to assess the possible benefits of ghrelin administration to humans (12–18, 28–31). Indeed, these studies include only one non-randomized phase I clinical trial (15). In this study, we performed a phase I/II trial to investigate the pharmacokinetics, safety and efficacy of ghrelin with regards to induction of hormone secretion and appetite in healthy volunteers.

Materials and methods

Healthy volunteers

Eighteen male volunteers between 21 and 25 years old participated in our phase I/II study, which was conducted at Kyoto University Hospital (Kyoto, Japan). The volunteers were judged to be healthy by standard clinical and laboratory investigations including chest X-ray, 12-lead electrocardiogram, prick allergen testing and blood pressure responses to postural changes. In addition, they were not suffering from any medical diseases or receiving medical treatment and had no past history of malignant disorders. They were randomly assigned to three groups: low- and high-dose ghrelin groups and a placebo group (see below). The baseline characteristics of each group of volunteers are summarized in Table 1. There were no significant differences in age, body mass index (BMI) or other parameters between the three groups. The study protocol was approved by the ethics committee on human research at the Kyoto University Graduate School of Medicine (project proposal number 400), and the studies were performed according to Good Clinical Practice (GCP) standards. Written informed consent was obtained from the volunteers prior to enrolment.

Study design

The study was randomized, double-blinded and placebo-controlled. Over a week-long screening period, eligible volunteers received either a bolus intravenous injection of ghrelin (1 μg/kg for the low-dose group or 5 μg/kg for the high-dose group) or a placebo injection (3.75% D-mannitol). Treatment assignments were randomized centrally to a 1:1:1 ratio, in blocks of three. The randomization code was generated by a statistician from the Department of Clinical Trial Design and Management and was maintained there until all data had been collected and the study was unblinded. Neither the investigators nor the volunteers were aware of the treatment assignments in the study.

Study drug

Human ghrelin was synthesized as previously described (1, 12, 15, 17). The acylated peptide was dissolved in 3.75% D-mannitol to yield a final concentration of 40 or 400 μg/ml. These solutions were then filtered and stored at −20 °C in sterile vials. Examinations conducted by the Japan Food Research Laboratories (Tokyo, Japan) found no traces of endotoxin in the ghrelin solutions, and a pyogen test based on the Japanese Pharmacopoeia was negative.

Assessment of safety

Overall safety was assessed on the following day and at 8 days post-infusion. At all study visits, vital signs (pulse rate and blood pressure), haematology, blood chemistry and urinalysis were measured. All volunteers were hospitalized during the night of drug administration.

Measurement of plasma ghrelin, GH, insulin-like growth factor-I (IGF-I), glucose and insulin concentrations

On infusion days, subjects were fasted overnight and had two intravenous cannulae (one for infusion and one for blood sampling) implanted at 0900 h ($t = -45$ min). Blood samples for hormone and glucose analyses were drawn from an indwelling catheter fixed in a forearm vein at −15, 0, 15, 30, 45, 60 and 90 min after drug administration. Pulse rate and blood pressure readings were taken at every blood drawing. N-RIAs and C-RIAs (radioimmunoassay (RIA) for the ghrelin N- and C-terminals, respectively) for plasma ghrelin were performed using anti-human ghrelin-(1–11) and -(13–28) sera respectively, as described previously (32, 33). The anti-human ghrelin-(1–11)

Table 1 Summary of volunteer baseline characteristics (means±S.D.).

Variable	Placebo ($n = 6$)	1.0 μg/kg ghrelin ($n = 6$)	5.0 μg/kg ghrelin ($n = 6$)
Age (years)	23.3±1.5	22.8±1.7	22.0±0.0
BMI (kg/m^2)	20.1±1.9	22.0±2.0	20.9±0.8
Weight (kg)	62.8±6.0	64.0±8.4	63.2±5.7
Serum GH (ng/ml)	6.2±6.2	0.8±1.0	1.9±3.9
Serum IGF-I (ng/ml)	275.7±67.6	262.8±98.5	287.8±62.3
Plasma glucose (mg/dl)	82.8±3.4	84.8±7.1	78.7±6.6
Serum insulin (U/ml)	4.1±2.2	3.4±2.0	4.3±1.2
Plasma total ghrelin (fmol/ml)	221.6±46.3	149.2±66.8	188.0±62.6
Plasma active ghrelin (fmol/ml)	18.3±5.4	12.0±5.3	20.7±10.1*

* $n = 5$; the active ghrelin level of one patient was eliminated because the sample was stored improperly.

antiserum specifically recognizes the *n*-octanoylated portion of ghrelin and does not recognize des-*n*-octanoylated ghrelin, while the anti-human ghrelin-(13–28) antiserum recognizes both *n*-octanoyl-modified and des-*n*-octanoylated ghrelin equally. Thus, by using both assays we were able to measure the total ghrelin plasma concentration and the concentration of the active form only. Serum GH, IGF-I and insulin were measured by immunoradiometric assay (IRMA) (FALCO Biosystems Ltd, Kyoto, Japan). IGF-I was measured before infusion, and also 1 and 8 days after the infusion.

Assessment of hunger sensation

Visual analogue scales rating hunger (possible scores 0–10 cm) were completed pre-infusion at −15 min, at infusion (0) and 15, 30, 45, 60 and 90 min after the administration of the study drug (34, 35). Positions on the scale were measured in centimetres and millimetres by an investigator who was unaware of the treatment groups. The change (Δ) in the visual analogue score at time t was calculated by subtracting the visual analogue score at 0 min from the score at time t in the same subject.

Pharmacokinetic and statistical evaluations

The pharmacokinetic evaluation for ghrelin was performed using the one-compartment model (36). The peak plasma concentration (C_{max}) was calculated with PK Solutions version 2 (Summit Research Services, CO, USA) and S-PLUS Version 4.0 (Mathematical Systems Inc., Japan). The area under the curve (AUC) for the entire 90 min blood-sampling period was calculated by the trapezoidal method. The $t_{1/2}$ was calculated as ln 2/z, where z is the terminal elimination rate constant. Pharmacokinetic parameters were analysed descriptively with the calculation of geometric means and 95% confidence intervals (CI). Comparisons between groups administered with different study drugs were performed using the Wilcoxon rank sum test with the Bonferroni correction. Other statistical analyses were performed with SAS version 8 (SAS Institute Inc., Cary, NC, USA). A two-tailed P value was used, with the required level of significance being $P < 0.05$.

Results

Pharmacokinetics

Mean plasma concentration-vs-time profiles of ghrelin after administration are shown in Fig. 1. Descriptive statistics of the pharmacokinetic parameters of ghrelin are summarized in Table 2. After administration of 1 and 5 μg/kg ghrelin, total plasma ghrelin concentrations reached 1058.7 and 6597.9 fmol/ml respectively, and had an elimination $t_{1/2}$ of 27–31 min. Active ghrelin at 15 min accounted for 42.2–52.3% of total ghrelin, and displayed a $t_{1/2}$ of 9–13 min, roughly one-third that of total ghrelin. The active ghrelin levels of one patient administered with 5 μg/kg ghrelin were eliminated from the analysis because his samples were stored improperly.

Assessment of safety

Several adverse events were reported by ghrelin-injected subjects (Table 3), but all of these complaints

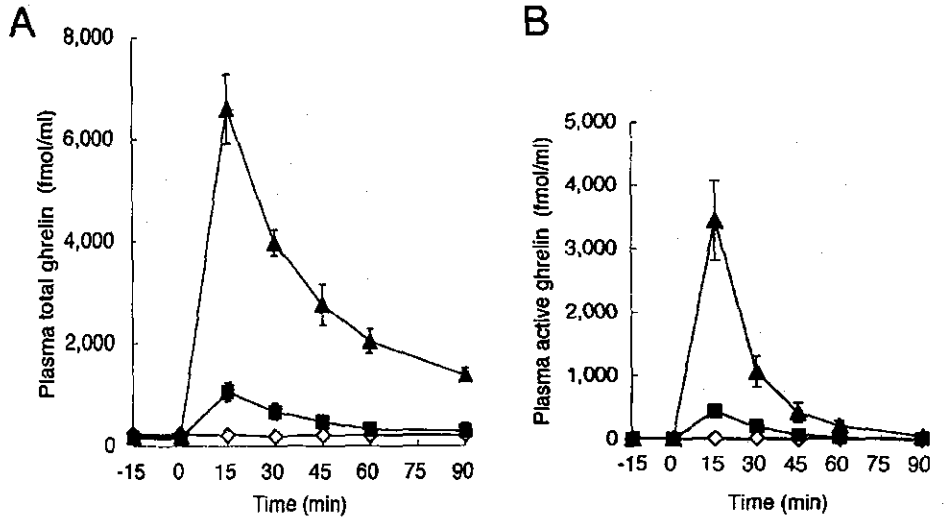

Figure 1 Pharmacokinetics of intravenous bolus administration of ghrelin. Plasma ghrelin concentrations were measured by C- and N-RIA for total (A) and active ghrelin (B) respectively. ▲, 5 μg/kg ghrelin; ■, 1 μg/kg ghrelin; □, placebo. All values are expressed as means±95% CI ($n = 6$ (A) and $n = 5$ (B)).

Table 2 Pharmacokinetic parameters of intravenous bolus administration of ghrelin. Results are given as means with 95% confidence intervals in parentheses.

	$C_{max,0-90\,min}$ (fmol/ml)	$AUC_{0-90\,min}$ (10^3fmol min/ml)	$AUC_{0-90\,min}$ (10^3fmol min/ml)*	$t_{1/2}$ (min)
Total ghrelin				
Placebo ($n = 6$)	230.7 (187.8, 273.5)	18.92 (16.51, 21.34)		
1.0 μg/kg ghrelin ($n = 6$)	1058.7 (882.6, 1234.8)	45.91 (35.64, 56.17)	54.6 (43.80, 65.41)	31.33 (20.49, 42.18)
5.0 μg/kg ghrelin ($n = 6$)	6597.9 (5919.0, 7276.6)	268.51 (244.10, 292.91)	327.87 (299.03, 356.71)	26.92 (24.65, 29.19)
Active ghrelin				
Placebo ($n = 6$)	23.8 (19.4, 28.2)	1.91 (1.58, 2.23)		
1.0 μg/kg ghrelin ($n = 6$)	447.2 (387.0, 507.5)	12.27 (10.27, 14.26)	18.92 (16.70, 21.14)	12.90 (8.97, 16.83)
5.0 μg/kg ghrelin ($n = 5$)	3454.0 (2714.5, 4194.6)	80.67 (66.74, 94.59)	143.68 (107.99, 179.36)	9.56 (7.37, 11.74)

* $AUC_{0-90\,min}$ in one-compartment model.

Table 3 Adverse events.

Adverse event	Placebo ($n = 6$)	1.0 μg/kg ghrelin ($n = 6$)	5.0 μg/kg ghrelin ($n = 6$)
Abdominal discomfort	0	1	0
Abdominal pain	0	0	1
Diarrhoea	0	0	1
Malaise	1	0	0
Fever	0	0	1
Glucosuria	1	1	0
Leukocytosis	0	0	1
Proteinuria	1	0	0
Hypoesthesia	1	0	0
Somnolence	0	2	0
Throat redness	0	0	1
Hyperhidrosis	0	0	1
Flushing	0	1	1
Total	4	5	7
Number of subjects	3	2	2
(Incidence, 95% CI)	(0.50, 0.12 ~ 0.88)	(0.33, 0.04 ~ 0.78)	(0.33, 0.04 ~ 0.78)

were transient and well tolerated. One subject administered with 1 μg/kg ghrelin experienced mild abdominal discomfort and a flushed sensation for a few minutes; another subject injected with 5 μg/kg ghrelin indicated a flushed sensation and a marked episode of perspiration in the upper part of the trunk that lasted for nearly an hour. Two subjects injected with 1 μg/kg ghrelin experienced somnolence. One subject administered with 5 μg/kg ghrelin manifested fever, pharyngeal redness and diarrhoea with abdominal pain and leukocytosis; however, specialist infectious disease physicians and investigators diagnosed him as having acute bacterial enterocolitis that was unrelated to the medication. No significant changes in heart rate or blood pressure were recorded following the injections. Ghrelin did not significantly alter the clinical blood chemistries or complete blood count, to the best of our ability to detect. Although mild glucosuria was found in a low-dose ghrelin subject on the day after the injection, it disappeared after 1 week and was also found in a placebo subject; this subject also returned to normal after 1 week. Another placebo subject showed mild proteinuria on the day after the injection, but did show any abnormal urinalysis after 1 week. Since both subjects did not show glucosuria at the screening or 1 week after injection, they were judged to be healthy. Abnormal urinary findings were not found in high-dose subjects. In total, the number of subjects that experienced adverse effects was not significantly different among the three groups (Table 3).

GH and IGF-I release

The GH responses to ghrelin are shown in Fig. 2A and Table 4. Ghrelin strongly stimulated GH release. No significant difference between the low- and high-dose ghrelin groups was observed in the peak or AUC GH release, suggesting that a submaximal-dose ceiling effect occurs even at 1 μg/kg ghrelin in normal young males. Administration of ghrelin did not induce a significant increase in plasma IGF-I (Fig. 2B).

Plasma glucose and serum insulin

Ghrelin administration resulted in significantly higher plasma glucose levels 15 min after injection with either 1 or 5 μg/kg ghrelin, although no dose dependency was observed (Fig. 3A). At 90 min after injection of 5 μg/kg ghrelin, plasma insulin demonstrated a statistically significant decrease (Fig. 3B). No significant

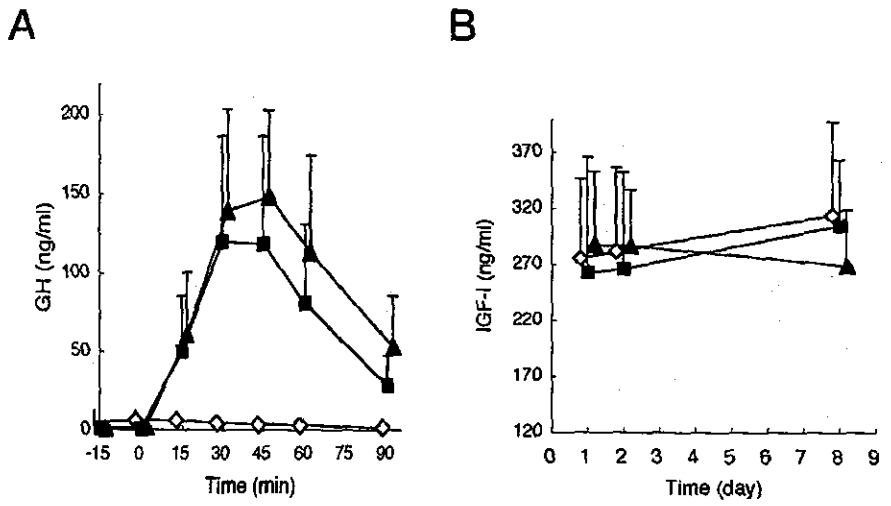

Figure 2 Serum GH (A) and IGF-I (B) levels following ghrelin administration. ▲, 5 μg/kg ghrelin; ■, 1 μg/kg ghrelin; ◇, placebo. All values are expressed as means±S.D. ($n = 6$).

Table 4 GH, glucose and insulin responses to ghrelin administration (means±S.D.).

	GH (ng/ml)		$\Delta AUC_{0-90\,min}$	
	$C_{max,0-90\,min}$*	$AUC_{0-90\,min}$†	Glucose (mg/dl)‡	Insulin (U/ml)§
Placebo ($n = 6$)	6.91±6.2	344.2±307.1	−82.5±138.4	27.9±82.0
1.0 μg/kg ghrelin ($n = 6$)	124.2±63.9	6547.3±3688.4	152.5±187.2	−26.5±54.5
5.0 μg/kg ghrelin ($n = 6$)	153.2±52.2	8564.4±3630.6	166.25±455.8	−86.3±81.4

*$P = 0.005$: placebo vs 1.0 μg/kg ghrelin; placebo vs 5.0 μg/kg ghrelin.
†$P = 0.005$: placebo vs 1.0 μg/kg ghrelin; placebo vs 5.0 μg/kg ghrelin.
‡Not significant (NS): placebo vs 1.0 μg/kg ghrelin; placebo vs 5.0 μg/kg ghrelin.
§NS: placebo vs 1.0 μg/kg ghrelin; placebo vs 5.0 μg/kg ghrelin.

differences were observed in the ΔAUC of glucose or insulin levels following ghrelin injection (Table 4).

Hunger sensation

Ghrelin administration tended to increase the hunger sensation in a dose-dependent manner, particularly within 45 min of injection (Fig. 4A). However, the difference between groups, based on evaluation by change in visual analogue score, did not reach statistically significance levels even at 15 min (Fig. 4B). In the placebo group, two of the six subjects did not show any change in hunger score. In contrast, all of the subjects in both the low- and high-dose ghrelin groups reported changes in their scores (data not shown).

Discussion

We performed a double-blind, randomized, placebo-controlled trial on 18 young healthy volunteers to investigate the pharmacokinetics, safety and efficacy of ghrelin in inducing hormone secretion and appetite. Since the number of subjects studied may be too low to obtain thorough information about these effects of ghrelin, we adopted this study design to minimize the disadvantage or bias that is caused by the rather low number of subjects. This is the first report to simultaneously analyse the pharmacokinetics of both total and active ghrelin, and is a larger clinical trial of ghrelin administration than the only previous clinical trial (15). The total plasma ghrelin concentrations recorded at 15 min following administration of either 1 or 5 μg/kg ghrelin (3.2 and 20 ng/ml respectively) appeared to be comparable to those recorded during a previous trial which examined the administration of 10 μg/kg ghrelin (~60 ng/ml) (15). Roughly 42.2–52.3% of the total ghrelin at 15 min was active ghrelin. The elimination $t_{1/2}$ of total plasma ghrelin was 27–31 min, nearly three times as long as that of active ghrelin, reflecting the rapid degradation of acylated active ghrelin in the plasma (33).

Several adverse effects were reported following ghrelin administration in our study, including abdominal discomfort, flushing, somnolence and hyperhidrosis (Table 3). Previous studies reported similar adverse

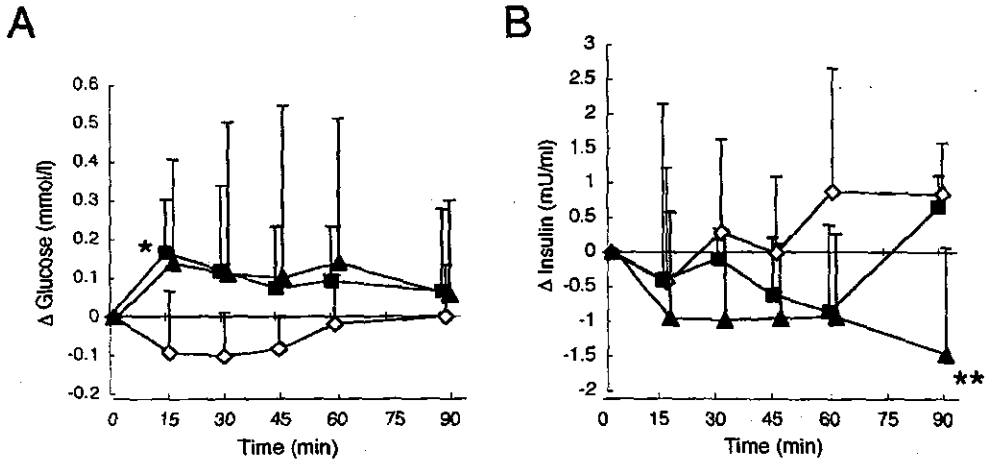

Figure 3 Changes in plasma glucose (A) and serum insulin (B) concentrations after ghrelin administration. $^{*}P < 0.05$, 1 μg/kg ghrelin vs placebo. $^{**}P < 0.05$, 5 μg/kg ghrelin vs placebo or 1 μg/kg ghrelin. *All values are expressed as means±S.D. ($n = 6$).

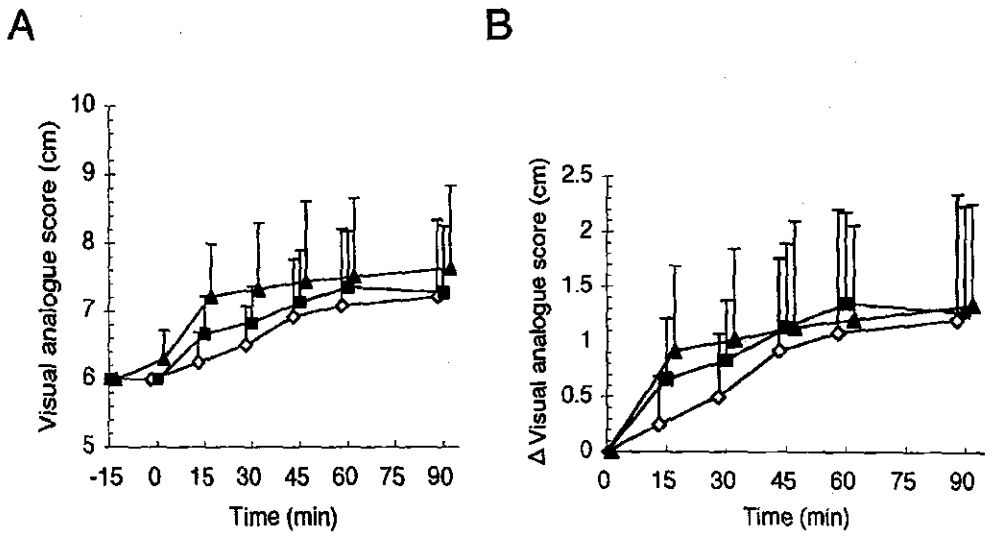

Figure 4 Changes in hunger scores following ghrelin administration. (A) Absolute visual analogue scores; (B) change in visual analogue scores. *All values are expressed as means±S.D. ($n = 6$).*

effects, but listed bowel movements and warm sensations instead of abdominal discomfort and flushing (Table 5). However, these are probably two different descriptions of the same phenomena. Because these adverse effects have been observed in multiple studies, it is likely that they are directly ghrelin related. All of these complaints, however, were transient and well tolerated. Ghrelin did not significantly alter clinical blood chemistries or complete blood count as far as we were able to discern.

In this study, 1 μg/kg of ghrelin appeared to result in a ceiling effect on the GH response in young men: $C_{max,0-90\,min}$ of GH was 124.2±63.9 ng/ml. This observation is in contrast to previous studies that were performed with the same size of groups (each group, $n = 6$); the 1 μg/kg dose was observed to elicit a significantly lower response than the 5 μg/kg dose (12, 17). The GH response to the 5 μg/kg dose appears to be slightly higher than those of the previous studies; $C_{max,0-90\,min}$ of GH was 153.2±52.2 vs 107.7.0±23.9 (12) or 109.8±26.2 ng/ml (17) (Table 4). Although the reasons for this discrepancy are unclear, it may be explained by the fact that the volunteers in this study were younger than those in previous studies (22 vs. 31 years). In fact, age-related variations in GH response to ghrelin or GHS in humans have been reported (30, 37). To confirm this, it is necessary to carry out further studies using more subjects, including elderly people.

There are only three reports by two groups concerning the effects of ghrelin on glycaemia and insulin secretion in humans (28, 30, 38). Broglio *et al.* (28)

Table 5 Adverse events in previous studies administering ghrelin.

References	No. of subjects	Sex (M/F)	Dose of ghrelin	Adverse events*
Takaya *et al.* (12)	6	M	1.0 or 5.0 μg/kg	Bowel movement (2), warm sensation (2)
Arvat *et al.* (13)	4	M	1.0 μg/kg	Hunger sensation (3)
Peino *et al.* (14)	6	M	3.3 or 6.6 μg/kg	Hyperhidrosis (2)
Nagaya *et al.* (15)	6	M	10 μg/kg	Warm sensation (4), somnolence (4)
Arvat *et al.* (16)	7	M	1.0 μg/kg	Hunger sensation (3)
Broglio *et al.* (28)	11	M	1.0 μg/kg	Hunger sensation (6)
Micic *et al.* (29)	6	M	1.0 μg/kg	(No adverse events)
Broglio *et al.* (30)	34	17 M/17 F	1.0 μg/kg	Hunger sensation (12)
Weikel *et al.* (31)	7	M	4 × 50 μg	(No adverse events)
Ukkola (39)	8	4 M, 4 F	3.3 μg/kg	(No adverse events)

* Parentheses shows the number of subjects who experienced adverse events.

found that administration of 1 or 3.3 μg/kg ghrelin led to a transient inhibition of insulin secretion coupled with hyperglycaemic effects in normal male adults. However, in those studies, glucose remained persistently elevated from 15 to 165 min, and absolute insulin levels were reduced from 30 min with a nadir at 45 min. In addition, there were significant changes observed in glucose and insulin AUC. Arosio *et al.* (38) also found a significant increase in glucose concentrations at 15 and 30 min, and a significant decrease (41% decrease from the baseline) in insulin concentrations at 60 min. Our results differ greatly from these previous studies, for we found that the ghrelin-induced glucose increase was much shorter and less pronounced, while the insulin decrease occurred later and to a lesser degree. Since the previous studies did not observe a significant change in insulin levels following ghrelin administration in young women, this insulin response may vary depending upon the type of group tested (30). Moreover, the effect of ghrelin on insulin secretion has been inconsistent in both human and animal models (39), as both inhibitory (28, 30, 40) and stimulatory (41–43) effects have been reported. Further studies are necessary to clarify the effect of ghrelin on glucose metabolism.

Ghrelin is the first circulating hormone demonstrated to stimulate appetite in man. Wren *et al.* (18) showed that a 17 ng/kg per min intravenous infusion of ghrelin for 270 min enhanced appetite and food intake in humans. The total amount of ghrelin infused in this study was 4.5 μg/kg. Similarly, in this study, ghrelin administration tended to increase the hunger sensation in a dose-dependent manner, particularly in the early phase after injection, although the difference between groups did not reach statistically significant levels. In addition, while two of the six subjects in the placebo group did not show any change in hunger score, all of the subjects in both the low- and high-dose groups reported increases in hunger. To confirm this orexigenic effect of ghrelin, a study using larger-sized groups or a cross-over study will be necessary. Although Weikel *et al.* (31) reported that the subjects in their study did not feel a change in appetite after 4 × 0.66 μg/kg of ghrelin administered hourly as intravenous boluses, that dose may have been too low to stimulate appetite. Because tests for appetite are subjective and variable among subjects, only double-blind tests such as this study may yield reliable findings.

In summary, we have confirmed the safety and hormonal effects of ghrelin in a randomized, double-blind test. No serious adverse effects were found. Ghrelin markedly stimulated GH release, slightly modulated blood glucose and insulin levels, and tended to increase the hunger sensation in a dose-dependent manner. These results suggest that ghrelin may have therapeutic and diagnostic potential in patients with disorders related to GH secretion and appetite.

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Genomic Structure and Characterization of the 5′-Flanking Region of the Human Ghrelin Gene

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Ghrelin, an endogenous ligand for the GH secretagogue receptor, induces GH secretion, food intake, and positive energy balance. Although ghrelin exhibits a variety of hormonal actions, the mechanisms regulating ghrelin expression and secretion remain unclear. To understand regulation of human ghrelin gene expression, we examined the genomic structure of approximately 5,000 bp of the 5′-flanking region of the human ghrelin gene. We performed rapid amplification of cDNA ends to estimate transcriptional start sites, indicating that there are two transcriptional initiation sites within the human ghrelin gene. Both transcripts were equally expressed in the human stomach, whereas the longer transcript was mainly expressed in a human medullary thyroid carcinoma (TT) cell line. Functional analysis using promoter-reporter constructs containing the 5′-flanking region of the gene indicated that the sequence residing within the −349 to −193 region is necessary for human ghrelin promoter function in TT cells. Within this region existed several consensus sequences for a number of transactivating regulatory proteins, including an E-box site. Destruction of this site decreased to 40% of the promoter activity. The upstream region of the promoter has two additional putative E-box sites, and site-directed mutagenesis suggested that these are also involved in promoter activation. Electrophoretic mobility shift assays demonstrated that the upstream stimulatory factor specifically bound to these E-box elements. These results suggest a potential role for upstream stimulatory factor transcription factors in the regulation of human ghrelin expression. (*Endocrinology* 145: 4144–4153, 2004)

VARIOUS PEPTIDE HORMONES produced by endocrine cells of the digestive tract control important physiological functions, including growth and repair of gut epithelium, motility of the gut wall, gastric emptying, glycemia, and exocrine pancreatic secretion (1). Ghrelin, an endogenous ligand for the GH secretagogue receptor, is a 28-amino acid peptide discovered in the stomach (2). Ghrelin is produced in X/A-like cells, whose functions have yet to be clarified (3). In addition to the stomach, ghrelin is expressed in the arcuate nucleus of the hypothalamus, pituitary, pancreas, kidney, placenta, and testes (2, 4–9). Ghrelin exerts a variety of actions, including GH secretion, food intake, vagal control of gastric function, cardiovascular effects, and control of energy balance (2, 10–18). Plasma ghrelin levels are elevated by fasting and suppressed after feeding. Ghrelin secretion is also suppressed by somatostatin (19–21). It remains controversial, however, whether plasma insulin or glucose affects plasma ghrelin levels (21–23) and whether ghrelin expression is altered by leptin (24, 25). Thus, the regulatory mechanisms governing ghrelin expression and secretion remain to be clarified.

We recently reported significant production of ghrelin by a human medullary thyroid carcinoma (hMTC) cell line, TT cells (26). The TT cell line is the most suitable model system developed to date for human thyroid parafollicular C cells, the origin of hMTC (27–30). TT cells were found to produce a variety of hormones, some of which are products of gastrointestinal endocrine and/or neuroendocrine cells. Thus, the TT cell line is an excellent tool to study the regulation of ghrelin gene expression.

To understand the regulation of human ghrelin gene expression, we identified the transcriptional initiation site, examined the promoter activity of 5 kb in the 5′-flanking region of the human ghrelin gene, and characterized proteins binding to the regulatory elements within this region. Transcription factors of the basic/helix-loop-helix (bHLH) family specifically bind to the putative E-box within the human ghrelin promoter to participate in the regulation of human ghrelin gene expression.

Abbreviations: bHLH, Basic/helix-loop-helix; CT, calcitonin; hMTC, human medullary thyroid carcinoma; Luc, luciferase; LZ, leucine zipper; NF-κB, nuclear factor κB; nt, nucleotide; RACE, rapid amplification of cDNA ends; TK, thymidine kinase; USF, upstream stimulatory factor.

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Materials and Methods

Cell culture

The hMTC cell line, TT cells (29, 30), were cultured as described previously (26). The human hepatoma cell line, HepG2 cells, obtained from American Type Culture Collection (Manassas, VA), were cultured

in DMEM (Invitrogen Life Technologies, Carlsbad, CA) with 4 mM L-glutamine and 2.1 g/liter sodium bicarbonate supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD) at 37 C in a humidified atmosphere containing 5% CO_2.

RNA isolation and rapid amplification of cDNA ends (RACE)

Polyadenylated RNA was extracted from TT cells using a FastTrack 2.0 kit (Invitrogen Life Technologies). Human stomach polyadenylated RNA was purchased from BD Clontech (Palo Alto, CA). The transcriptional start of the human ghrelin gene was estimated by 5′-RACE using a 5′-Full RACE Core Set (Takara Shuzo Co., Shiga, Japan) according to the manufacturer's instructions (31, 32). Polyadenylated RNA from TT cells was reverse transcribed, then subjected to nested PCR. PCR products were subcloned into the pGEM-T vector (Promega Corp., Madison, WI) and sequenced using a BigDye Terminator cycle sequencing kit FS and 3100 genetic analyzer (Applied Biosystems, Foster, CA).

RT-PCR

cDNA was synthesized using a First-Strand cDNA Synthesis Kit (Amersham Biosciences, Little Chalfont, UK) and 1 μg polyadenylated RNA according to the manufacturer's instructions. Resulting cDNAs were subjected to PCR using the sense and antisense primers specified in Table 1. All primers were designed to recognize separate exons to eliminate the possibility of DNA contamination.

Quantitative PCR

Quantitative PCR was performed as described previously (4, 33). Primers and probes are designated in Table 2 and Fig 3A. Probes were labeled with fluorescent (6-carboxy-fluorescein) and nonfluorescent quencher (minor groove binder) dyes. cDNAs (1 ng) were amplified using the following conditions: 50 C for 2 min, 95 C for 10 min, and 40 cycles of 95 C for 15 sec and 60 C for 1 min, then continuous incubation at 25 C. Quantitative PCRs were performed, recorded, and analyzed by using the an ABI7700 PRISM Sequence Detection System (Applied Biosystems).

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

The PCR primers used were based on the sequence data of a bacterial artificial chromosome (CITB-187P1, GenBank accession no. AC008116 mapped to 3p26–25) (34): sense, 5′-GGGGAGAGAGGGTCTCCAGGCAGCTGCCTC-3′ [nucleotide (nt) 47534–47563]; and antisense, 5′-AAGAATAGAAGTGGGGAAATGAAAGCATTC-3′ (nt 52531–52560). Genomic sequences from human leukocytes was used as a template. PCR products were subcloned into pCR II-TOPO vector (Invitrogen Life Technologies) to obtain the 5027-bp (−5059 to −33, the translation start site was set at +1) fragment of the 5′-flanking region of the human ghrelin gene. The sequences were confirmed as described above.

TABLE 1. List of primers for RT-PCR

	No. in Fig. 2A
Sense primers	
5′-AAGGAGTCGAAGAAGCCACCA-3′	(1)
5′-CTGCAACCCAGCTGAGGCCATGCC-3′	(2)
5′-AGAGGCACATGAGAAGGGGAGGC-3′	(3)
5′-ACCTCCGCCAGGAACTGC-3′	(4)
Antisense primers	
5′-GCCAGATGAGCGCTTCTAAACTTA-3′	

Plasmid construction

To analyze the function of the 5′-flanking region of the human ghrelin gene, we generated various deletion mutants of the ghrelin promoter by PCR. The *MluI* and the *Hin*dIII or *Xho*I site overhang the oligonucleotides used as sense and antisense primers, respectively. PCR products were subcloned into the *MluI-Hin*dIII site of a reporter plasmid, pGL3-basic vector [Promega Corp.; −4110, −2109, −1509, −1109, −780, −349, and −192/−33 GHRE-luciferase (Luc) and −2109, −1509, −1109, and −780/−490 GHRE-Luc] or the *MluI-XhoI* site of pGL3-promoter vector (Promega) (−2109, −1509, −1109, and −780/−490 GHRE-simian virus 40 Luc), to create a fusion with the Luc gene. The correct orientation of these deletion mutant constructs was confirmed by sequencing.

Mutations were created using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's instruction. −1509/−33 GHRE-Luc was used as the template for PCR amplification. For mutagenesis, each sequence of E-boxes was replaced as follows: CAGGTG to tcGGTG for E1, CACCTG to tcCCTG for E2; and CATCTG to tcTCTG for E3 (the mutated bases are in *lower case*). Mutated construct was isolated from each reaction and verified by sequencing.

The putative transcription factor binding sites on the 5′-flanking region of the human ghrelin gene were identified by computational analysis using the DNASIS (Hitachi Software Engineering Co., Tokyo, Japan) and TFSEARCH databases (www.cbrc.jp/research/db/TFSEARCHJ.html), based on the TRANSFAC databases (35).

Transient transfection and Luc assay

TT and HepG2 cells were plated at 5–8 × 10^5 and 1 × 10^5 cells/well in 12-well tissue culture plates (Corning Inc., Corning, NY), respectively. Cells were maintained in 1 ml antibiotic-free medium for 1 d before transfection. Transient transfections were performed using Lipofectamine Plus reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Transfections included 800 ng experimental reporter gene and 80 ng pRL-thymidine kinase (TK), which contained the cDNA encoding *Renilla* Luc (Promega Corp.) as an internal control for transfection efficiency. After transfection, cells were grown in antibiotic-free medium and harvested after 48 h. Luc activities were determined using the Dual-Luciferase Reporter Assay System (Promega Corp.), and luminescence was measured with a Wallac 1420 ARVOsx multilabel reader (PerkinElmer Life Sciences, Tokyo, Japan). Firefly Luc activity was normalized to *Renilla* Luc activities in each well. Each experiment was performed at least three times with triplicate samples.

Preparation of cell extracts and EMSA

Nuclear extracts were prepared from TT cells using a nuclear extract kit (Active Motif, Carlsbad, CA), according to the manufacturer's instruction. EMSAs were conducted using a LightShift chemiluminescent EMSA kit (Pierce Chemical Co., Rockford, IL) with slight modifications of the original manufacturer's instruction. The double-strand oligonucleotide probe was end labeled using a biotin 3′ end DNA labeling kit (Pierce Chemical Co.). Biotinylated probe (200 fmol) was incubated with approximately 10 μg nuclear protein and 0.5 μg poly(dI-dC) in the presence or absence of competing oligonucleotide in 10× binding buffer (containing 100 mM Tris, 500 mM KCl, and 10 mM dithiothreitol, pH 7.5). After 30-min incubation at room temperature, DNA-protein complexes were separated by electrophoresis on a 6% DNA retardation gel (Invitrogen Life Technologies) at 4 C in 0.5× Tris-borate, EDTA buffer (containing 89 mM Tris-borate and 2 mM EDTA, pH 8.0). For supershift assay experiments, binding reactions were incubated for 45 min at room temperature with antibodies before the addition of labeled probes. The antibodies used in supershift assay experiments were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). After electrophoresis, samples were transferred onto nylon membranes (Amersham Bio-

TABLE 2. List of primers and probes for quantitative PCR

Probe	Forward primer	Reverse primer	Amplicon size (bp)
1-43T	1-23F	1-77R	
5′-CTCCCCCAGGCCCA-3′	5′-CAGCTGCCTGGAGACCCTC-3′	5′-CTCAGCTGGGTTGCAGACAG-3′	55
2-30T	2-11F	2-78R	
5′-TCTGCAACCCAGCTGA-3′	5′-GGAACTGCAGGCCCACCT-3′	5′-AGGCTGCAGACGGTCCCT-3′	68

sciences) and fixed by UV irradiation. Biotinylated DNA was detected using a Fujix Lumino-image analyzer (LAS-1000, Fuji Photo Film Co., Ltd., Tokyo, Japan).

Results

Identification of the transcription initiation site

To identify the transcriptional initiation site within the human ghrelin gene, we performed 5′-RACE using polyadenylated RNA isolated from TT cells. In TT cells, two distinct clones were obtained with a 30-bp difference in length (Fig. 1A). Sequencing of these PCR products indicated the presence of two different transcriptional initiation sites, one located at −80 and the other at −555 (each transcript was named Transcript-A and Transcript-B, respectively) (Fig. 1 B). These results indicated that the short first exon, which is only 20 bp in length, is present in the noncoding region of the human ghrelin gene.

The presence of these two transcripts was confirmed by RT-PCR analysis of TT cells and human stomach RNA. PCR products of the expected sizes were detected by agarose gel electrophoresis (Fig. 2B), suggesting that both transcripts were present in TT cells and human stomach.

Tissue specificity of transcription in the human ghrelin gene

To examine how these two transcripts were expressed in different tissues, we performed quantitative PCR. In the human stomach, both transcripts were equally expressed, whereas in TT cells Transcript-B was expressed at much lower levels (Fig. 3B). These results suggest tissue specificity in ghrelin gene expression. The finding that the total amount of transcripts isolated from the human stomach was greater than that isolated from TT cells was compatible with previous reports of ghrelin contents estimated by Northern blotting or RIA (3, 26, 36).

Genomic analysis of the 5′-flanking region of the human ghrelin gene

The 5059-bp fragment contained a partial sequence of the 5′-flanking region of the human ghrelin gene (Fig. 4). No typical GC or CAAT box was identified. A TATA box-like sequence, a TATATAA element, was identified 24 bp upstream (−585 to −579) of the transcriptional initiation site of Transcript-B. A comparison of the 5′-flanking sequence of human ghrelin with the initiator consensus sequence Py-Py-A-N-A/T-Py-Py identified putative initiator elements located at positions −557 to −551 and −82 to −76, in agreement with the transcriptional initiation sites estimated by 5′-RACE.

Functional analysis of the 5′-flanking region of the human ghrelin gene

To identify the regulatory regions important for expression of the human ghrelin gene, a series of 5′ and/or 3′ deletion constructs of the promoter were subcloned into the pGL3-Basic vector, then cotransfected with pRL-TK into TT cells (Fig. 5A). Figure 5B exhibits the effects of these deletions on Luc reporter activity in TT cells. Luc activity increased by deleting the region from −4110 to −1509 and decreased after

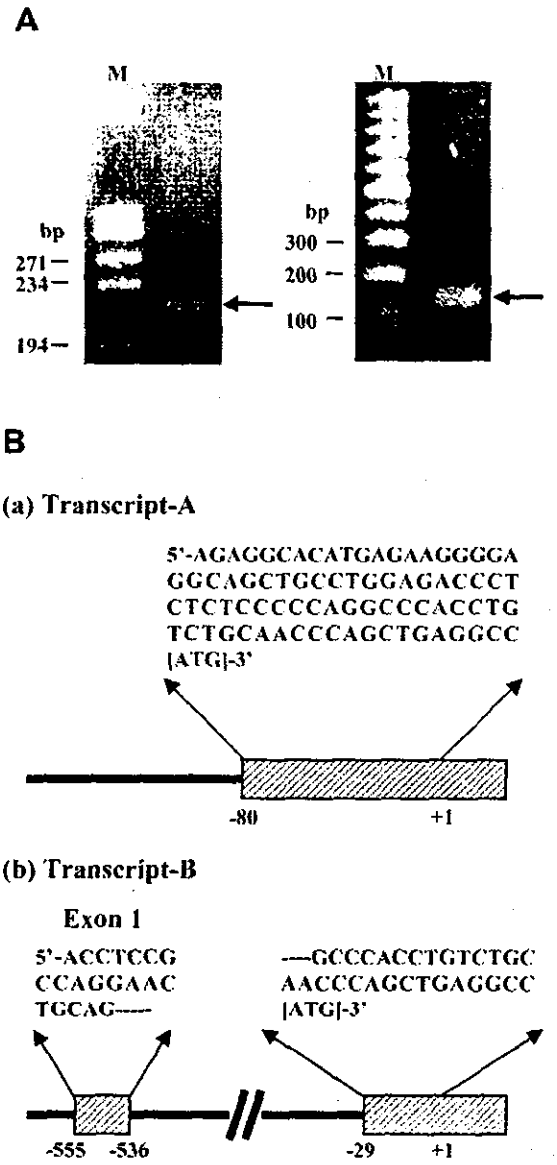
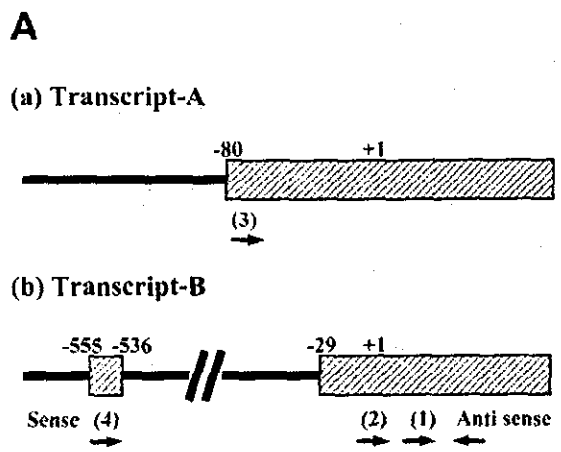


FIG. 1. 5′-RACE of the human ghrelin gene. A, Polyadenylated RNA isolated from TT cells was reverse transcribed as described in *Materials and Methods*. The resultant cDNAs were subjected to 5′-RACE. The secondary products obtained by nested PCR using TT cell cDNAs contained two band species, as visualized by agarose gel electrophoresis (*arrows*). M, Molecular size marker. B, Sequence and genomic location of the 5′-flanking region of the human ghrelin gene. *Bold* and *fine bars* indicate exons and introns, respectively. As shown in (a) and (b), the human ghrelin gene has two transcriptional initiation sites (named Transcript-A and Transcript-B, respectively). The nucleotide sequences indicated are within the 5′-untranslated region.

deletion of −1509 to −1109. Although Luc activity was restored by additional deletion of −780 to −349, it was markedly decreased by deletion of −349 to −192. To elucidate the significance of the transcription factor binding sites between −193 and −349, the 3′ truncations (to −490 bp) of some of the 5′ deletion series were also created. As shown in the *middle part* of the Fig. 5B, all promoter activity was lost. However, because this deletion also removes the alternative



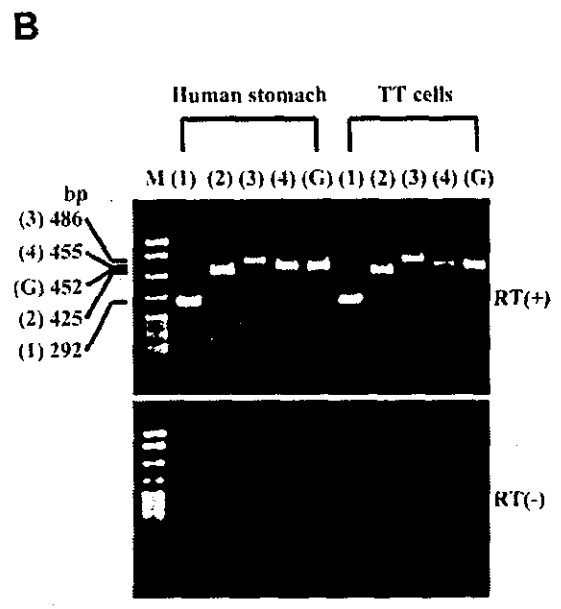
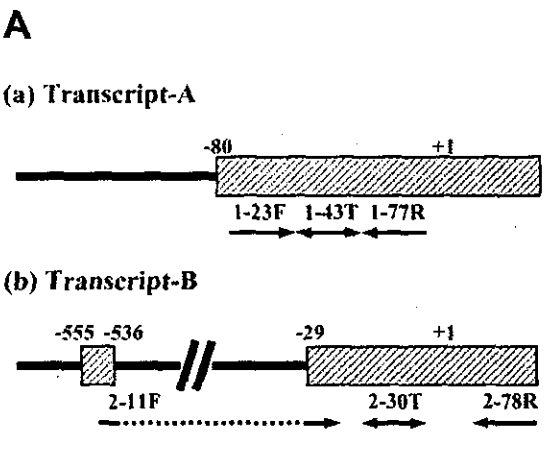


FIG. 2. RT-PCR analysis of the human ghrelin gene transcripts containing 5′-untranslated region. A, Schematic diagram representing the positions of primers used for RT-PCR. The sequences of the sense primers, from (1)–(4), are shown in Table 1. B, Electrophoretic analysis of RT-PCR products using polyadenylated RNA from TT cells and the human stomach. The number in the *parentheses* corresponds to the sense primer used in the reaction. Glyceraldehyde-3-phosphate dehydrogenase (G) was used as a positive control for PCRs.

transcription start site at −80 bp (*i.e.* Transcript-A), and essentially all the ghrelin transcripts in the TT cells used in the transfection assay originate here (Fig. 3B), we next examined the promoter activity using heterologous promoter. A series of 3′ deletion constructs of the promoter were subcloned into the pGL3-promoter vector (in the *bottom part* of Fig. 5A), then cotransfected into TT cells. The Luc activity was restored by the simian virus 40 promoter and the pattern of the activity was similar to the corresponding native constructs, indicating that the initiator/transcription start site here is mainly active in this cell type. These results suggest the presence of activating sequences within the −1509 to −1110 and −349 to −193 regions.



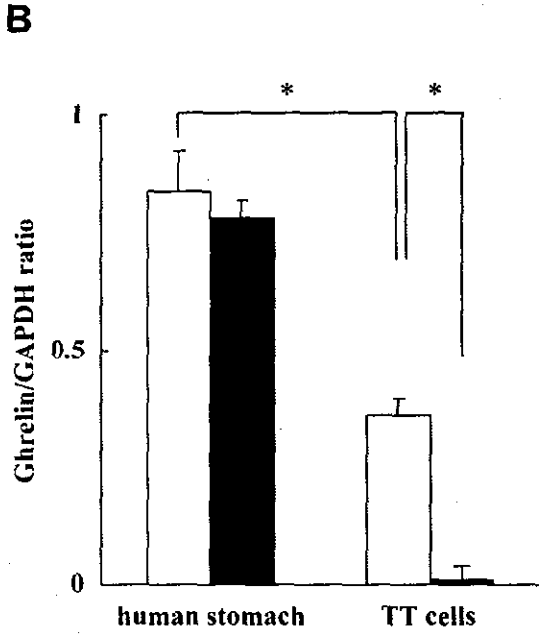


FIG. 3. Quantitative PCR analysis of the human ghrelin gene transcripts containing 5′-untranslated region. A, Schematic diagram representing the positions of primers and probes used for quantitative PCR (sequences are shown in Table 2). B, Ghrelin expression levels in the human stomach and TT cells. Polyadenylated RNA from the human stomach and TT cells were reverse transcribed and subjected to quantitative PCR. Ghrelin expression was normalized to glyceraldehyde-3-phosphate dehydrogenase expression in each sample. □, Transcript-A; ■, Transcript-B. The data represent the mean ± SE for triplicate samples. *, Statistically significant differences ($P < 0.01$) measured by Student-Newman-Keuls tests.

To examine the cell specificity of human ghrelin promoter function, −1509/−33 GHRE-Luc or −349/−33 GHRE-Luc were transiently transfected into TT and HepG2 cells. The Luc activity of both constructs was very low in HepG2 cells in comparison with TT cells (Fig 5C). These results indicate that these promoter/regulatory regions contain elements essential for cell-specific expression.

A computational analysis of the −349 to −193 regions upstream of the human ghrelin gene revealed the presence

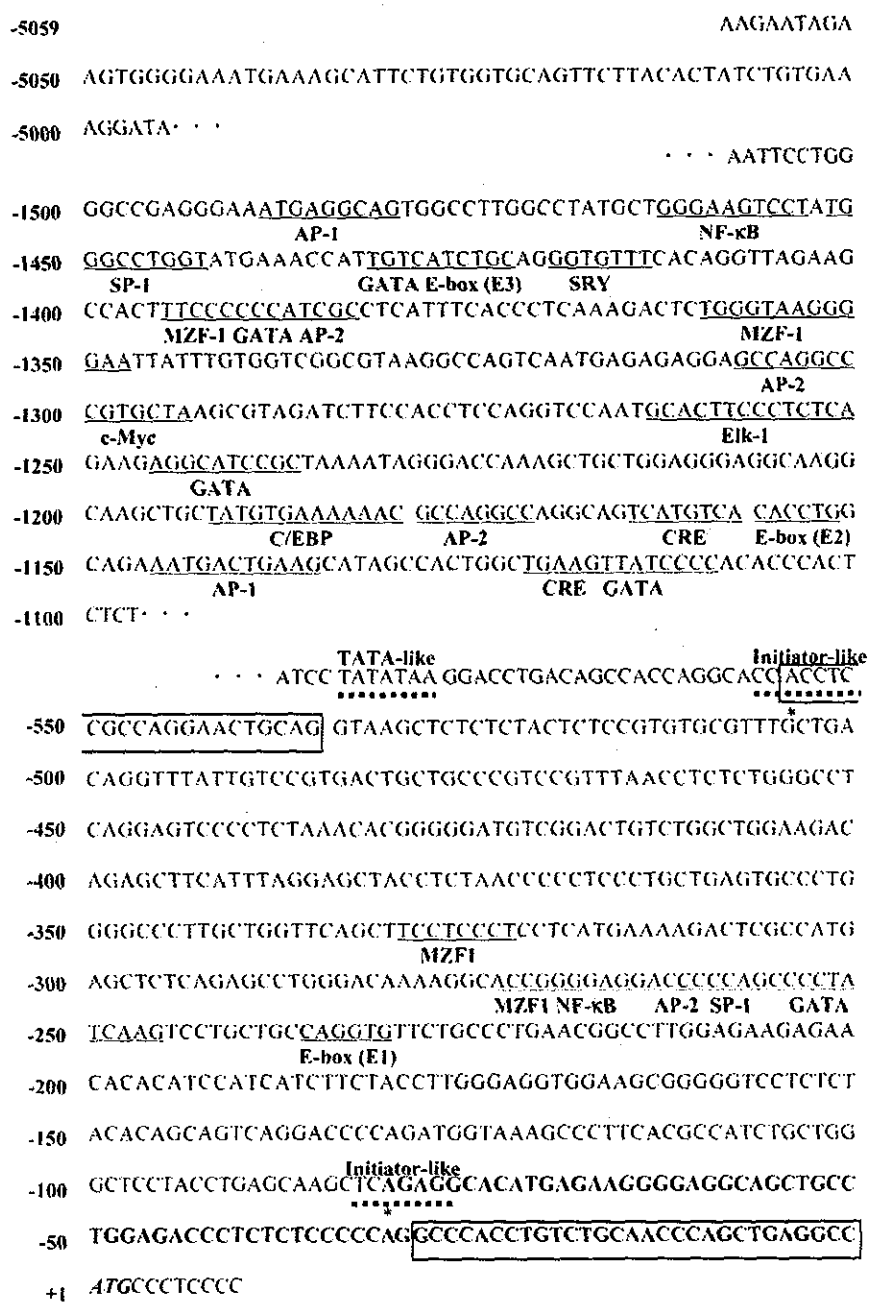
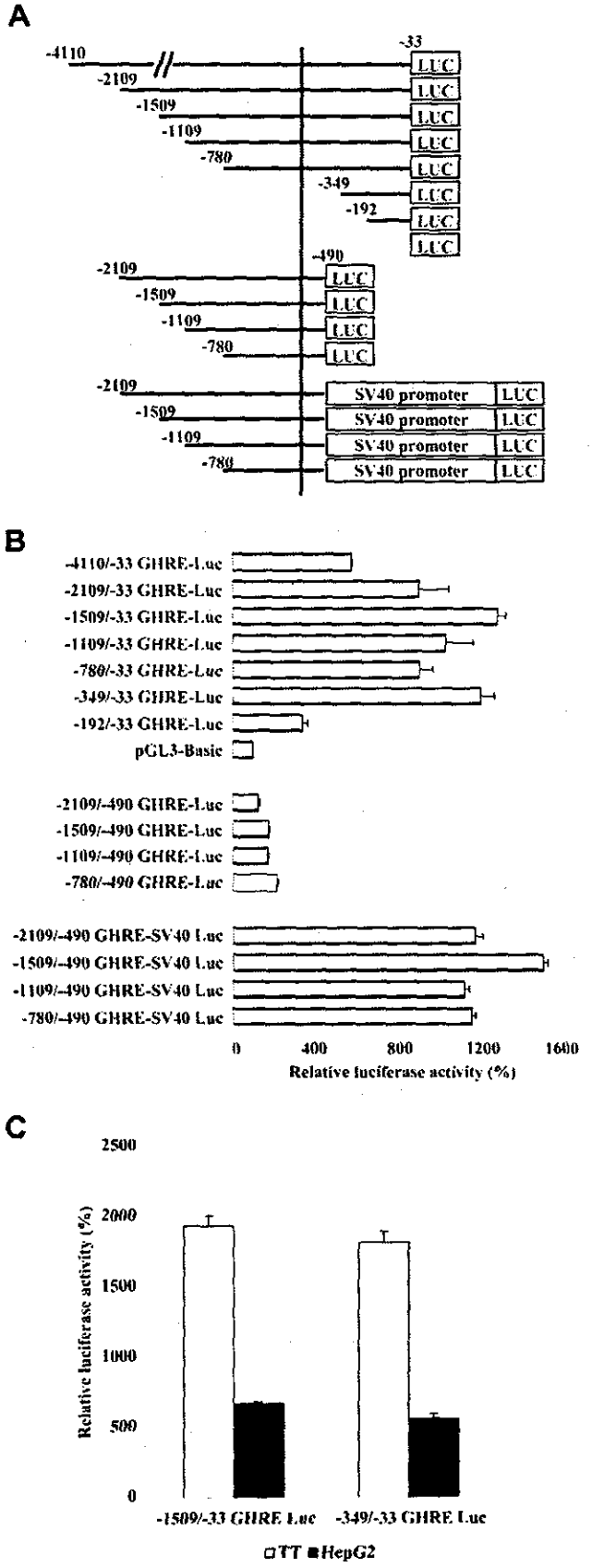


FIG. 4. The nt sequences of the 5′-flanking region of the human ghrelin gene. The translational start site was set at +1. The 5′ ends of the 5′-RACE products are denoted by *asterisks*. The sequences shown in *bold letters* indicate Transcript-A. The sequences enclosed by an *open box* indicate Transcript-B. *Dotted-lined* sequences indicate TATA box-like or initiator-like sequences. *Underlined sequences* indicate putative binding sites for transcription factors.

of several consensus binding sequences for a number of transactivating regulatory proteins (Fig. 4). We predicted a consensus E-box motif at −236 to −231. Sequence analysis identified additional putative *cis*-acting elements for several transcription factors, including two myeloid zinc finger protein-1 sites (−330 to −323, and −274 to −267) and sites for NF-κB (−270 to −261), activator protein-2 (−263 to −254), stimulating protein 1(−262 to −253), and GATA (−255 to −246).

Activation of transcription by E-box

To elucidate the role of the E-box (E1) in the −349 to −193 region necessary for human ghrelin promoter activity in TT cells, we examined the activation of gene expression using site-directed mutagenesis. The putative E-box sequence of E1 site CAGGTG was mutated to TCGGTG using −1509/−33 GHRE-Luc as a template (Fig. 6A). The Luc activity exhibited considerably reduced activity (60% of −1509/−33 GHRE-Luc levels; Fig. 6B). Because progressive 5′ deletion analysis of upstream of the promoter showed less, but a significant, decrease in activity, and the additional putative E-box sites were detected at −1157 to −1152 (E2) and −1429 to −1424 (E3) among other transcription factors (Fig. 4), we next examined the effects of these two distal E-box elements. As shown in Fig. 6B, sequential disruption of the sites decreased Luc activity in an additive manner. Mutations of both distal



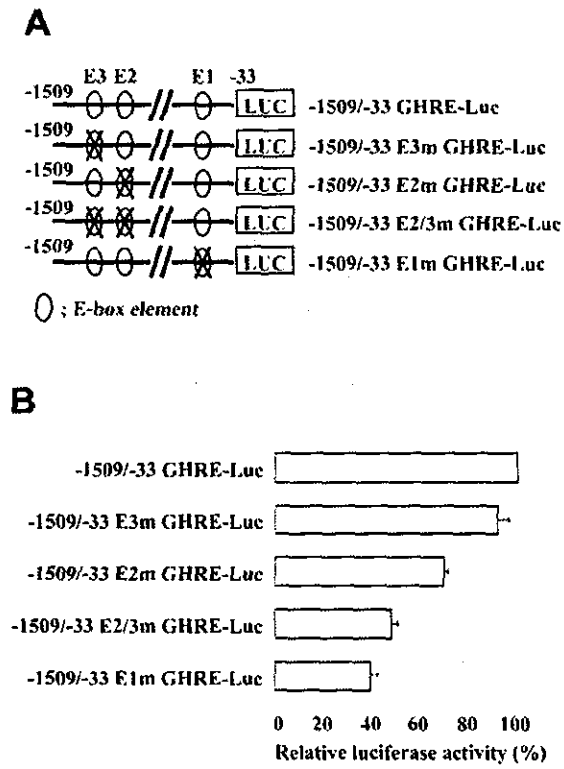


FIG. 6. Activation of the human ghrelin promoter activity by E-boxes. A, Schematic diagram representing wild-type and site-specific mutations of the human ghrelin promoter, introduced into the upstream region of the Luc gene. A *cross* represents the site-specific mutation of the putative E-box. B, Each construct was transiently cotransfected with pRL-TK into TT cells. Promoter activity was normalized to *Renilla* Luc activity, then expressed as a percentage of the activity of −1509/−33 GHRE-Luc. The data represent the mean ± SE for triplicate samples. Similar results were obtained in all independent experiments.

E-box sites decreased promoter activity by 50%. These results indicated that both proximal and distal E-boxes within the human ghrelin promoter are required for the enhancer activity.

Putative E-boxes in the human ghrelin promoter bind upstream stimulatory factor 1 (USF1)/USF2

As transcription factors of the bHLH family are important regulators of cell fate determination and differentiation in

FIG. 5. The human ghrelin promoter activity. A, Schematic diagram representing deletions of the human ghrelin gene introduced upstream of the Luc gene, containing variable of 5′ and/or 3′ ends. Exon 1 is represented by *vertical bars*. B, Each construct was transiently cotransfected with pRL-TK into TT cells. Promoter activity, after normalization to *Renilla* Luc activity, was expressed as a percentage of the activity of promoterless pGL3-basic. The data represent the mean ± SE for triplicate samples. Similar results were obtained in all independent experiments. C, Cell specificity of human ghrelin promoter activity. −1509/−33 GHRE-Luc or −349/−33 GHRE-Luc were transiently transfected into TT and HepG2 cells. Promoter activity was normalized to *Renilla* Luc activity, then expressed as a percentage of the activity of promoterless pGL3-basic. The data represent the mean ± SE for triplicate samples. Similar results were obtained in all independent experiments.

enteroendocrine cells (37), we examined the role of bHLH transcription factors in the regulation of human ghrelin gene expression. Computational analysis predicted that these putative E-box sites bind the bHLH-leucine zipper (LZ) transcription factor USF1. To determine the partner nuclear proteins for these elements in TT cells, oligonucleotides containing the sequences were used for EMSA. We first examined the E1 sequences. Utilization of the oligonucleotide (Fig. 7 A, WT E1) generated several DNA/protein complexes (Fig. 7 B, lane 2). The formation of two of these complexes is specific, because they were competed by a 25-fold excess of WT E1, but not by a mutant oligonucleotide MUT E1 (Fig. 7B, lanes 3–6).

To examine the binding of E1 to USF, antibodies against USF1 and USF2 were used for supershift assay experiments.

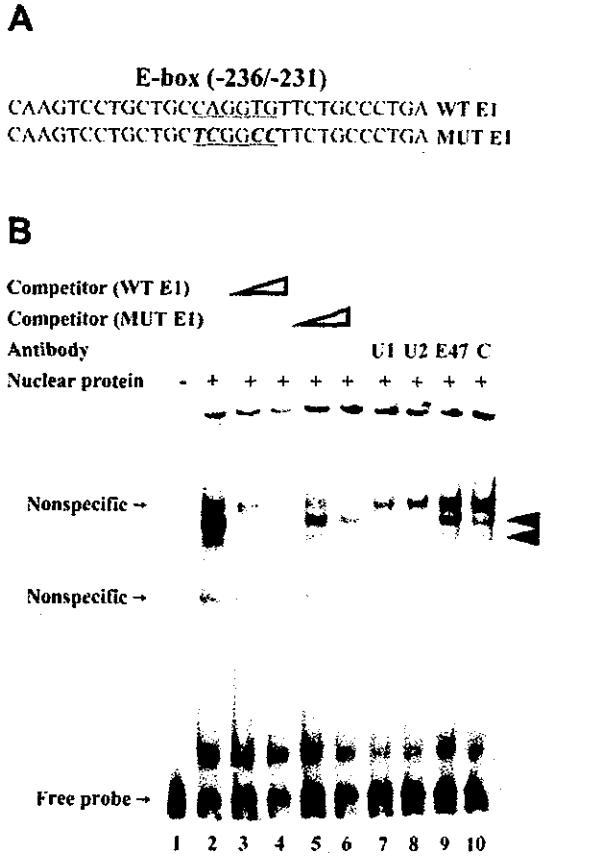


FIG. 7. Specific binding of the transcription factor USF to the −236 to −231 E-box element (E1) in the human ghrelin promoter using nuclear proteins from TT cells. A, Sequences of the double-stranded oligonucleotides used in EMSA. WT E1, Wild-type human ghrelin sequence identical to the −249 to −220 region, containing the E-box at −236 to −231. MUT E1 contains a mutated E-box (the E-box element is *underlined*; the mutated base pairs are indicated by *italic bold letters*). B, Oligonucleotide WT E1 was used as the probe for EMSA either without competitor (lane 2) or in the presence of 25- and 50-fold molar excesses of unlabeled WT E1 (lanes 3 and 4, respectively) and MUT E1 (lanes 5 and 6, respectively) oligonucleotides. The specific complex formed from TT cell nuclear extract and WT E1 is indicated by an *arrowhead*. Supershift assay experiments were performed using 1 μl (200 μg/0.1 ml) antibody against USF1 (U1; lane 7), USF2 (U2; lane 8), or E47 (E47; lane 9) and 5 μl (200 μg/0.5 ml) normal rabbit IgG (C; lane 10).

Addition of antibody against USF1 resulted in the complete disappearance of the two complexes (Fig. 7B, lane 7). Addition of antibody against USF2 also completely dissociated them (Fig. 7B, lane 8). Antibody against E47, which recognizes a similar E-box sequence, or normal rabbit IgG did not disrupt them (Fig. 7B, lanes 9 and 10). These results suggest that the putative E-box specifically binds USF1/USF2; the complexes probably contain a USF1/USF2 heterodimer. Similarly, we next examined the E2 and E3 sequences. In EMSA using oligonucleotides containing the E2 and E3 sequences, one of DNA/protein complexes proved to be specific using specific competitors and antibodies, respectively (Fig. 8). These results suggest a potential role for USF transcription factors in the regulation of human ghrelin gene expression.

Discussion

In this study we analyzed the 5′-flanking region of the human ghrelin gene to identify a set of protein-DNA interactions within the human ghrelin promoter. By 5′-RACE using polyadenylated RNA isolated from TT cells, we identified two transcription initiation sites, located at −80 (Transcript-A) and −555 (Transcript-B). This analysis indicated the presence of a short noncoding first exon, only 20 bp in length, within the human ghrelin gene presented at the latter transcript (Transcript-B). Tanaka *et al.* (38) reported that the mouse ghrelin gene contains five exons and four introns, with a similar first exon of 19 bp, and they postulated a possible function for a TATATAA sequence within the mouse ghrelin promoter. They predicted that the presence of a short noncoding first exon is a feature common to mammalian ghrelin genes, as an upstream region in the reported human ghrelin sequence shows high sequence homology to the exon 1 region of mouse ghrelin (38). In contrast, Kishimoto *et al.* (39) reported recently that the 5′ end of cDNA of the human ghrelin gene was −32 bp from the translation start site as a major transcription start site, suggesting the absence of such a noncoding exon. However, the cDNA library they used was commercially based, and there is a possibility that it may not necessarily express a full-length cDNA. Although the expression levels were different, both longer transcripts (Transcripts A and B) were actually expressed in TT cells and stomach in our study. Taken together, there are at least two transcription initiation sites, one of which contains a short first exon, and the promoter usage may be regulated in a cell-specific manner.

Our functional analysis of the 5′-flanking region of the human ghrelin gene demonstrates that Luc activity exhibited the biphasic changes (Fig. 5B) different from those reported by Kishimoto *et al.* (39). This discrepancy probably results from differences in the cells used for transfections. Ghrelin-producing X/A-like cells (3), primarily found in the oxyntic gland and infrequently in the pyloric gland and small intestine, represent a major endocrine cell population in the human oxyntic gland. These cells possess round, compact, and electron-dense neurosecretory granules (40, 41). The ECC10 cell line (42), derived from a human gastric carcinoid tumor, had characteristic neurosecretory granules, well to moderately developed microvilli, and demosomal junctions. Neuron-specific enolase and carcinoembryonic antigen,

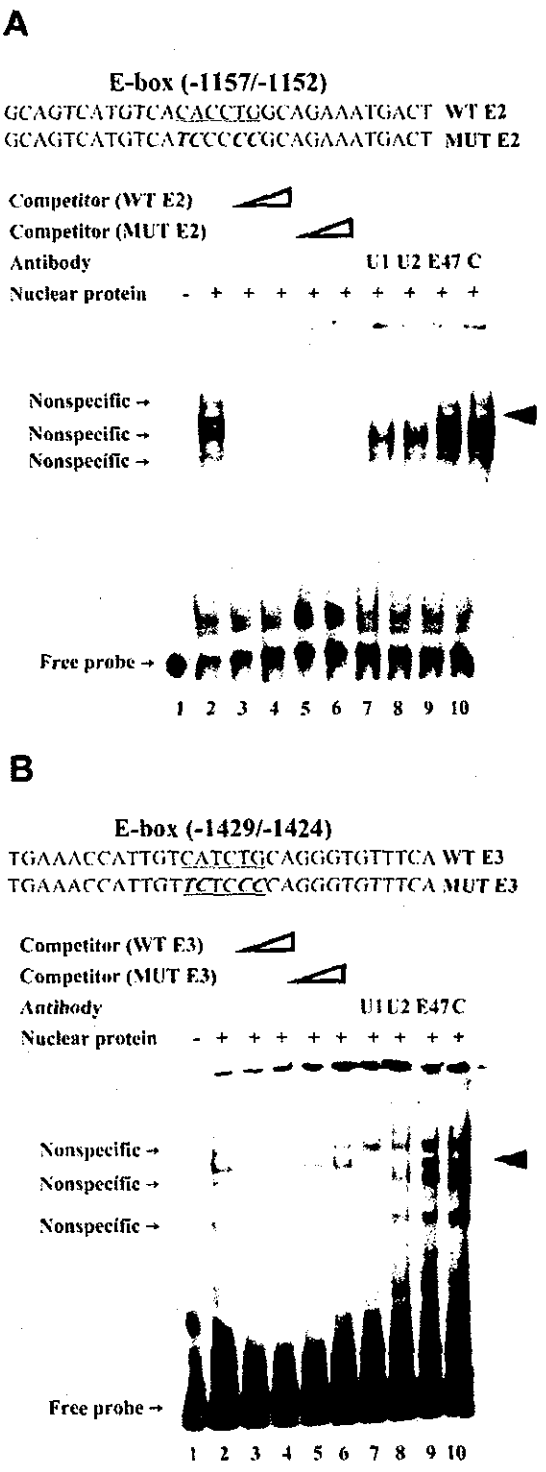


FIG. 8. Specific binding of the transcription factor USF to the distal E-box element (E2 and E3) in the human ghrelin promoter. Sequences of the double-stranded oligonucleotides used in the EMSA are shown on the *top* of each figure. A, WT E2 represents wild-type human ghrelin sequence identical to the −1169 to −1140 region, containing the E-box at −1157 to −1152. MUT E2 contains a mutated E-box (the E-box element is *underlined*; the mutated base pairs are indicated by *italic bold letters*). B, WT E3 represents wild-type human ghrelin sequence identical to the −1441 to −1412 region, containing the E-box at −1429 to −1424. MUT E3 contains a mutated E-box. Representative EMSAs were shown on the *bottom* of each figure. Oligonucleotide WT E2 or E3 was used as the probe for EMSA either without competitor (lane 2) or in the presence of 25- and 50-fold molar excesses of unlabeled WT E2 or E3 (lanes 3 and 4, respectively) and MUT E2 or E3 (lanes 5 and 6, respectively) oligonucleotides. The specific complex formed from TT cell nuclear extract and WT E2 or E3 is indicated by *an arrowhead*. Supershift assay experiments were performed using 1 μl (200 μg/0.1 ml) antibody against USF1 (U1; lane 7), USF2 (U2; lane 8), or E47 (E47; lane 9) and 5 μl (200 μg/0.5 ml) normal rabbit IgG (C; lane 10).

however, are not detected in these cells. In addition, no amines or peptide hormones could be detected in ECC10 cells by immunocytochemical and cytochemical studies, indicating that ECC10 cells belong to the variant type that is used in the classification of small cell lung carcinoma cell lines. The ECC10 cell line is supposed to arise via neoplastic neometaplasia from adenocarcinoma cells to endocrine cells. The TT cell line possesses a considerable number of secretory granules, a well developed rough endoplasmic reticulum, and a prominent Golgi apparatus. In addition to calcitonin (CT), TT cells produce a variety of hormones, namely CT gene-related peptide, ACTH, neurotensin, enkephalin, PTH-related peptide, gastrin-releasing peptide, serotonin, synaptophysin, neuron-specific enolase, calbindin, tyrosine hydroxylase, and chromogranin A, all of which are expressed in a wide variety of endocrine and neuroendocrine tissues (43–45). Additional marker proteins have been detected in the cytosol (carcinoembryonic antigen) and as part of the cytoskeleton (α-tubulin and cytokeratin). However, the fact that TT cells expressed Transcript-B, which contains a noncoding short exon, at lower levels than Transcript-A and functional analysis of the human ghrelin promoter using TT cells (Fig. 5B) support the idea that the transcriptional initiation site located at −80 is more important for transcriptional regulation of ghrelin in TT cells. These results also suggest that a TATA box-like sequence, TATATAA, located at −585 does not function significantly, at least in TT cells, similar to the previous report using ECC10 cells (39). Although the stomach equally expressed both transcripts, there are no functional stomach cell lines to determine whether the TATATAA element located at the 5′-flanking region of the human ghrelin gene may function in the tissue.

In both invertebrates and vertebrates, transcription factors of the bHLH family regulate cell fate determination and differentiation in a variety of cell types (46, 47). In the digestive tract, endocrine cell development is also regulated by members of the bHLH transcription factor family. The member of the neurogenin family, neurogenin 3, is transiently expressed in endocrine progenitors during digestive tract development. This molecule controls endocrine cell fate specifications in multipotent endodermal progenitors of the digestive tract (37, 48, 49). In the mouse intestine, loss of Math1 leads to depletion of secretory cell lineages, including enteroendocrine cells (50). BETA2/NeuroD controls terminal differentiation of the enteroendocrine secretin-producing cells through a coordination of secretin gene transcription with cell cycle arrest (51–53).

USF proteins, members of the bHLH-LZ family of transcription factors, were first identified by their role in the

regulation of adenovirus major late promoter transcription (54, 55). USF proteins contain 43-kDa (USF1) and 44-kDa (USF2) polypeptides, encoded by separate genes (56, 57). USF proteins primarily bind as dimers to consensus sequences containing the CACGTG motif termed an E-box (54, 57, 58). USF proteins are ubiquitously expressed, although different ratios of USF homo- and heterodimers are found in different cell types (59). Although the biological functions of USF are poorly understood, these proteins regulate the expression of a wide variety of genes, including CT/CT gene-related peptide (60), TGFβ2 (61), and Pdx-1, a critical player in pancreatic development (62, 63). USF proteins have also been reported to bind other sequences, including CGCGTG (64), CCCGTG (65), CAGCTG (60, 66), CACCTG (67), and CACATG (68, 69). By EMSA, we demonstrated that USF proteins also bind the CAGGTG and CATCTG motifs within the human ghrelin promoter. Thus, USF proteins are capable of binding to a variety of E-box motifs to regulate gene expression.

The data presented here indicate that putative E-boxes in the human ghrelin promoter specifically bind the USF1/USF2 heterodimer. USF proteins play a potential role in the regulation of human ghrelin expression. As the transcriptional activity was not completely lost upon mutations of the E-box sites (Fig. 6), other transcription factors may participate, together with USF proteins, to exert full activity of the promoter. Indeed, the LZ domains of several members of the bHLH-LZ family participate in various interactions with additional transcription factors (70, 71). USF proteins can interact with transcription factor for RNA polymerase II D when bound to the TATA box motif (54). Thus, it is possible that human ghrelin promoter activity is regulated by modulation of these essential protein-protein interactions. We are investigating the possibility of other putative *cis*-acting elements for several transcription factors functioning in this pathway.

In summary, the bHLH-LZ transcription factors USF1 and USF2 specifically bind to E-boxes in the human ghrelin promoter as a heterodimer, probably playing a role in the regulation of human ghrelin expression. Although the molecular mechanism of human ghrelin transcriptional regulation remains unclear, the present study contributes greatly to our understanding of the controls governing ghrelin secretion.

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Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy subjects using a new direct ELISA assay

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ABSTRACT Two forms of ghrelin, acylated and desacyl, circulate in plasma. Although acylation is thought to be essential for ghrelin biological activities, recent studies have suggested that desacyl ghrelin may also possess biological activity. A new commercial ELISA system has now enabled us to measure plasma levels of each of these two ghrelin forms separately. This assay system directly measures levels using small amounts of plasma. To evaluate the utility of this assay system, we measured the plasma levels of the two forms of ghrelin in healthy volunteers. Although acylated ghrelin levels were equivalent to those measured previously by RIA, desacyl ghrelin levels were lower than those expected from the total ghrelin levels previously determined by RIA. The ratios of acylated to desacyl ghrelin significantly correlated with previously determined acylated, but not desacyl, ghrelin levels. After BMI adjustment, the levels of acylated, but not desacyl, ghrelin plasma levels were higher in female subjects than those in males. Several metabolic and hormonal parameters significantly correlated with either plasma acylated or desacyl ghrelin levels. These findings indicate that separate measurements of the two ghrelin form levels may provide valuable information on their structure, gender differences, and physiological implications.

Ghrelin, a 28 amino acid peptide, exhibits a variety of biological activities, including the stimulation of both GH release and food intake (1-3). Plasma ghrelin levels are elevated after fasting and reduced following feeding (4, 5). Plasma ghrelin concentrations negatively correlate with increasing body mass index (BMI); levels are decreased in obese subjects and increased in anorexia nervosa patients (5-8). In addition, low ghrelin plasma levels are associated with insulin resistance, hypertension, and the prevalence of type 2 diabetes (9), prompting the hypothesis that circulating ghrelin levels are related to appetite, energy homeostasis, and glucose metabolism.

A portion of ghrelin possesses a unique fatty acid modification, an *n*-octanoylation, at Ser 3 (1). Of the two circulating ghrelin forms, acylated and unacylated (desacyl), the acylated form is thought to be essential for ghrelin biological activity. Desacyl ghrelin, however, has recently been reported to influence cell proliferation and adipogenesis (10-13). To better understand the roles of the individual ghrelin forms, it will be important to measure plasma levels of both acylated and desacyl ghrelin separately.

Previous measurements of circulating ghrelin levels in humans, however, have been performed using an antiserum which recognizes both forms, providing a measure of total ghrelin levels. Commercial radioimmunoassays (RIAs) from both Phoenix Pharmaceuticals (cat. No. RK-031-30) and Linco Research (cat. No. GHRT-89HK) can only measure total ghrelin levels (14). Although our RIA assays, N-RIA and C-RIA, can measure acylated ghrelin and total ghrelin, respectively (15), our determinations of ghrelin concentrations by N-RIA were limited to patients with renal diseases (16) and anorexia nervosa (17). Although an assay analyzing acylated ghrelin levels is now commercially available from Linco (cat. No. GHRA-88HK), few studies examining ghrelin levels in human plasma samples using this assay have yet been reported (18). The recent availability of a new enzyme-linked immunosorbent assays (ELISAs) that can measure both acylated and desacyl forms of ghrelin from Mitsubishi Kagaku Iatron, Inc. (Tokyo, Japan) provides the first assay that can specifically measure the desacyl form oghrelin in small quantities of plasma (50 μl/well). In addition, this assay system does not require the complicated and time-consuming purification of ghrelin using a Sep-Pak C_{18} cartridge. In this study, we measured the plasma levels of acylated and desacyl ghrelin in healthy volunteers using these new ELISA kits. To explore the physiological implications of these two ghrelin forms, we examined the gender differences in plasma ghrelin levels and their correlations with hormonal and metabolic parameters.

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Materials and Methods

Subjects

Sixteen male and 20 female volunteers between 21 and 61 years of age participated in our study. All of the subjects are Japanese and did not suffer from any known medical conditions. None of the subjects were currently taking medication. The study protocol was approved by the ethics committee on human research at Kyoto University Graduate School of Medicine and Kansai Medical School. Written informed consent was obtained from all subjects prior to enrollment.

Measurement of the concentrations of plasma ghrelin and glucose and serum nonesterified fatty acid (NEFA), GH, IGF-I, insulin, and leptin

Blood samples for hormone and glucose analyses were drawn

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from a forearm vein in the morning after overnight fasting. Plasma samples were prepared as previously described (1). Blood samples were immediately transferred to chilled polypropylene tubes containing EDTA-2Na (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Inc., Kyoto, Japan: 500 kallikrein inactivator U/ml), centrifuged at 4°C. To the separated plasma was immediately added 1N HCl (10% volume of plasma volume). The acylated and unacylated forms of ghrelin were measured using two commercially available ELISA kits, Active Ghrelin ELISA Kit and Desacyl-Ghrelin ELISA Kit, respectively, according to the manufacturer's protocol (Mitsubishi Kagaku Iatron, Inc.) (19, 20). The minimal detection limits of acylated and desacyl ghrelin in this assay system were 2.5 and 12.5 fmol/ml, respectively. The intra- and interassay coefficiencies of variation were 6.5% and 9.8%, respectively, for acylated ghrelin and 3.7% and 8.1%, respectively, for desacyl ghrelin. Plasma glucose was measured using the glucose oxidase method. NEFA was measured enzymatically. Serum GH, IGF-1, and insulin were measured by immunoradiometric assay (IRMA), while serum leptin was measured by radioimmunoassay (RIA) (Mitsubishi Kagaku Bio-Clinical Laboratories, inc., Tokyo, Japan). Insulin resistance was calculated according to the homeostatis model of assessment-insulin resistance (HOMA-IR), calculated as insulin (μU/ml) x blood glucose (mmol/l) /22.5 (21).

Statistical analysis

Data are expressed as the mean ± SD. Statistical evaluations were performed using Student *t*-test, Pearson's rank correlation, and multiple-regression analyses as appropriate. P values less than 0.05 was considered statistically significant.

Results

Plasma ghrelin concentrations in volunteers

We examined the anthropometric, hormonal, and metabolic parameters in volunteers (Table 1). The mean levels of plasma acylated and desacyl ghrelin in the 36 volunteers were 15.9 ± 9.4 and 66.1 ± 45.3 fmol/ml, respectively. There was an excellent correlation between acylated and desacyl ghrelin levels ($r = 0.840$, $p < 0.0001$). The ratio of acylated to desacyl ghrelin (A/D ratio) and the ratio of acylated to[acylated + desacyl] ghrelin levels (A/[A + D] ratio) were 24.9 ± 8.4% and 19.6 ± 5.4 %, respectively (Table 1). The levels of acylated ghrelin correlated significantly with the A/D and A/[A + D] ratios ($r = 0.371$, $p < 0.05$), while desacyl ghrelin did not ($r = -0.097$, $p = 0.577$).

Plasma ghrelin levels in female subjects were significantly higher than those in male subjects for both acylated (male, 10.9 ± 6.1; female, 19.9 ± 9.8) and desacyl ghrelin (male, 49.1 ± 23.5; female, 79.8 ± 53.9), giving final plasma concentrations 1.8- and 1.6-fold higher in females for acylated and desacyl ghrelin, respectively. BMI, GH, glucose, leptin, and NEFA levels, however, also differed significantly between the genders. As BMI is known to correlate negatively with plasma ghrelin levels (5, 7, 8, 22, 23), we performed a BMI adjustment by multiple regression analysis. The significant gender difference persisted for plasma levels of acylated ($p < 0.05$), but not desacyl ($p = 0.201$), ghrelin levels.

Correlations of ghrelin concentrations with various parameters in control subjects

BMI, serum insulin levels, plasma glucose levels, and HOMA-IR negatively correlated with plasma levels of both acylated and desacyl ghrelin, while GH exhibited a positive correlation (Table 2). Age, IGF-1, and leptin levels did not correlate with either ghrelin form.

Discussion

The mean levels of plasma acylated ghrelin in 36 volunteers, 15.9 ± 9.4 fmol/ml, determined using new commercial ELISAs, were comparable with those previously determined for patients with normal renal function (14.7 ± 5.8 fmol/ml) (16) and healthy women (21.2 ± 3.1 fmol/ml) (17) by RIA. In contrast, the mean levels of plasma desacyl ghrelin, 66.1 ± 45.3 fmol/ml, were lower than expected from the total ghrelin levels measured by RIA (145.0 ± 33.5 to 223.4 ± 22.7 fmol/ml (5, 23, 24). The ratio of acylated to [acylated + desacyl] ghrelin (A/[A + D] ratio), 19.6 ± 5.4%, determined here was also lower than that of acylated to total ghrelin, 8.3 ±

Table 1. Plasma ghrelin concentrations and various parameters in healthy subjects

Parameters	All (n = 36)	Male (n = 16)	Female (n = 20)	Male vs Female (P value in *t*-test*)
Age (yr)	33.5 ± 9.0	34.7 ± 7.1	32.6 ± 10.3	0.486
BMI (kg/m^2)	21.7 ± 2.8	23.4 ± 3.0	20.3 ± 1.9	**0.001**
GH (ng/ml)*	4.10 ± 6.38	0.29 ± 0.24	7.15 ± 7.27	**0.001**
IGF-1 (ng/ml)	263.1 ± 71.0	258.25 ± 57.8	266.9 ± 81.4	0.722
Insulin (μU/ml)	7.1 ± 4.3	7.95 ± 5.5	6.5 ± 3.0	0.311
Glucose (mg/dl)	88.4 ± 6.6	92.4 ± 4.9	85.3 ± 6.1	**0.001**
HOMA-IR	1.6 ± 1.0	1.8 ± 1.3	1.4 ± 0.7	0.180
Leptin (ng/ml)	7.2 ± 5.2	5.4 ± 2.8	8.7 ± 6.2	0.054
NEFA (mEq/l)	0.43 ± 0.16	0.37 ± 0.14	0.48 ± 0.16	**0.030**
active ghrelin (fmol /ml)	15.9 ± 9.4	10.9 ± 6.1	19.9 ± 9.8	**0.003**
des-acyl ghrelin (fmol /ml)	66.1 ± 45.3	49.1 ± 23.5	79.8 ± 53.9	**0.041**
A/[A + D] ratio	19.6 ± 5.9	18.0 ± 5.6	20.8 ± 5.1	0.129
A/D ratio	24.9 ± 8.4	22.6 ± 8.8	26.8 ± 7.8	0.139

*: $p < 0.05$. Bold values: $P < 0.05$.

Systeme International (SI) units for GH, micrograms per liter (conversion factor, 1.0); for IGF-I to nanomoles per liter (0.131); for glucose, millimoles per liter (0.05551); for insulin, picomoles per liter (6.945); for leptin, nanomoles per liter (0.08).

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