Original Article

A Functional Variant in the Human Betacellulin Gene Promoter Is Associated With Type 2 Diabetes

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Betacellulin (BTC) plays an important role in differentiation, growth, and antiapoptosis of pancreatic β-cells. We characterized about 2.3 kb of the 5′-flanking region of human *BTC* gene and identified six polymorphisms (−2159A>G, −1449G>A, −1388C>T, −279C>A, −233G>C, and −226A>G). The G allele in the −226A>G polymorphism was more frequent in type 2 diabetic patients (n = 250) than in nondiabetic subjects (n = 254) (35.6% vs. 27.8%, P = 0.007), and the −2159G, −1449A, and −1388T alleles were in complete linkage disequilibrium with the −226G allele. The frequencies of the −279A and −233C alleles were low (7.0 and 2.0% in diabetic patients), and no significant differences were observed. In the diabetic group, insulin secretion ability, assessed by the serum C-peptide response to intravenous glucagon stimulation, was lower in patients with the −226G allele (G/G, 2.96 ± 0.16 ng/ml; G/A, 3.65 ± 0.18 ng/ml; A/A, 3.99 ± 0.16 ng/ml at 5 min after stimulation; P = 0.008). Furthermore, in vitro functional analyses indicated that both the −226G and the −233C alleles caused an ~50% decrease in the promoter activity, but no effects of the −2159A>G, −1449G>A, −1388C>T, and −279C>A polymorphisms were observed. These results suggest that the −226A/G polymorphism of the *BTC* gene may contribute to the development of diabetes. *Diabetes* 54:3560–3566, 2005

Impaired insulin secretion and insulin resistance are major defects observed in type 2 diabetic patients. When it becomes impossible for pancreatic β-cells to secrete the amount of insulin corresponding to the demand in peripheral tissues, blood glucose levels are elevated in diabetic patients. This insufficient insulin secretion is associated with an insufficient β-cell mass in the pancreatic islet and/or functional defects of the β-cells. Several recent reports have shown the importance of the β-cell mass in the pathophysiology of type 2 diabetes (1–4). Regulation of the β-cell mass appears to involve a balance of β-cell replication, neogenesis (the development of new islets from pancreatic ducts), and apoptosis. Some factors that are associated with the regulation of the β-cell mass have been identified (5), and we hypothesized that functional gene polymorphisms in these factors might be associated with the development of diabetes.

Betacellulin (BTC), a member of the epidermal growth factor (EGF) family, was purified from the conditioned medium of a cell line derived from mouse pancreatic β-cell tumors (6). Its primary translational product was composed of 178 amino acid residues, which contained a signal sequence, transmembrane, and cytoplasmic domains in addition to an EGF-like domain (7). The expression of BTC is predominantly found in the pancreas and in the intestine (8). In particular, BTC is expressed in α-, β-, and duct cells in a normal adult pancreas and in primitive duct cells of the fetal pancreas (9). BTC converts the rat pancreatic acinar cell line (AR42J cells) to insulin-expressing cells together with activin A (10) and has the potential for the growth of a rat insulinoma cell line, INS-1 cells (11). BTC promotes the neogenesis of β-cells and accelerates the improvement of glucose tolerance in mice with diabetes induced by selective alloxan perfusion (12). BTC also improves glucose metabolism by promoting the conversion of intraislet precursor cells to β-cells in streptozotocin-treated mice (13). Furthermore, the activation of the EGF receptor by BTC induces an inhibitory effect on apoptosis (14). These observations suggest that BTC plays an important role in differentiation, growth, and antiapoptosis of the pancreatic β-cells.

We previously screened gene polymorphisms in the protein coding exons of the human *BTC* gene in type 2 diabetic patients (15). The frequencies of polymorphisms identified, however, were similar between the diabetic patients and the control subjects. In this study, to examine the role of polymorphisms in the promoter of the human *BTC* gene, we characterized the 5′-flanking region of the human *BTC* gene and screened gene polymorphisms in the promoter in type 2 diabetic patients.

RESEARCH DESIGN AND METHODS

Characterization of the 5′-flanking region of the human *BTC* gene. To assess the portions of the 5′-flanking region of the human *BTC* gene required for promoter activity, a series of deletions of the region were fused to a luciferase reporter gene. The 5′-flanking regions of the human *BTC* gene spanning −2,330 to 160 bp, −881 to 160 bp, −669 to 160 bp, −350 to 160 bp, and −152 to 160 bp numbered relative to the translation start site, were amplified by PCR using Pfu DNA polymerase (Stratagene, La Jolla, CA), and were subcloned into the *Sma*I site of pGL3-basic firefly luciferase reporter vector (Promega, Madison, WI) in the 5′-3′ orientation. The sequences of constructs were confirmed by bidirectional sequencing. We transiently trans-

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BTC, betacellulin; CPR, C-peptide response; EGF, epidermal growth factor.

TABLE 1
Clinical characteristics of the subjects enrolled in the present study

	Nondiabetic subjects	Type 2 diabetic patients
n (male/female)	254 (67/187)	250 (135/115)
Age (years)	75.1 ± 8.0	63.1 ± 11.3
BMI (kg/m^2)	21.8 ± 3.7	23.5 ± 3.2
A1C (%)	5.0 ± 0.4	7.4 ± 1.3
Age at diagnosis (years)	—	45.5 ± 9.4
Mode of treatment (D/OHA/Ins) (%)	—	18.8/40.8/40.4

Data are means ± SD unless otherwise indicated. D, diet; Ins, insulin; OHA, oral hypoglycemic agent.

fected 0.1 μg each of these constructs with 0.01 μg pRL-SV40 vector (renilla luciferase under control of SV40 promoter), as an internal control for transfection efficiency, into βTC3 cells using FuGENE6 transfection reagent (Roche Diagnostics, Mannheim, Germany). The βTC3 cells were seeded into 12-well culture plates and were maintained in Dulbecco's modified Eagle's medium containing 4,500 mg/l glucose, 10% fetal bovine serum, and antibiotics (100 units/ml penicillin G sodium and 100 mg/ml streptomycin sulfate). After 48 h, we collected the cells and measured luciferase activity using Dual-luciferase Reporter Assay System (Promega). The relative luciferase activity for each construct was calculated as a fold increase over the activity for the promoterless control vector (pGL3-basic). The data presented represent the means of three independent transfection experiments per construct.

Screening and genotyping of polymorphisms in the promoter region of the human *BTC* gene. About 2.3 kb of the promoter region was amplified with a PCR and sequenced in DNA samples from 20 Japanese type 2 diabetic patients. PCR was carried out using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA). Sequencing was carried out using Big Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems) on an automated DNA capillary sequencer (model 310; Applied Biosystems). Polymorphisms identified in this screening were designated according to their location from the translation start site. Sequence information of the primers and the conditions for PCR-direct sequencing are shown in Supplemental Table 1 in the online appendix (available at http://diabetes.diabetesjournals.org).

Polymorphisms identified were genotyped in 250 Japanese type 2 diabetic patients and 254 nondiabetic subjects using PCR direct sequencing. We calculated linkage disequilibrium coefficients (D′ and Δ^2) using the Graphical Overview of Linkage Disequilibrium program (http://www.sph.umich.edu/csg/abecasis/GOLD). All of the type 2 diabetic patients in this study were recruited from patients attending the outpatient clinic of the Wakayama Medical University Hospital. All patients were evaluated for their insulin secretion ability by the serum C-peptide immunoreactivity response (CPR) to intravenous glucagon stimulation. Because renal function affects serum CPR levels, patients with an elevated serum creatinine level (>1.2 mg/dl) were not included. Diabetes was diagnosed according to the criteria of the World Health Organization, and patients who were glutamic acid decarboxylase antibody positive and/or had started insulin therapy within 3 years of the diagnosis of diabetes were excluded from this study. Nondiabetic subjects were chosen using the following criteria: age of >60 years, HbA_{1c} (A1C) of <5.6%, fasting plasma glucose of <110 mg/dl, and no family history of diabetes. The clinical characteristics of type 2 diabetic patients and nondiabetic subjects are shown in Table 1. All of the participants gave their written informed consent before participating in the study. This study was approved by the ethics committee of the Wakayama Medical University.

Assessment of the insulin secretion ability in type 2 diabetic patients. We assessed the insulin secretion ability in type 2 diabetic patients using their serum CPR response to intravenous glucagon stimulation (16). After an overnight fast, glucagon (1 mg/body) was injected intravenously, and serum CPR levels were measured before (CPR0′) and 5 min after (CPR5′) injection, and then, the increment of CPR for 5 min (ΔG5′) was calculated. The patients treated with drugs were instructed not to take their morning oral hypoglycemic agents or insulin on the day of test.

Functional properties of polymorphisms on the promoter activity of the human *BTC* gene. The −279C>A, −233G>C, and −226A>G gene polymorphisms were introduced into the pGL3-reporter vector containing the 5′-flanking region spanning from −669 to 160 bp using QuikChange site-directed mutagenesis kit (Stratagene) for generating five constructs, pGL3-(−669/160)CGA, pGL3-(−669/160)CGG, pGL3-(−669/160)AGA, pGL3-(−669/160)CCA, and pGL3-(−669/160)CCG. Four gene polymorphisms, −2159A>G, −1449G>A, −1388C>T, and −226A>G, were also introduced into the pGL3-reporter vector containing the 5′-flanking region spanning from −2,330 to 160 bp using the same kit for generating three constructs, pGL3-(−2330/160)AGCA, pGL3-(−2330/160)GATA, and pGL3-(−2330/160)GATG. These constructs were transfected into the βTC3 cells, and the relative luciferase activity for each construct was calculated as described above.

Statistical analysis. Results are presented as means ± SE unless otherwise indicated. The proportion of genotypes or alleles was compared by a χ^2 test. Group differences of continuous variables were compared using an unpaired *t* test or a one-way ANOVA followed by a post hoc analysis with a Fisher's protected least-significant difference test. Categorical variables were compared with a χ^2 test. The data of serum CPR levels were log transformed before analyzing. These analyses were performed with the StatView program for Windows (version 5.01; SAS Institute, Cary, NC). A P value of <0.05 was considered to be statistically significant.

RESULTS

Characterization of the 5′-flanking region of the human *BTC* gene. The relative luciferase activities of the reporter constructs in the βTC3 cells are shown in Fig. 1. The construct containing from −350 to 160 bp demonstrated a fivefold increase in activity compared with the promoterless construct ($P < 0.001$), whereas only background activity was obtained with the construct containing from −152 to 160 bp. Furthermore, a significant increase in activity was observed with the addition of the region between −669 and −350 bp ($P = 0.020$). Conversely, the further addition of the region between −881 and −669 bp attenuated the promoter activity ($P = 0.002$). Finally, the promoter activity of the construct containing the region up to −2,330 bp was significantly higher than that of the construct containing the region up to −669 bp ($P < 0.001$). These data suggest that the basal promoter of the human *BTC* gene is located within the −200-bp region between −350 and −152 bp relative to the translation start site, the positive regulatory elements are located within the regions between −669 and −350 bp and between −2,330 and −881 bp, and the negative regulatory element(s) is located within the region between −881 and −669 bp.

Polymorphisms in the promoter region of the human *BTC* gene. About 2.3 kb of the promoter region was screened in 20 Japanese type 2 diabetic patients, and six polymorphisms were identified. These polymorphisms include a G for A substitution at −2,159 bp (−2159A>G), an A for G substitution at −1,449 bp (−1449G>A), a T for C substitution at −1,388 bp (−1388C>T), an A for C substitution at −279 bp (−279C>A), a C for G substitution at −233 bp (−233G>C), and a G for A substitution at −226 bp (−226A>G). All polymorphisms were numbered relative to the translation start site. The reference single nucleotide polymorphism ID numbers of −2159A>G, −1449G>A, −1388C>T, and −226A>G are rs11733938, rs13121979, rs13101336, and rs2278862, respectively.

We genotyped these polymorphisms in 250 type 2 diabetic patients and 254 nondiabetic subjects. The allelic distributions were in the Hardy-Weinberg equilibrium expectations, and the −2159G, −1449A, −1388T, and −226G alleles were in complete linkage disequilibrium with each other ($D' = 1.00$, $\Delta^2 = 1.00$). For the −226A>G polymorphism, the G allele was significantly more frequent in type 2 diabetic patients than in nondiabetic subjects ($P = 0.007$). The odds ratios (OR) of subjects with the G/G or A/G genotype were 1.90 (95% CI 1.05–3.41, $P = 0.031$) and 1.49 (1.02–2.16, $P = 0.038$) compared with those having the A/A genotype, respectively. In the −279C>A and −233G>C polymorphisms, no significant differences were observed

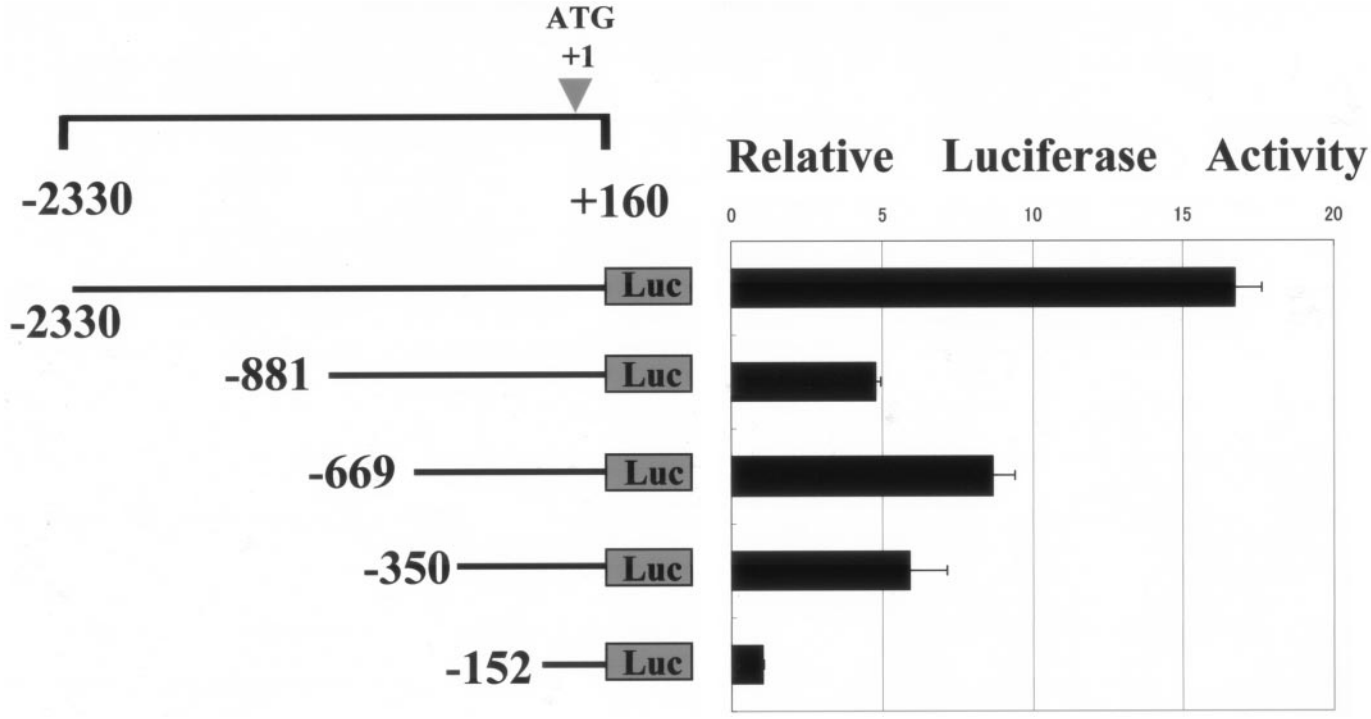

FIG. 1. Deletion analysis of the 5′-flanking region of the human *BTC* gene. The 5′-flanking region was progressively deleted, fused to a firefly luciferase reporter (pGL3-basic) vector, and transfected into βTC3 cells. Cells were cotransfected with a *Renilla* luciferase control (pRL-SV40) vector. The firefly luciferase activity of each construct was normalized in comparison with coexpressed *Renilla* luciferase activity and expressed as the fold increase relative to the activity of the promoterless pGL3-basic vector. All constructs are numbered relative to the translation start site. The data presented represent the means of three independent transfection experiments per construct. Data are means ± SE. The differences in the averaged activities were compared using a one-way ANOVA followed by post hoc test.

in genotypic and allelic distribution between diabetic patients and nondiabetic subjects (Table 2).

Relationship between the −226A>G polymorphism and the insulin secretion ability in type 2 diabetic patients. As shown in Table 3, there were no significant differences in sex, age, age at diagnosis of diabetes, duration of diabetes, maximum BMI, and present BMI among the three groups of type 2 diabetic patients according to the −226A>G genotypes. However, the insulin secretion ability evaluated by the serum CPR response to glucagon stimulation was significantly lower in patients with the G allele (G/G, 2.96 ± 0.16 ng/ml; G/A, 3.65 ± 0.18 ng/ml; and A/A, 3.99 ± 0.16 ng/ml for CPR5′; $P = 0.008$ by ANOVA. G/G, 1.39 ± 0.11 ng/ml; G/A, 1.87 ± 0.11 ng/ml; and A/A, 2.13 ± 0.11 ng/ml for ΔG5′; $P = 0.002$.) (Fig. 2). The fasting serum CPR levels were also lower in patients with the G allele (G/G, 1.58 ± 0.10 ng/ml; G/A, 1.76 ± 0.07 ng/ml; and A/A, 1.86 ± 0.07 ng/ml; $P = 0.182$), and the A1C levels and the percentage of patients with insulin treatment were higher in patients with the G allele, but these differences were not statistically significant.

Effects of polymorphisms on the promoter activity of the human *BTC* gene. To investigate whether the −279C>A, −233G>C, and −226A>G gene polymorphisms would affect the promoter activity of the human *BTC* gene, we constructed four plasmids containing the 5′-flanking region spanning from −669 to 160 bp, pGL3-(−669/160)CGA, pGL3-(−669/160)CGG, pGL3-(−669/160)AGA, and pGL3-(−669/160)CCA vectors. In transient transfection into the βTC3 cells, the pGL3-(−669/160)CGA vector induced luciferase activity sevenfold greater, relative to the promoterless pGL3-basic vector (Fig. 3). On the other hand, the pGL3-(−669/160)CGG vector had an ~50% decrease in the activity compared with the pGL3-(−669/

TABLE 2
Comparison of genotypic and allelic distributions of gene polymorphisms between type 2 diabetic patients and nondiabetic subjects

Polymorphisms	Genotypes				Alleles		
−226A>G	A/A	A/G	G/G		A	G	
Type 2 diabetes	106 (42.4)	110 (44.0)	34 (13.6)	$P = 0.032$	322 (64.4)	178 (35.6)	$P = 0.007$
Nondiabetic	136 (53.5)	95 (37.4)	23 (9.1)		367 (72.2)	141 (27.8)	
−233G>C	G/G	G/C	C/C		G	C	
Type 2 diabetes	240 (96.0)	10 (4.0)	0 (0)	$P = 0.439$	490 (98.0)	10 (2.0)	$P = 0.419$
Nondiabetic	247 (97.2)	7 (2.8)	0 (0)		501 (98.6)	7 (1.4)	
−279C>A	C/C	C/A	A/A		C	A	
Type 2 diabetes	215 (86.0)	35 (14.0)	0 (0)	$P = 0.643$	465 (93.0)	35 (7.0)	$P = 0.655$
Nondiabetic	222 (87.4)	32 (12.6)	0 (0)		476 (93.7)	32 (6.3)	

Data are *n* (%). The results of −2159A>G, −1449G>A, and −1388C>T are not shown because the −2159G, −1449A, and −1388T alleles are in complete linkage disequilibrium with the −226G allele.

TABLE 3
Clinical characteristics and biochemical data of type 2 diabetic patients classified according to their genotypes of the −226A>G polymorphism

	−226A >G genotype			
	G/G	G/A	A/A	*P*
n (male/female)	34 (16/18)	110 (58/52)	106 (61/44)	0.488
Age (years)	62.4 ± 2.0	64.3 ± 1.1	62.2 ± 1.1	0.377
Age at diagnosis (years)	45.6 ± 1.5	45.4 ± 0.9	45.6 ± 1.0	0.976
Duration of diabetes (years)	16.7 ± 1.8	18.6 ± 1.0	16.4 ± 1.0	0.265
Maximum BMI (kg/m^2)	27.0 ± 0.6	26.5 ± 0.3	27.0 ± 0.3	0.554
BMI (kg/m^2)	23.4 ± 0.5	23.3 ± 0.3	23.7 ± 0.3	0.662
A1C (%)	7.7 ± 0.2	7.5 ± 0.1	7.2 ± 0.1	0.072
Mode of treatment (D/OHA/Ins) (*n*)	6/11/17	18/47/45	23/44/39	
Insulin (%)	50.0	40.9	37.7	0.389

Data are shown as means ± SE unless otherwise indicated. *P* values are compared by one-way ANOVA or χ^2 test for existence of insulin treatment. D, diet; Ins, insulin; OHA, oral hypoglycemic agents.

160)CGA vector (3.84 ± 0.23 vs. 6.98 ± 0.33, $P < 0.001$). Furthermore, the pGL3-(−669/160)CCA vector had an ~50% decrease in the activity compared with the pGL3-(−669/160)CGA vector (3.73 ± 0.23 vs. 6.98 ± 0.33, $P < 0.001$). The relative luciferase activity of the pGL3-(−669/160)AGA vector was similar to that of the pGL3-(−669/160)CGA vector (7.30 ± 0.43 vs. 6.98 ± 0.33, $P = 0.533$). Because not only the −226A>G polymorphism but also the −233G>C polymorphism affected the promoter activity of the human *BTC* gene, we further constructed pGL3-(−669/160)CCG vector, but the relative luciferase activity of the pGL3-(−669/160)CCG vector was similar to those of the pGL3-(−669/160)CGG and pGL3-(−669/160)CCA vectors. We next examined the effect of −2159A>G, −1449G>A, and −1388C>T polymorphisms on the promoter activity of the human *BTC* gene, because the −226G allele was in complete linkage disequilibrium with −2159G, −1449A, and −1388T alleles. Four polymorphisms, −2159A>G, −1449G>A, −1388C>T, and −226A>G, were introduced into pGL3-reporter vector containing the 5′-flanking region spanning from −2,330 to 160 bp for generating three plasmids, pGL3-(−2330/160)AGCA, pGL3-(−2330/160)GATA, and pGL3-(−2330/160)GATG. The pGL3-(−2330/160)GATA vector induced luciferase expression ~14-fold greater, relative to the promoterless pGL3-basic vector, and the pGL3-(−2330/160)GATG had an ~50% decrease in activity compared with the pGL3-(−2330/160)GATA vector (14.4 ± 1.20 vs. 8.02 ± 0.50, $P < 0.001$) (Fig. 4). On the other hand, the relative luciferase activity of the pGL3-(−2330/160)GATA vector was similar to that of the pGL3-(−2330/160)AGCA vector (14.4 ± 1.20 vs. 16.4 ± 0.81, $P = 0.131$). These results suggest that −2159A>G, −1449G>A, and −1388C>T polymorphisms do not affect the transcription of the human *BTC* gene.

DISCUSSION

In this study, our purpose was to investigate the role of gene polymorphisms in the promoter of the human *BTC* gene in type 2 diabetic patients. Because the promoter of the human *BTC* gene had not been well studied, we initially characterized about 2.3 kb of the 5′-flanking region of the human *BTC* gene in a pancreatic β-cell line. We then screened gene polymorphisms in this region and found that the G allele of the −226A>G polymorphism was more frequent in type 2 diabetic patients than in nondiabetic subjects. However, the possibility that this

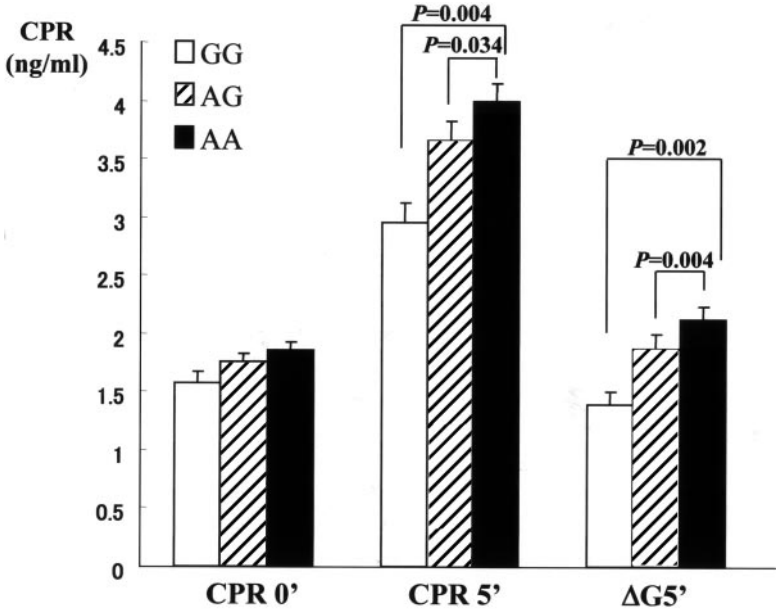

FIG. 2. The effect of the −226A>G polymorphism on fasting C-peptide immunoreactivity (CPR) levels and CPR response to glucagon stimulation in type 2 diabetic patients. The serum CPR levels at 5 min after injection (CPR 5′) and the increment of CPR for 5 min (ΔG5′ = CPR 5′ − CPR 0′) were significantly lower in patients with the G allele. Data are means ± SE. The differences in the averaged CPR levels were compared using a one-way ANOVA followed by post hoc test.

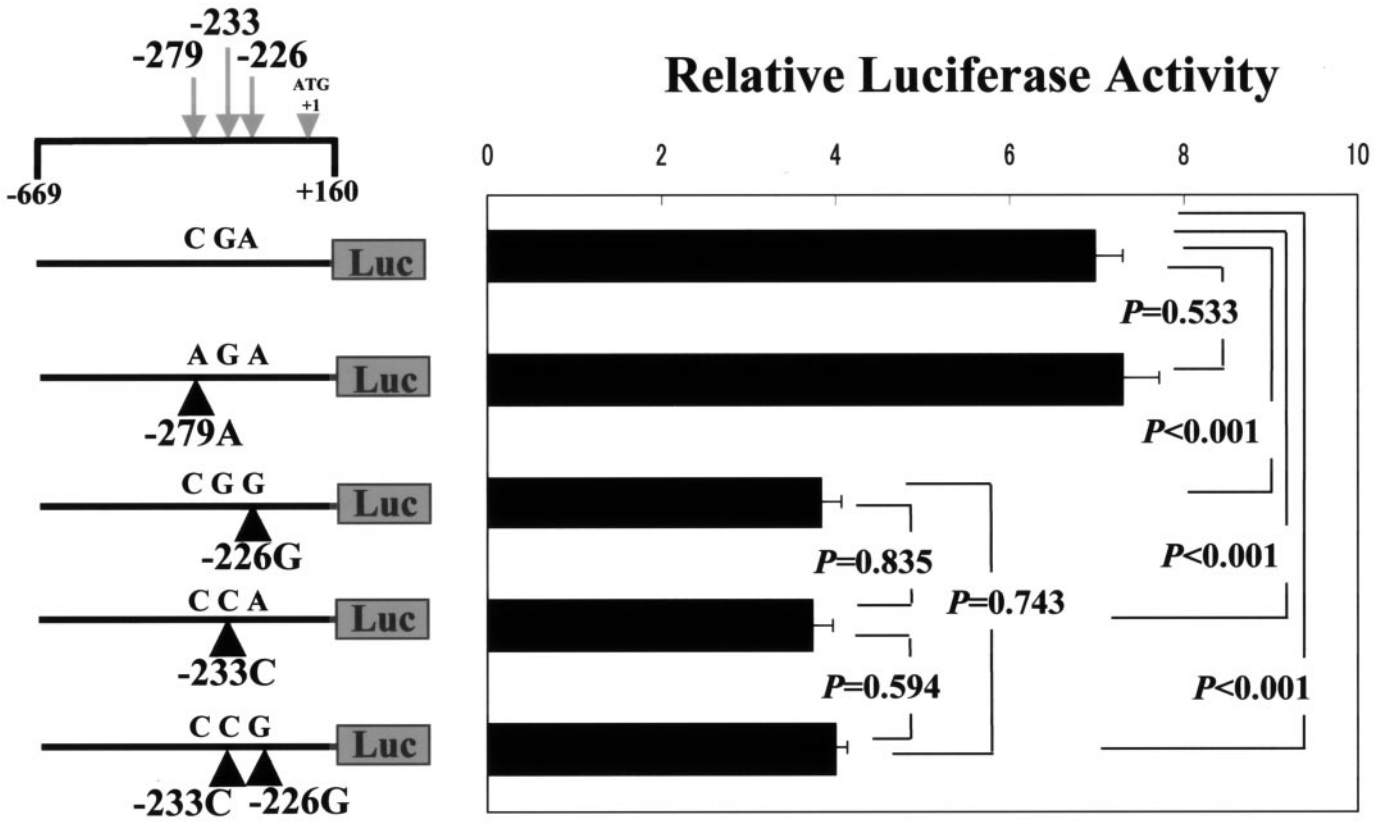

FIG. 3. The effects of the −279C>A, −233G>C, and −226A>G polymorphisms on the promoter activity of the human *BTC* gene. Five plasmids containing the 5′-flanking region, spanning from −669 to 160 bp, with each of five different combinations for the −279C>A, −233G>C, and −226A>G polymorphisms upstream of a luciferase gene transcriptional unit were constructed and transiently transfected into the βTC3 cells. The data presented represent the means of three independent transfection experiments per construct. Data are means ± SE. The differences in the averaged activities were compared using a one-way ANOVA followed by post hoc test.

result is a false-positive finding should be considered, because the sample size of our case-control analysis is small and the *P* values obtained are modest (17). Therefore, we further investigated the effect of the −226A>G polymorphism on clinical profiles of patients and the functional properties of all polymorphisms identified. We first examined the relationship between the G allele of the −226A>G polymorphism and insulin secretion ability in type 2 diabetic patients, because BTC plays an important role in differentiation, growth, and antiapoptosis of the pancreatic β-cells (10–14). Glucagon is a potent stimulus for the pancreatic β-cells, and the serum CPR to intravenous glucagon stimulation has been used to evaluate residual insulin secretion in diabetic patients (16,18). We, therefore, used this test to evaluate the insulin secretion ability in our patient group and observed that the serum CPR to glucagon was significantly lower in patients with the G allele. On the other hand, the A1C level was higher, but not significantly so, in patients with the G allele. The chronic hyperglycemia itself also causes the impairment of β-cell function. However, we think that this may not affect the result, because it has been reported that the serum CPR to glucagon was little influenced by the chronic hyperglycemia (16,19). Moreover, although an influence of oral hypoglycemic agents on the serum CPR should be considered, the percentages of patients treated with oral drugs were almost similar among the groups (G/G, 32%; G/A, 42.7%; A/A, 41.5%; $P = 0.550$). We next examined the functional properties of polymorphisms. A promoter with the −226G allele had an ~50% decrease in the activity. The −226G allele was also in complete linkage disequilibrium with the −2159G, −1449A, and −1338T alleles, but no effects of the −2159A>G, −1449G>A, and −1388C>T polymorphisms on the promoter activity were observed. Furthermore, although we could not observe significant difference in the frequency of −233C allele in our case-control study, a promoter with the −233C allele also had an ~50% decrease in the activity. The −233C allele frequency was only 2.0% in diabetic patients and 1.4% in nondiabetic subjects. The power of our study may be not enough to detect the difference in case-control analysis because of the low frequency of C allele. In subjects with the G/C genotype on the −233G>C polymorphism, 6 of 10 subjects in diabetic group and 2 of 7 subjects in nondiabetic group had the A/G genotype on the −226A>G polymorphism. Subjects with the G/G genotype on the −226A>G polymorphism were not observed in both groups. On the basis of these results, we conclude that a decreased expression of BTC may be associated with the development of type 2 diabetes.

BTC is a member of the EGF family. The addition of BTC promoted proliferation, regeneration, and neogenesis of pancreatic β-cells in both a cell line (11) and diabetic mice developed by treatment with the β-cell toxin (12,13). Furthermore, BTC has an antiapoptotic effect via transactivation of the EGF receptors, and the effect is greater than that by EGF (14). Several reports suggest that a decreased β-cell mass is observed in type 2 diabetic patients (1–4) and plays an important role in the pathogenesis of type 2 diabetes. The β-cell mass is regulated by a balance be-

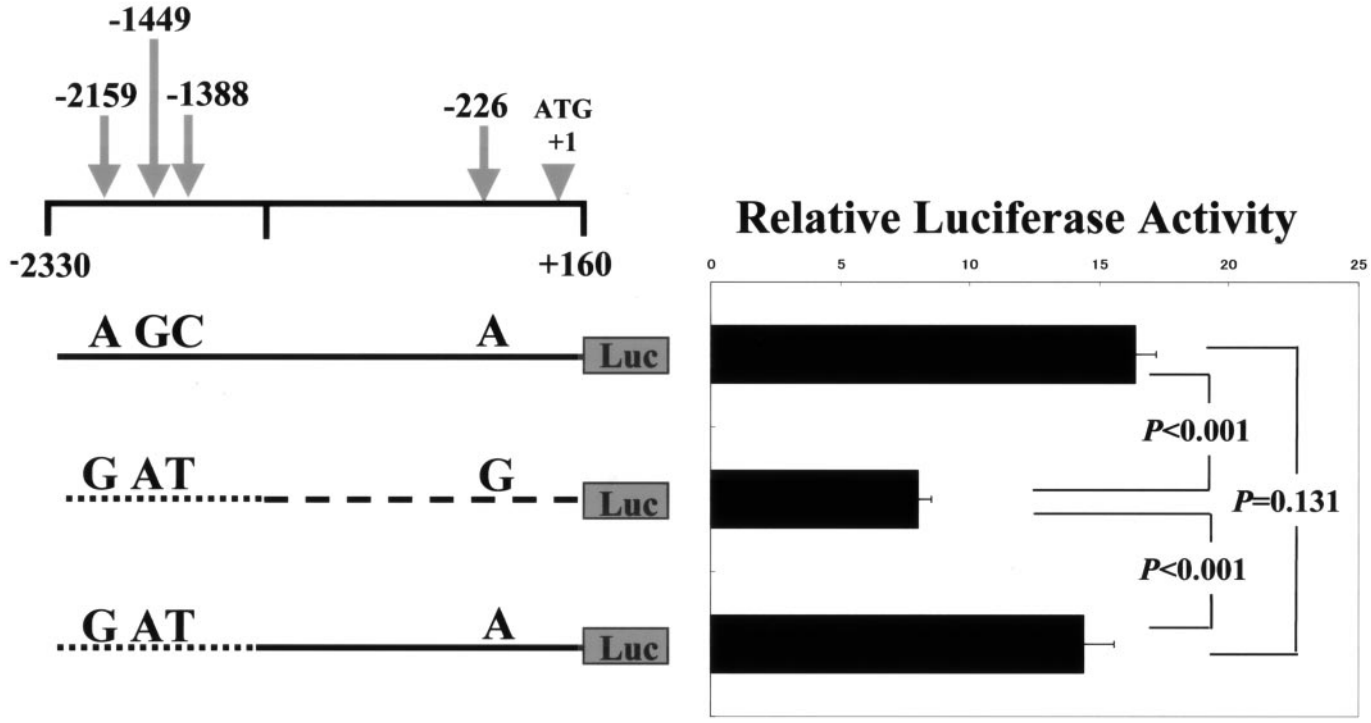

FIG. 4. The effects of the −2159A>G, −1449G>A, and −1388C>T polymorphisms on the promoter activity of the human *BTC* gene. Three plasmids containing the 5′-flanking region, spanning from −2,330 to 160 bp, with each of three different combinations for the −2159A>G, −1449G>A, −1388C>T, and −226A>G polymorphisms upstream of a luciferase gene transcriptional unit were constructed and transiently transfected into the βTC3 cells. No effects of the −2159A>G, −1449G>A, and −1388C>T polymorphisms in the promoter activity were observed. The data presented represent the means of three independent transfection experiments per construct. Data are means ± SE. The differences in the averaged activities were compared using a one-way ANOVA followed by post hoc test.

tween an input (β-cell neogenesis from pancreatic ducts and β-cell replication within islets) and an output (β-cell apoptosis). Because BTC accelerates the input and decelerates the output, a decreased expression of BTC affected by gene polymorphism may accelerate a decrease in β-cell mass in type 2 diabetic patients. *BTC*-null mice have been generated by gene targeting (20). The mice were viable and grew normally. No morphological changes were observed in the newborn pancreases; also, adult $BTC^{+/+}$ and $BTC^{-/-}$ males responded similarly to glucose challenge tests. Their actual data, however, were not shown in the report. In addition to the BTC dysfunction, other genetic and environmental factors, including aging, may be required for the development of overt diabetes.

The sequences surrounding the −226A>G polymorphism were analyzed for potential transcription factor binding sites using TFSEARCH (available at http://www.rwcp.or.jp/papia/). The sequences were similar to the consensus of a caudal-type homeo box (Cdx)-binding site in reverse orientation, and a G for A substitution of the −226A>G polymorphism resulted in disrupting the consensus. Cdx is the family of homeodomain proteins related to the *Drosophila* "caudal" gene, which is required for anterior-posterior regional identity. In the mouse, Cdx1 and Cdx2/3, which are homologs of the *Drosophila* caudal gene, also have functions in anteroposterior patterning and posterior axis elongation (21). In addition, Cdx1 and Cdx2/3 are expressed in the intestine and regulate intestine-specific gene expression (22). The expression of Cdx2/3 is also found in both α- and β-cells (23) and is one of the islet-enriched transcription factors (24). BTC is also predominantly expressed in the endocrine pancreas and intestine (8). This evidence suggests that Cdx protein is a good candidate of the transcription factor, which binds to the region surrounding the −226A>G polymorphism. The −233G>C polymorphism, which is 7 bp upstream from the −226A>G polymorphism, also affected the promoter activity of the *BTC* gene. Because the effects of the −233G>C polymorphism and the −266A>G polymorphisms were not additive, both polymorphisms may affect the binding of an identical transcription factor. The −233G>C polymorphism, however, did not influence the consensus sequence of Cdx binding site on the analysis using TFSEARCH.

It has been recently reported that the Cys7Gly polymorphism in the *BTC* gene is associated with type 2 diabetes in African Americans (25). Our previous study, however, showed that the minor allele frequency was similar between type 2 diabetic patients (1.8%) and nondiabetic subjects (1.8%) in Japanese (15), and the frequency in Japanese patients was lower than in African Americans (32%). Furthermore, no significant difference in the allele frequency between patients and nondiabetic subjects was observed in Caucasians (25). On the other hand, the −226G allele frequency was higher in type 2 diabetic patients than in nondiabetic subjects both in African Americans (25 vs. 20%, $P = 0.14$) and Caucasians (37 vs. 34%, $P = 0.35$) (25), although their differences were not statistically significant. Further studies will be needed to understand the ethnic difference in susceptibility to type 2 diabetes.

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