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Cloning and characterization of the 5′-flanking region of the human ghrelin gene

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Abstract

Ghrelin, a novel growth hormone releasing peptide, was recently isolated from stomach. We have cloned and characterized the 5′-flanking region, containing from −2000 to −1 upstream from the translation start site of the human ghrelin gene. There was neither typical GC nor CAAT box but there were a TATATAA element and putative binding sites for several transcription factors. Ghrelin promoter was activated only in human stomach derived ECC10 cells among several cell lines examined. Functional analysis showed that promoter activity was increased by deletion of nucleotides from −2000 to −605 whereas it was decreased by further deletion and that the TATATAA element is not functioning. Glucagon and its second messenger cAMP enhanced the promoter activity, suggesting that stimulated transcription of ghrelin gene by glucagon might be responsible for increased ghrelin production during fasting at least in part. These initial characterizations will facilitate further studies of the regulatory mechanisms for ghrelin gene expression.

Keywords: Ghrelin; Growth hormone; Stomach; Promoter; Transcription; Glucagon

Ghrelin is an acylated 28-amino acid peptide recently identified in human and rat stomachs as an endogenous ligand for the GH secretagogue receptor [1]. Ghrelin increases secretion of GH, food intake, and controls energy balance [1–3]. It has been reported that ghrelin produced in the X/A cells of the stomach is a major source of plasma ghrelin [4,5]. Other than stomach, ghrelin is expressed in the pituitary gland, arcuate nucleus of the hypothalamus, pancreas, kidney, and placenta [1,5–8]. Also, some tumors produce ghrelin [9,10]. Papotti et al. [10] reported that RT-PCR showed ghrelin mRNA in all gastrointestinal carcinoids examined.

It is well known that plasma ghrelin levels were increased during fasting and decreased by refeeding. However, factors which regulate the production of ghrelin have not been clarified. While there is a report to show that ghrelin expression in the stomach is up-regulated by insulin and leptin [11], it is also reported that ghrelin expression in the stomach was down-regulated by insulin [12] and leptin [13]. Therefore, it seems that insulin and leptin are not the main regulators for ghrelin production.

To understand the transcriptional regulation of the human ghrelin gene expression, we have cloned and characterized the 5′-flanking region of the human ghrelin gene.

Materials and methods

Materials and cell culture. All chemicals were obtained from Sigma. Fetal calf serum (FCS), horse serum, RPMI1640, Dulbecco's modified Eagle's medium (DMEM), and Ham's F-10 were obtained from Life Technologies (Tokyo, Japan). The human gastric carcinoid derived cell line ECC10 cells, which were obtained from Riken Cell Bank (Cell No. RCB0983), human gastric adenocarcinoma derived MKN1, and MKN 45 cells were maintained in RPMI1640 with 10% calf serum. Rat pituitary derived GH3 cells were maintained in Ham's F-10 medium supplemented with 15% horse serum and 2.5% FCS. COS7, CHO,

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PC3, F9, HeLa, and JEG3 cells, which were derived from monkey kidney, Chinese hamster ovary, human prostate gland, mouse testis, human cervix, and human trophoblast, respectively, were maintained in DMEM with 10% calf serum.

Cloning of the 5′-flanking region of the human ghrelin gene. We have cloned the 5′-flanking region of the human ghrelin gene with polymerase chain reaction (PCR)-based gene walking method (Human Genome Walker kit; Clontech, Palo Alto, CA). The 5′-flanking region of the human ghrelin gene was amplified from the five Human Genome Walker genomic libraries with two consecutive rounds of PCR using the adaptor primers AP1 and AP2 and the gene-specific reverse primers GSP1 (5′-CAGGAGGCTGCAGACGGTCCC-3′, +32 to +13) and GSP2 (5′-TGGGGAGGGCATGGCCTCAGC-3′, +12 to −9) (the translation start site was set at +1) (see Fig. 1A). The gene-specific oligonucleotide primers were synthesized based on sequences of the human ghrelin cDNA (GenBank Accession No. AB029434). The PCR product was subcloned into the pT7 blue vector (Novagen, Madison, WI) and sequenced with a DNA autosequencer (ABI prism377A; Perkin–Elmer). Because the sequence of the PCR product was found on a *Homo sapiens* chromosome 3 clone RP11-1082A18 (GenBank Accession No. AC090841), we amplified the 5′-flanking region using GSP3 (5′-GTTTTTCTTCCAGCGAAATAAAG -3′, −2000 to −1978 according to the draft sequence) and GSP4 (5′-GGCCTCA GCTGGGTTGCAGAC-3′, −1 to −21). Human Genome Walker genomic library (No. 1) was used as a template. The PCR product was subcloned into the pT7 blue vector and sequenced.

Rapid amplification cDNA ends. The 5′-end of the human ghrelin cDNA was determined with the rapid amplification of cDNA end (5′-RACE) method (see Fig. 3A). Human stomach Marathon Ready cDNA (Clontech) was amplified with PCR using the adaptor primer AP1′ and cDNA specific primer GSP5 (5′-GGCTCAGGAAGCTGG AGCCTG-3′). The reaction involved 1 min of denaturation at 94 °C, followed by 5 cycles consisting of 30 s of denaturation at 94 °C and 4 min of annealing and extension at 72 °C, 5 cycles consisting of 30 s of denaturation at 94 °C and 4 min of annealing and extension at 70 °C, and 25 cycles consisting of 20 s of denaturation at 94 °C and 4 min of annealing and extension at 68 °C. The PCR products were diluted to one-fiftieth and then subjected to the secondary PCR with the nested primers AP2′ and GSP6 (5′-CCATGGCCAAGTCCAGCCAGA-3′) using the same protocol. The PCR products were sequenced after being subcloned into the pT7 blue vector.

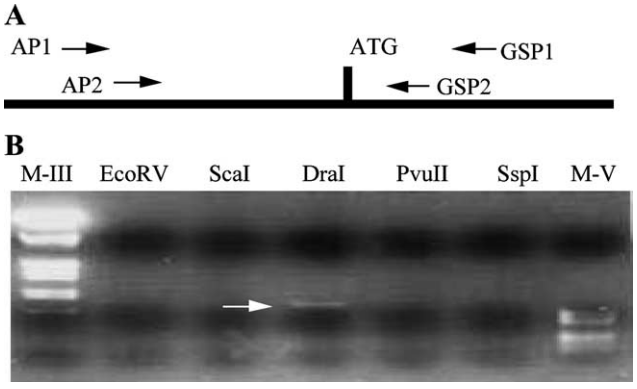

Fig. 1. Cloning of the 5′-flanking region of human ghrelin gene. (A) Schematic diagram representing the positions of primers used for the PCR-based gene walking method. The 5′-flanking region of the human ghrelin gene was amplified from the five Human Genome Walker genomic libraries with two consecutive rounds of PCR using the adaptor primers and the gene-specific reverse primers. (B) Results of agarose gel (1%) electrophoresis and ethidium bromide staining of amplification product are shown. We obtained a PCR product extending to the first *Dra*I site (arrow) containing from −605 to −1 of the human ghrelin gene (the translation start site was set at +1). DNA molecular size markers III and V were run in the left and the right lanes, respectively.

Reporter gene construction and transient expression assays. A fragment of the 5′-flanking region of the human ghrelin gene (−2000 to −1) was subcloned into a reporter plasmid, pGL3-Basic vector (Promega, Tokyo, Japan), to be fused to the luciferase gene (−2000-Ghrelin-Luc). Deletion mutant plasmids were generated by PCR (−1000, −605, −301, −200, and −150-Ghrelin-Luc). Two mutant forms of −605-Ghrelin-Luc, which have mutated TATATAA element (−585 to −579), were made as described previously [14] using −605-Ghrelin-Luc as a template. Mutant sense primer 1 (5′-GCCAGTCATCCTGTCTGAGG ACCTGACAG-3′) and mutant anti-sense primer 1 (5′-CTGTCAGGT CCTCAGACAGGATGACTGGC-3′), or mutant sense primer 2 (5′-GCCAGTCATCCGACATGGGGACCTGACAG-3′) and mutant anti-sense primer 2 (5′-CTGTCAGGTCCCCATGTCGGATGACT GGC-3′) were used. The correct sequence of these mutant plasmids was confirmed by DNA sequencing. After transfection with Lipofectamine Reagent (Invitrogen, Tokyo, Japan), the cells were grown in a normal growth medium, or the medium containing 0.1% BSA instead of serum with vehicle or 10 nM glucagon, or the medium with vehicle or 1 mM CPT-cAMP, or the four medium containing different concentrations of glucose (50, 100, 200, and 400 mg/dl) for 24 h. Luciferase activity was determined in a Turner design luminometer TD-20/20 (Promega) using the dual luciferase assay system (Promega) and normalized with the luciferase activity of co-transfected pRL-CMV containing the cDNA encoding *Renilla* luciferase (Promega). In each transfection, 2 μg of the reporter plasmids and 20 ng pRL-CMV were co-transfected into ECC10, COS7, CHO, PC3, F9, HeLa, JEG3, or GH3 cells cultured in the 35-mm dishes.

Reverse transcription and amplification of cDNA. Total RNA was prepared from cultured ECC10 using Trizol according to the supplier (Life Technologies). The first strand cDNA was synthesized from 4 μg total RNA using random hexamers (GeneAmp RNA PCR Core Kit, Perkin–Elmer, Foster City, CA). PCR was carried out in a 50 μl reaction mixture containing 4 μl of the above first-strand cDNA, 5 μl of 10× PCR buffer, 1 μl of 10 mM dNTP mix, 4 pmol of each primer, and 2.5 U *Taq* DNA polymerase (Gibco). The sequences of primers for the amplification of the human glucagon receptor cDNA were 5′-TGG ATGGCGAGGAGATTGAG-3′ and 5′-GCGGACGAAGATGAAG AAGT-3′ [15]. The sequences of primers for the amplification of the human insulin receptor cDNA were 5′-ATGAACTCCTTCAATTAT AC-3′, and 5′-TGGTGGAAAGTACTCCTCCCG-3′ [16]. The sequences of primers for the amplification of the human leptin receptor cDNA were 5′-CAGAAGCCAGAAACGTTTGAG-3′ and 5′-AGC CCTTGTTCTTCACCAGT-3′ [17]. The sequences of primers for the amplification of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were 5′-CCCTTCATTGACCTCAACTA-3′ and 5′-GCCAGTGAGCTTCCCGTTCA-3′ [18]. The PCR involved 30 cycles consisting of 1 min of denaturation at 94 °C, 1 min of annealing at 58 °C, and 1 min of extension at 72 °C. Autoclaved water and Human liver Marathon Ready cDNA (Clontech) were used as a negative and a positive template, respectively. These PCR products were verified with DNA sequencing.

Statistical analysis. The data were expressed as means ± SE. Statistics were analyzed by one-way repeated measures analysis of variance with a significance level of 0.05.

Results

Cloning of the 5′-flanking region of the human ghrelin gene

We obtained a PCR product extending to the first *Dra*I site which contained −605 to −1 upstream from the

translation initiation site of the human ghrelin gene (Fig. 1B). Another PCR product which contained −2000 to −1 upstream from the translation initiation site of the human ghrelin gene was successfully amplified by GSP3 and GSP2. DNA sequences of the latter PCR product completely matched not only with the sequence, which are identified on a *Homo sapiens* chromosome 3 clone RP11-1082A18 (GenBank Accession No. AC090841),

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-2000 GTTTTTCTTCCAGCGAAATAAAGGATTCAGCCTCCCAGAGCTTTAAAGTCTTTCCAACTCTTTCATTCATTCTATGGTCTCTCTCTCCTCAAGAATCCTC
-1900 AGTGGCTGCCCTCCTGCAAAAAAAGCCCAAACTTCTTGGGCTAGCAGTTCCCAAGGCATCCATCACCTGGCCCCAAACCACCTTCTTATGTTTTTGTTAT
          AP2                                                AP2         bHLH
-1800 GACCCCATCGCCACCCTCCCCCAAGCCACCCAGCCTTCTCCATGCTGCCCTGATGTACTCTACCCACGCCTTCACCTCCCCTCTGCTTGCCGCAAAGCCC
        AP2            bHLH                                                AP2
-1700 TCCCTCCAAAGCCCTGCCCCGAGTTCACCACAGGCCTCTTTCCTGGAGCCTTTGCTAACATCCCTGCCGCAAGCCCCAGCCTAAGGTGAGCTCCTCTCCT
                                                                          NF-IL6
-1600 GAACTGCTAGAACTTCTCTGCAGACAACTCATTGGCCCTTAGGGGTTCCTGCCTGGGCACCCAGTTGGATGAAGCACTCCAGAAAGGAAGAAATTCCTGG
                                                                  bHLH                    PEA
-1500 GGCCGAGGGAAATGAGGCAGTGGCCTTGGCCTATGCTGGGAAGTCCTATGGGCCTGGTATGAAACCATTGTCATCTGCAGGGTGTTTCACAGGTTAGAAG
                                                                        bHLH
-1400 CCACTTTCCCCCCATCGCCTCATTTCACCCTCAAAGACTCTGGGTAAGGGGAATTATTTGTGGTCGGCGTAAGGCCAGTCAATGAGAGAGGAGCCAGGCC
            AP2                           HNF5                                                        AP2
-1300 CGTGCTAAGCGTAGATCTTCCACCTCCAGGTCCAATGCACTTCCCTCTCAGAAGAGGCATCCGCTAAAATAGGGACCAAAGCTGCTGGAGGGAGGCAAGG
-1200 CAAGCTGCTATGTGAAAAAACGCCAGGCCAGGCAGTCATGTCACACCTGGCAGAAATGACTGAAGCATAGCCACTGGCTGAAGTTATCCCCACACCCACT
                          AP2                 AP2
-1100 CTCTGGAGAGGATGATCAGGAGCAGTCTGCTCAACCGGGAGGTGGGACTCCTCCTCGGGAAGGTGTAGAATCACCAGCCTGGCTCCCTGCGGACTCCCGG
                                              NFkB
-1000 GGCTCACAGAGGCCAGAGCAGCAACAGCACATGGGAAACAACGGGGCGCTGGACTGGGGAGGTCTCAGAGCTCTCCTAGTGATGACAGCCTCATTTTACC
                                   bHLH        Myb
 -900 CAGGGAGAAAGGGCGAGTAAGCTAAGGTCACACAGCAACAAAGCTGCACCCAGACCCCAGAGCCACTCTCCTCCCTCCCTCCTCCACCAGGGCCATGCCC
                             half ERE
 -800 ACTTGGGGCACCCCGCCACCGTGTTCCAGGGACAGCTGGAGCACATGCTTCTTCCCTCGCCAACCCAGCAATTCCGCAGGGCATCTGACCTCCACTGTTG
                                        bHLH     bHLH            AP2                      half ERE
 -700 ACTTCTACCCAGAGGACAAGAACATTTTTAGTTCCCAAGGAATGTACATCAGCCCCACGGAAGCTAGGCCACCTCTGGGATGGGGTTGCTGGTTTAAAAC
                      half GRE                                AP2
 -600 AAACGCCAGTCATCCTATATAAGGACCTGACAGCCACCAGGCACCACCTCCGCCAGGAACTGCAGGTAAGCTCTCTCTACTCTCCGTGTGCGTTTGCTGA
                   TATA box like
 -500 CAGGTTTATTGTCCGTGACTGCTGCCCGTCCGTTTAACCTCTCTGGGCCTCAGGAGTCCCCTCTAAACACGGGGGATGTCGGACTGTCTGGCTGGAAGAC
 -400 AGAGCTTCATTTAGGAGCTACCTCTAACCCCCTCCCTGCTGAGTGCCCTGGGGCCCTTGCTGGTTCAGCTTCCTCCCTCCTCATGAAAAGACTCGCCATG
                                    AP2
 -300 AGCTCTCAGAGCCTGGGACAAAAGGCACCGGGGAGGACCCCCAGCCCCTATCAAGTCCTGCTGCCAGGTGTTCTGCCCTGAACGGCCTTGGAGAAGAGAA
                                          AP2                 bHLH
 -200 CACACATCCATCATCTTCTACCTTGGGAGGTGGAAGCGGGGGTCCTCTCTACACAGCAGTCAGGACCCCAGATGGTAAAGCCCTTCACGCCATCTGCTGG
                                                                    bHLH                bHLH
 -100 GCTCCTACCTGAGCAAGCTCAGAGGCACATGAGAAGGGGAGGCAGCTGCCTGGAGACCCTCTCTCCCCCAGGCCCACCTGTCTGCAACCCAGCTGAGGCC
   +1 ATG                                                                              *
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Fig. 2. The nucleotide sequences of the 5′-flanking region of the human ghrelin gene. The translation start site was set at +1. The major 5′-end of the 5′-RACE products is denoted by an asterisk. The putative binding sites for several transcription factors were found.

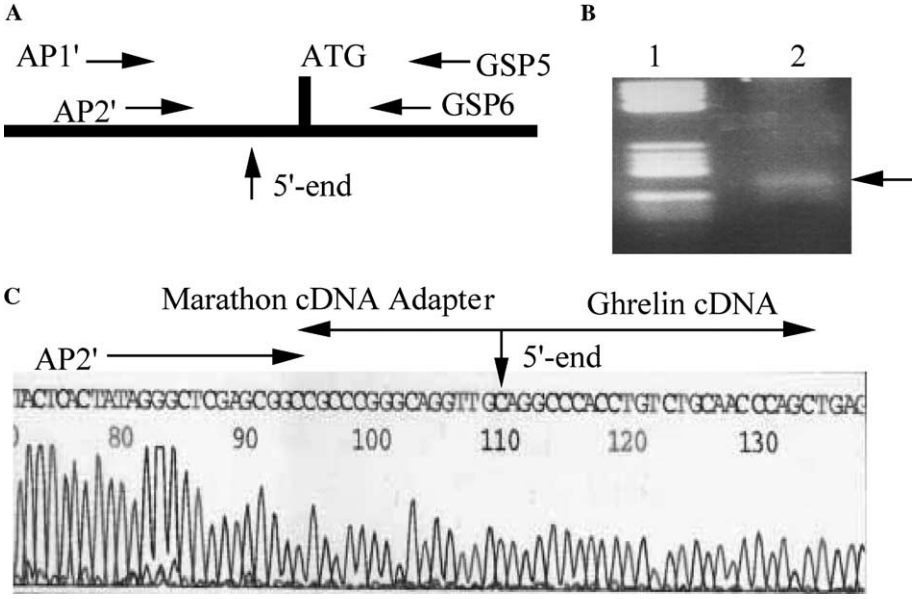

Fig. 3. 5′-RACE of the human ghrelin. (A) Schematic diagram representing the positions of primers used for 5′-RACE. (B) Human stomach Marathon cDNA was subjected to two sets of PCR. Results of agarose gel (1%) electrophoresis and ethidium bromide staining of amplification products are shown in lane 2. DNA molecular size marker V was run in lane 2. (C) Amplified products were subcloned and sequenced, and representative data are shown.

but also with DNA sequences of the former PCR product which contained −605 to −1 upstream from the translation initiation site of the human ghrelin gene (Fig. 2).

The 5′-end of cDNA of the human ghrelin gene was determined by the 5′-RACE. The human stomach cDNA was amplified by the second PCR using AP2′ and GSP5 and the PCR products were subcloned into the pT7 blue vector. All of the eight clones sequenced showed that the 5′-end of cDNA of the human ghrelin gene was −32 from the translation start site, suggesting that −32 seemed to be a major transcription start site (Fig. 3).

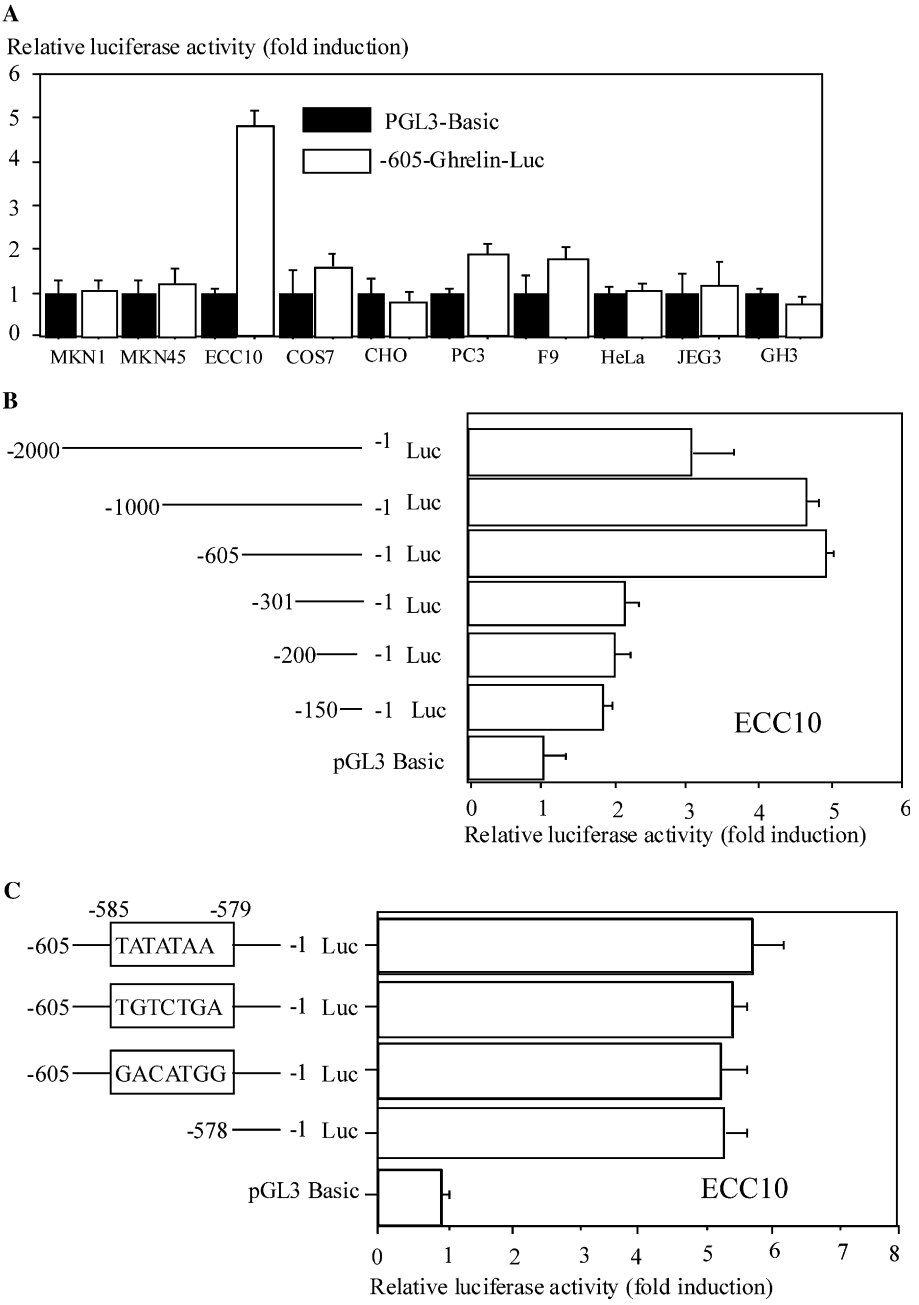

Fig. 4. Functional analysis of the human ghrelin promoter. (A) Cell specificity of the human ghrelin promoter activity. Plasmids PGL3-Basic containing −605 to −1 of the human ghrelin gene (−605-Ghrelin-Luc) were transiently transfected into several cell lines. (B) Deletion analysis of the human ghrelin promoter using ECC10. The schematic diagram on the left represents each deletion construct of the human ghrelin gene fused into the upstream region of the luciferase gene. (C) TATATAA element is not important for promoter activity of the human ghrelin gene. There is a TATA box like element (TATATAA) from −585 to 579. To clarify its role for the human ghrelin gene transcription, TATATAA was mutated to TGTCTGA or GACATGG, or deleted. (A–C) In each transfection, 2 μg of the reporter plasmids was transfected into ECC10, COS7, CHO, PC3, F9, HeLa, JEG3, or GH3 cells cultured in the 35-mm dishes. Twenty ng of pRL-CMV containing the cDNA encoding *Renilla* luciferase was co-transfected to normalize the luciferase activity in each transfection. Experiments were performed in triplicate and values (means ± SE) are expressed as multiples of induction relative to luciferase activity when promoterless PGL3-Basic was transfected.

There was no typical GC or CAAT box but there were a TATATAA element (−585 to −579) and putative binding sites for several transcription factors including activator protein-2 (AP2) (−1897 to −1890, −1851 to −1844, −1738 to −1731, −1393 to −1384, −1308 to −1301, −1179 to −1172, −1157 to −1152, −744 to −735, −648 to −641, −371 to −365, and −264 to −254), basic helix-loop-helix (bHLH) (−1837 to −1832, −1539 to −1534, −973 to −968, −768 to −763, −759 to −753, −236 to −231, −132 to −127, and −110 to −105), PEA-3 (−1516 to −1511), Myb (−962 to −957), NF-IL6 (−1636 to −1628), half-site for the estrogen response element (−876 to −871, −715 to −710), half-site for the glucocorticoid response element (−682 to −677), hepatocyte nuclear factor-5 (−1361 to −1354), and NF-κB (−1057 to −1048).

Functional analysis of the human ghrelin 5′-flanking region

Plasmids containing cloned 5′-flanking sequences of the human ghrelin gene fused to the luciferase gene were transfected into several cell lines. As illustrated in Fig. 4A, the human ghrelin promoter is activated only in ECC10 derived from human gastric carcinoid [19], but not activated in other examined cell lines including MKN1 and MKN45 which were derived from human gastric adenocarcinoma, suggesting that human ghrelin promoter has cell-specific activity.

To identify the important regulatory regions for the expression of human ghrelin gene, deletion mutants of the 5′-flanking sequences were constructed. Fig. 4B summarizes the effect of these deletions on the luciferase reporter activity in ECC10. The luciferase activity was increased by deletion from −2000 to −605, whereas it was decreased by further deletion. Because there was a TATATAA element from −585 to −579, we mutated or deleted this element to clarify its role for the activation of human ghrelin promoter. Neither mutation nor deletion of the TATATAA element decreased the promoter activity, suggesting that TATATAA element is not functioning (Fig. 4C).

Fasting causes the increase of both ghrelin and glucagon levels in peripheral circulation. Therefore, we examined the effect of glucagon and its second messen-

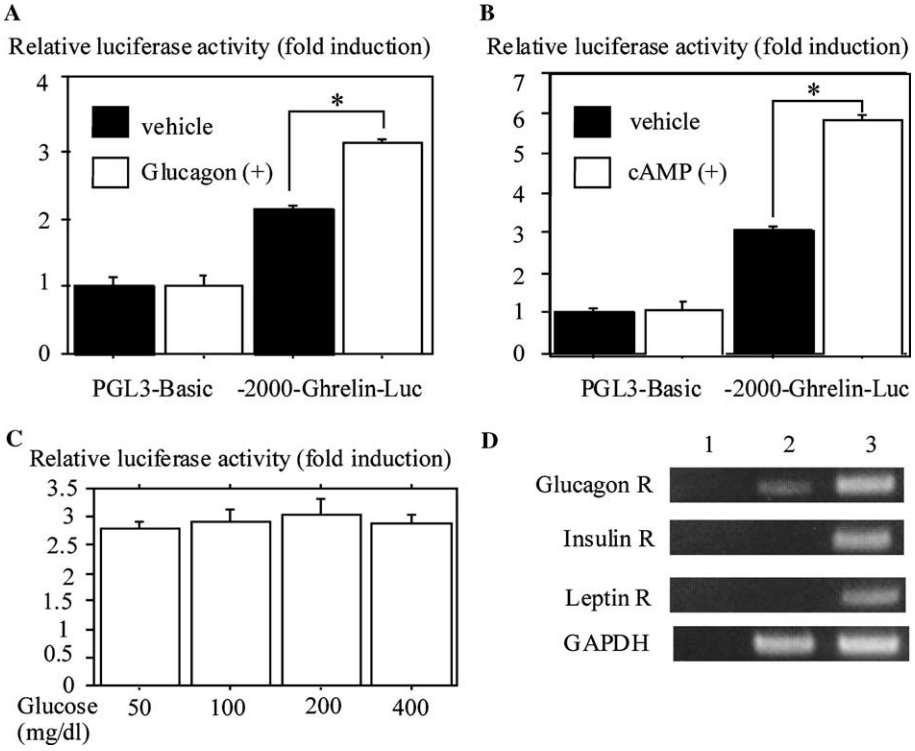

Fig. 5. (A) Effect of glucagon on the reporter activity of −2000-Ghrelin-Luc containing from −2000 to −1 of the human ghrelin gene. PGL3-Basic and −2000- Ghrelin-Luc were transfected into ECC10 and cultured in RPMI 1640 containing 0.1% BSA with or without 10 nM glucagon. (B) Effect of CPT-cAMP on the reporter activity of −2000-Ghrelin-Luc. PGL3-Basic and −2000-Ghrelin-Luc were transfected into ECC10 and cultured in RPMI 1640 containing 10% FCS with or without 1 mM CPT-cAMP. (C) Reporter activity of −2000-Ghrelin-Luc cultured in several different glucose concentrations. PGL3-Basic and −2000-Ghrelin-Luc were transfected into ECC10 and cultured in RPMI 1640 containing 10% FCS with glucose (50, 100, 200, or 400 mg/dl). (A–C) In each transfection, 2 μg of the reporter plasmids was transfected into the ECC10 cells cultured in the 35-mm dishes. Twenty nanograms of pRL-CMV containing the cDNA encoding *Renilla* luciferase was co-transfected to normalize the luciferase activity in each transfection. Each bar represents the means ± SE ($n = 5$) of luciferase activity expressed as fold induction vs. promoterless PGL3-Basic. Asterisks mean statistical significance vs. vehicle-treated control ($P < 0.05$). (D) Expression of mRNA of glucagon receptor, insulin receptor, leptin receptor, and G3PDH in ECC10 (lane 2) and in human normal liver (lane 3). Autoclaved water as a negative control was used (lane 1).

ger cAMP on the human ghrelin promoter activity. Glucagon and cAMP significantly increased the activity of human ghrelin promoter (Figs. 5A and B). In contrast, the concentration of glucose in the medium did not affect the promoter activity (Fig. 5C). ECC10 expressed the glucagon receptor but not the receptor of insulin nor leptin (Fig. 5D), suggesting that insulin and leptin seem not able to regulate ghrelin gene transcription directly, at least in our experimental conditions.

Discussion

This is the first report of the functional analysis of 5′-flanking region of the human ghrelin gene. Although Tanaka et al. [20] reported the presence of short noncoding first exon of mouse ghrelin gene and suggested a possible function of TATATAA element detected in mouse ghrelin promoter, we and another group [1] could not detect such a noncoding exon in the human ghrelin gene. Furthermore, here we showed that TATATAA element detected in the 5′-flanking region of the human ghrelin gene is not functioning at least under our experimental conditions. Since Tanaka et al. did not perform a functional analysis of the mouse ghrelin promoter, it still remains unclear whether TATATAA element is working in mouse ghrelin gene.

Although ECC10 is derived from human gastric carcinoid [19] but not from X/A cells which produce ghrelin in the normal stomach [5], RT-PCR showed ghrelin mRNA in ECC10 while not in MKN1 and MKN45 which are derived from human gastric adenocarcinoma (data not shown). Furthermore, among several cell lines examined, ghrelin promoter is activated only in ECC10 and not activated in other cell lines including MKN1 and MKN45, suggesting that human ghrelin promoter has cell-specific activity.

The plasma ghrelin levels were increased during fasting and decreased by refeeding. However, the factors regulating ghrelin production have not been clarified. We first proposed the possibility that glucagon is a positive regulator of ghrelin production. We also showed that cAMP-signaling up-regulates the ghrelin gene transcription. It is noteworthy that GHRH, which also activates a cAMP-signaling cascade, up-regulates ghrelin gene transcription in the pituitary gland [21]. However, the mechanism by which cAMP up-regulates ghrelin gene transcription remains unclear because there are no AP1 site and cAMP responsive element (CRE) in our cloned 5′-flanking region at least from −2000 to −1 upstream from the translation start site. It is possible that there is a cell-specific transcription factor that regulates ghrelin gene transcription and integrates cAMP stimulation to activate ghrelin gene transcription using transcriptional cofactors in the same manner as other cell specific transcription factors [14,22,23].

In addition to stimulating the secretion of GH, ghrelin also regulates the energy balance [1–3]. Therefore, several factors involved in the energy balance may regulate the ghrelin production. Ghrelin expression in the stomach is reported to be increased by insulin and leptin [11]. Later, there were also reports of contradictory results that gastric ghrelin expression was decreased by insulin [12] and leptin [13]. Therefore, insulin and leptin do not seem to be the main regulators for the production of ghrelin. Furthermore, in ECC10 cells neither leptin nor insulin seemed to regulate the ghrelin gene transcription at least directly through their receptors.

In summary, we have characterized the 5′-flanking region of the human ghrelin gene for the first time. These initial characterizations should facilitate further study of the mechanism involved in the transcriptional regulation of the human ghrelin gene expression in human health and disease.

Acknowledgments

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