretion. Thus, we need to take the alteration of GHRH-R expression into account to understand the regulatory mechanism of GH secretion.

The GHRH-R transcripts have a highly specific distribution in the anterior pituitary, even though the GHRH-R gene is also expressed in the rat hypothalamus (14). During the development of the anterior pituitary gland, the GHRH-R transcripts were detected at embryonic day 16 in somatotrophs following the gene activation of Pit-1, a pituitary-specific POU domain transcription factor (15). The GHRH-R are not expressed in the pituitary of Pit-1-defective Snell/Jackson dwarf mice (16, 17). Thus, it appears that Pit-1 is required for the GHRH-R gene expression. However, it remains unknown whether the Pit-1-binding site is present in the GHRH-R gene and whether it functions for the GHRH-R expression.

In the present study, we have cloned and characterized the 5'-flanking region of the human GHRH-R gene to elucidate the regulatory molecular mechanism of the human GHRH-R gene expression. Furthermore, we demonstrated that the 5'-flanking region contained a functional promoter and that its promoter activity was dependent on Pit-1.
EXPERIMENTAL PROCEDURES

Cloning of the 5'-Flanking Region of the Human GHRH-R Gene by PCR Amplification Method—We cloned 5'-flanking region of the human GHRH-R gene using a PCR-based method for DNA walking in uncloned genomic DNA. (PromoterFinder DNA walking kit (CLONTECH)) (18). Adaptor-ligated genomic DNA was amplified with two consecutive PCRs using two oligonucleotide primer pairs. One set of primers used in the primary PCR was the adaptor-specific primer ASP (5'-CCACCTCTCCTCCTGATTG-3'), corresponding to the synthetic adaptor and the gene-specific antisense primer GSP2 (5'-TTCCATGTGAGGCCCCAGCGTTGCT-3') corresponding to the 5'-end of the human GHRH-R cDNA (nucleotides position +5 to -19 according to Mayio (1)). Another set of primers for the secondary PCR was the adaptor specific primer ASP (5'-CTATGAAGGAGGCTGGGTT-3'), nested to ASP1, and primed with the cDNA end ligated with the gene-specific antisense primer GSP1 (5'-GAGAGACTCACTATAGGG-3'). The 5'-end of GSP1 to facilitate subcloning. PCR was performed according to the manufacturer's instructions. The PCR conditions were 32 cycles of 94 °C for 25 s and 67 °C for 4 min following 7 cycles of 94 °C for 25 s and 72 °C for 4 min. PCR products were analyzed by electrophoresis on a 1% agarose gel.

DNA Sequencing—PCR products were subcloned into pT7Blue vector (Invitrogen) or pBluescript SK+ (Stratagene) and sequenced with a DNA sequencer (model 373A or 310, Perkin-Elmer, Applied Biosystems).

Rapid Amplification of 5'-cDNA Ends—5'-RACE was performed using Marathon-Ready cDNA library (CLONTECH) derived from human pituitary gland poly(A) tails according to the manufacturer's instructions. In the first experiment, PCR was performed using ASP1 (5'-CCACCTCTCCTCCTGATTG-3', specific to the adaptor ligated to cDNA ends and GSP5 (5'-TGGTACAGGCACTCTCATCCTCTCA-3') corresponding to the coding region from +101 to +127 of the human GHRH-R cDNA (1). Cycling parameters used in this 5'-RACE protocol were 35 cycles of 94 °C for 30 s, 64 °C for 30 s and 68 °C for 2 min 30 s. PCR products were analyzed by electrophoresis on an ethidium bromide-stained 1.2% agarose gel. The PCR products were subcloned into pT7Blue vector and sequenced with a DNA sequencer.

RNAse Protection Analysis—RNAse protection assays were performed, based on the method of Gilman (19). A DNA fragment corresponding to nucleotides ~2207 to ~19 was inserted into pBluescript SK+ (+). The plasmid containing the DNA fragment from ~2207 to ~19 was linearized with BalI and used as a template for antisense cRNA probe. The antisense cRNA probe was synthesized by T3 RNA polymerase and [α-32P]CTP. The labeled probe (105 cpm) was hybridized to 4 µg of normal human pituitary gland poly(A)+ RNA (CLONTECH) in 30 µl of hybridization buffer containing 80% formamide, 40 µm PIPES, 400 µM NaCl, and 1 mM EDTA overnight at 50 °C. Following the hybridization step, RNases A and T1 were added to the hybridization mixture, which was incubated at 37 °C for 1 h. 25 µl of the protected hybridization step, RNases A and T1 were added to the hybridization mixture, which was incubated at 37 °C for 1 h. The protected fragment. This underlined HindIII site was added to the 5'-end of GSP1 to facilitate subcloning. PCR was performed according to the manufacturer's instructions. The PCR conditions were 32 cycles of 94 °C for 25 s and 67 °C for 4 min following 7 cycles of 94 °C for 25 s and 72 °C for 4 min. PCR products were analyzed by electrophoresis on a 1% agarose gel.

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Cell Culture—Cells from the rat pituitary-derived cell line, GH3, were cultured in Ham's F-10 medium supplemented with 15% (v/v) horse serum and 2.5% (v/v) fetal bovine serum. HeLa and BeWo cells were maintained in Dulbecco's modified Eagle's medium with 10% (v/v) calf serum. All culture media contained penicillin (100 units/ml) and kanamycin (100 µg/ml).

Protein Gene Construction and Transient Expression Assays—A 2189-nucleotide HindIII fragment of the 5'-flanking genomic sequence (from +19 to −1297, 2007 to −19, when the translation start site was numbered +1) of GHRH-R gene was subcloned into a promoterless luciferase reporter vector, pGL3 basic vector (Promega), and the resulting reporter gene was named as hGHRH-R-Luc(−120 to −19). This hGHRH-R-Luc(−2207 to −19) was digested with SacI, and after removing SacI/SacI fragment, the remaining vector was self-ligated with T3 DNA Ligase to form hGHRH-R-Luc(−1377 to −19). A DraI/HindIII fragment was prepared starting at +101 (hGHRH-R-Luc(−1377 to −19), digestion with Drai and HindIII. This fragment was inserted into the multicloning site of pGL3 basic vector digested with SmaI and HindIII to form hGHRH-R-Luc(−941 to −19). hGHRH-R-Luc(−399 to −19) was prepared by self-ligating after the digestion of hGHRH-R-Luc(−2207 to −19) with Bsal and SmaI. hGHRH-R-Luc(−310 to −19) was prepared by self-ligating after the digestion of hGHRH-R-Luc(−2207 to −19) with ApaI and SmaI. hGHRH-R-Luc(−190 to −19), hGHRH-R-Luc(−120 to −19), hGHRH-R-Luc(−100 to −19), and hGHRH-R-Luc(−100 to −19). These plasmids were introduced into GH3, HeLa, or BeWo cells using LipofectAce (Life Technologies, Inc.). 2 µg of GHRH-R-luciferase reporter construct, pG5 basic vector alone, or rat PRL-luciferase reporter construct, with or without RSV-Pit-1 expression vector, was transfected to the cells in 60-mm dishes. Cells were harvested 48 h after transfection, and luciferase activity was measured using Luciferase Assay System and Luminometer TD-20/20 (Promega). Protein concentration was measured by Bio-Rad protein assay based on the method of Bradford (29).
**FIG. 2. Analysis of the 5′-untranslated region of human GHRH-R mRNA.** A, analysis of the 5′-RACE products. The 5′-RACE experiment was performed using a pair of primers, AP1 and GSP5, as described under “Experimental Procedures.” Marathon-Ready cDNA library (CLON-TECH) derived from human pituitary gland was used as a template. 5′-RACE products were analyzed on an ethidium bromide-stained 1.2% agarose gel. Lanes 1 and 2, molecular weight standards. Lane 3, RACE products. bp, base pairs. B, nucleotide sequence of the 5′-noncoding region of the human GHRH-R gene. The 5′-flanking region is shown in uppercase letters. The coding region is shown in lowercase letters. Numbers are relative to the adenosine of the initiation codon. The 5′-ends of all the RACE products are indicated with solid circles. A solid triangle indicates the major transcription start site determined by RNase protection assay.

DNase-I Footprint Analysis—For footprint studies, recombinant Histagged Pit-1 was produced in *Escherichia coli* and partially purified with nickel-nitrilotriacetic acid resin (Qiagen) according to the manufacturer's instructions. His-tagged Pit-1 appeared to have same function compared with recombinant Pit-1 previously described (20). The DNA fragment used for DNase-I footprint analysis of the promoter and enhancer regions of GHRH-R gene was prepared by digestion of *Hin*36I and *Bsu*III flanking region with BglII and partially purified His-tagged Pit-1 was produced in *Escherichia coli* tagged Pit-1 was produced in *Escherichia coli*.

**FIG. 3. RNase mapping of the transcription start sites of the human GHRH-R gene.** A, autoradiogram of RNase protection assay. RNase protection assay was performed as described under "Experimental Procedures." A uniformly labeled probe corresponding to bases −399 to −19 was hybridized to 4 μg of human pituitary poly(A)+ RNA or 20 μg of yeast tRNA and digested with RNases A and T1. The protected fragments were analyzed by electrophoresis on 6% polyacrylamide/50% urea sequencing gel. Lane 1, human pituitary poly(A)+ RNA. Lane 2, tRNA. Lanes M, bacteriophage M13 mp18 single strand DNA sequence when primer (−40) was used. The major protected band is indicated by an arrow. bp, base pairs. B, schematic representation of the probe used for RNase protection assay and protected fragment. The major transcription start site is indicated by a bent arrow. Numbers are relative to the adenosine of the initiation codon.

**RESULTS**

**Cloning of 5′-Flanking Region of the Human GHRH-R Gene by PCR-based Method—**First, we cloned 5′-flanking region of the human GHRH-R gene as described under "Experimental Procedures." We obtained three genomic fragments, which were about 6, 2.7, and 0.9 kb in size, respectively. The nucleotide sequence of the 0.9-kb fragment was identical to that of 3′-side of the 2.7-kb fragment. In addition, restriction mapping of those fragments indicated that the 6-kb fragment contained the 2.7- and 0.9-kb fragments. The complete sequence of the 2.7-kb genomic fragment is shown in Fig. 1.

According to the analysis of the 5′-flanking sequence of the human GHRH-R gene by MacDNASIS Pro software (Hitachi), no typical mammalian TATA boxes were present, but a CAAT box was present at nucleotides −399 to −393. In addition, there were several putative regulatory elements similar to consensus elements, AP-1 (−1558 to −1552), AP-2 (−241 to −230), SP1 (−1179 to −1171), CRE (−524 to −517), GREs (−983 to −969, −1506 to −1492, and -2474 to -2460), two EREs (−662 to −648 and −1887 to −1873), and eleven putative Pit-1-binding elements. The locations of one nucleotide mismatched Pit-1-binding site consensus are as follows: nucleotides −129 to −123, −166 to −160, −171 to −165, −434 to −428, −839 to −833, −1049 to −1043, −1138 to −1132, −1336 to −1330, −1631 to −1625, −1816 to −1810, and −1881 to −1875.

**Analysis of the 5′-Flanking Region of the Human GHRH-R Gene**—To analyze the 5′-end of the human GHRH-R cDNA, we performed 5′-RACE experiments. The 5′-RACE analysis using ASP1 and GSP5 as primers showed that about 300-nucleotide band was amplified (Fig. 2A). From the sequence analysis of these products, the 5′-end of the longest clone was found to extend to 137 nucleotides upstream from the translation start site. The 5′-ends of all the RACE products are indicated with solid circles in Fig. 2B. To confirm the results of 5′-RACE experiments, RNase protection assays were performed. RNase mapping with a 381-nucleotide labeled cRNA probe comple-
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FIG. 4. Promoter activity of the 5′-flanking region of the human GHRH-R gene in heterologous cells. The human GHRH-R 5′-flanking 2.2-kb fragment (~2207 to ~19) was subcloned into the promoterless luciferase plasmids, pGL3 basic vector. Two μg of these expression plasmids were transfected into GH3, HeLa, or BeWo cells, and luciferase activity was measured as described under “Experimental Procedures.” rPRL-Luc, a reporter plasmid containing rPRL gene, was used as a positive control. Each value was normalized for total protein. Results represent the means ± S.E. for three transfections from a representative experiment.

FIG. 5. Expression analysis of the 5′-flanking region of the human GHRH-R gene in GH3 cells. A, 2 μg of reporter plasmids were transfected into GH3 cells by lipofection method and harvested 2 days after transfection. rPRL-Luc, a reporter plasmid containing rPRL gene, was used as a positive control. Luciferase activity was measured as described under “Experimental Procedures.” Each value was normalized for total protein. Results represent the means ± S.E. for three transfections from a representative experiment.
significantly lower luciferase activity than wild hGHRH-Luc(−310,−19), and hGHRH-Luc(−310,−19) containing mutations in both P2 upper and P2 lower showed a marked reduction in activity, which was almost the same as that of the pGL3 basic vector (Fig. 5B). These findings, consistent with the results from the deletion study, suggested that P1, P2 upper, and P2 lower contribute to stimulation of GHRH-R expression.

On the other hand, when hGHRH-Luc(−130,−19) was used as the reporter plasmid, a P1 mutation did not change the luciferase activity, suggesting that P1 may work in the reporter plasmid with a longer 5'-region (Fig. 5B). The drop in luciferase activity due to deletion of the region between −130 to −120, which was observed in the deletion study, could not be due to the loss of Pit-1 binding because inactivation of the Pit-1 site by mutation has no effect in the context of a short upstream region. In BeWo cells, co-transfection of the deletion mutants down to −310 and RSV-Pit-1 expression vector increased luciferase activity compared with control. However, deletion to −130 resulted in a decrease in the activity in BeWo cells even when Pit-1 expression vector was co-transfected (Fig. 6). Results of these transfection studies indicated that the region from −310 to −130 was important for Pit-1-dependent GHRH-R gene expression in BeWo cells.

**DNase-I Footprint Analysis**—The region from −310 to −130, which was important for the Pit-1-dependent expression of GHRH-R gene, contains DNA sequence P2 (−171 to −160), similar to the Pit-1-binding element. To confirm that Pit-1 binds to the sequence, DNase-I footprint studies were used. Recombinant Pit-1 protected the region from −176 to −155, in which P2 was included, in a dose-dependent fashion. However, Pit-1 did not protect P1 (−129 to −123), which is also similar to the Pit-1-binding element (Fig. 7).

**Mobility Shift Assays for Analysis of Pit-1 DNA Binding**—To further examine Pit-1 binding to P1 and P2, a mobility shift assay was used. A radiolabeled oligonucleotide probe spanning −175 to −142 (P2 probe) formed two specific DNA-protein complexes when incubated with Pit-1, because P2 has two putative Pit-1-binding elements. The two complexes were specifically competed by excess unlabeled oligonucleotide. The consensus sequence of Pit-1 was mutated in the P2 probe, the upper DNA protein complex disappeared, confirming that the element was important for binding of Pit-1. Another radiolabeled oligonucleotide probe spanning −145 to −112 (P1 probe) formed only one DNA-protein complex when incubated with the same amount of Pit-1. When the consensus sequence of Pit-1 was mutated in the P1 probe, the binding of Pit-1 to P1 was reduced, confirming that the site was a Pit-1-binding element. However, the amount of DNA-protein complex was smaller than that when P2 probe was used, suggesting that the binding affinity of P1 to Pit-1 was weaker than that of P2 (Fig. 8). The finding appeared to be consistent with the result observed in the DNase-I footprint analysis that Pit-1 did not protect P1 (Fig. 7).

**DISCUSSION**

In the present study, we cloned the 5'-flanking region of the human GHRH-R gene by the PCR-based DNA walking method and determined transcription start site with RNase protection assay and 5'-RACE. Furthermore, we analyzed its function and Pit-1 dependence using transient transfection assay and deter-
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No intron was found in the 5′-flanking region of the human GHRH-R gene, because the sequence of the 5′-RACE products was completely identical to that obtained from the analysis of genomic DNA flanking to GHRH-R coding region. In addition, these results suggest the accuracy of the PCR-based method. The structure of the GHRH-R gene resembles that of recently characterized human vasoactive intestinal peptide receptor gene (25), which belongs to the same subfamily of G protein-coupled receptors, in respect to lack of intron in its 5′-untranslated region. Typical TATA homologies were not present in the 5′-flanking sequence upstream from the transcription start site. A number of genes are known to lack obvious TATA boxes. Such TATA-less promoters can be divided into two classes. One class consists of GC-rich promoters (26), found in housekeeping genes, which usually contain several transcription start sites spread over a fairly large region and several Sp1-binding sites (27, 28). Another class has no apparent TATA boxes and is not GC-rich (27). Many of these promoters are not constitutively active but rather are regulated during differentiation and development (27). In the human GHRH-R gene, GC content appeared not so rich, and neither a CAAT box nor a typical Sp1-binding site was at the site appropriate to the start site. Thus, the human GHRH-R promoter may belong to the latter class.

Despite the absence of typical TATA homologies, analysis of the 2.7-kb nucleotide sequence of the 5′-flanking region revealed that it contained a number of putative regulatory cis-acting elements. Of particular interest, we identified several sequences corresponding to Pit-1-binding consensus (TATTNCAT) (Fig. 1). As described previously, the GHRH-R gene expression is considered to be controlled by Pit-1 (15–17). In fact, we demonstrated that our cloned 5′-flanking region of the gene increased luciferase activity in Pit-1-expressing GH3 cells and that the 5′-flanking region increased luciferase activity in non-Pit-1-expressing BeWo cells exclusively when RSV-Pit-1 expression vector was co-transfected. The region −310 to −130 was required for Pit-1-dependent promoter activity in BeWo cells according to the deletion mutant analysis. In the region, there are two Pit-1-binding elements, P2 upper and P2 lower. The mutation of P2 upper caused 50% reduction in the luciferase activity, and the mutation of both P2 upper and P2 lower suppressed the activity to basal levels in GH3 cells. These findings demonstrated that P2 is a functional site for GHRH-R.
gene expression. Another important region for GHRH-R gene expression was located from −130 to −120. Although the Pit-1-binding site (P1) was present in the region, mutation of P1 did not affect the luciferase activity when hGHRH-Luc−130,−19) was used as a reporter plasmid. This finding was consistent with the results that P1 is a low affinity binding site (P1) was present in the region, mutation of GHRH-R gene expression, because P1 mutation decreased luciferase activity by 40% when hGHRH-Luc−310,−19) was used as a reporter gene. P1 may function in combination with P2 or other cis-elements, located from −310 to −130.

On the other hand, the hGHRH-Luc−2207,−19) exhibited two times lower luciferase activity than serial deletion mutant constructs. The luciferase activity appeared to drop between −2207 and −1377 (Fig. 5A). The results might imply that the region −2207 to −1377 serves as a significant repressor in GH3 cells.

In summary, we have cloned and characterized the 5′-flanking region of the human GHRH-R gene. The 5′-flanking region contained a functional promoter, and its promoter activity was dependent on Pit-1. The binding site of Pit-1 was located from −176 to −155 judging from DNase-I footprint analysis, and the region containing the Pit-1-binding sites was important for Pit-1-dependent expression of GHRH-R gene. Characterization of this 5′-flanking region will further clarify the regulatory mechanism of human GHRH-R gene expression.

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