

# A Functional Polymorphism in the Promoter of UCP2 Enhances Obesity Risk but Reduces Type 2 Diabetes Risk in Obese Middle-Aged Humans

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Obesity is frequently associated with type 2 diabetes. We previously observed an association of a functional G/A polymorphism in the uncoupling protein 2 (UCP2) promoter with obesity. The wild-type G allele was associated with reduced adipose tissue mRNA expression *in vivo*, reduced transcriptional activity *in vitro*, and increased risk of obesity. On the other hand, studies in animal and cell culture models identified pancreatic  $\beta$ -cell UCP2 expression as a main determinant of the insulin secretory response to glucose. We therefore ascertained associations of the -866G/A polymorphism with  $\beta$ -cell function and diabetes risk in obesity. We show here that the pancreatic transcription factor PAX6 preferentially binds to and more effectively *trans* activates the variant than the wild-type UCP2 promoter allele in the  $\beta$ -cell line INS1-E. By studying 39 obese nondiabetic humans, we observed genotype differences in  $\beta$ -cell function; wild-type subjects displayed a greater disposition index (the product of insulin sensitivity and acute insulin response to glucose) than subjects with the variant allele ( $P < 0.03$ ). By comparing obese subjects with and without type 2 diabetes, we observed genotype-associated differences in diabetes prevalence that translated into a twofold age-adjusted risk reduction in wild-type subjects. Thus, the more common UCP2 promoter G allele, while being conducive for obesity, affords relative protection against type 2 diabetes. *Diabetes* 51:3331–3335, 2002

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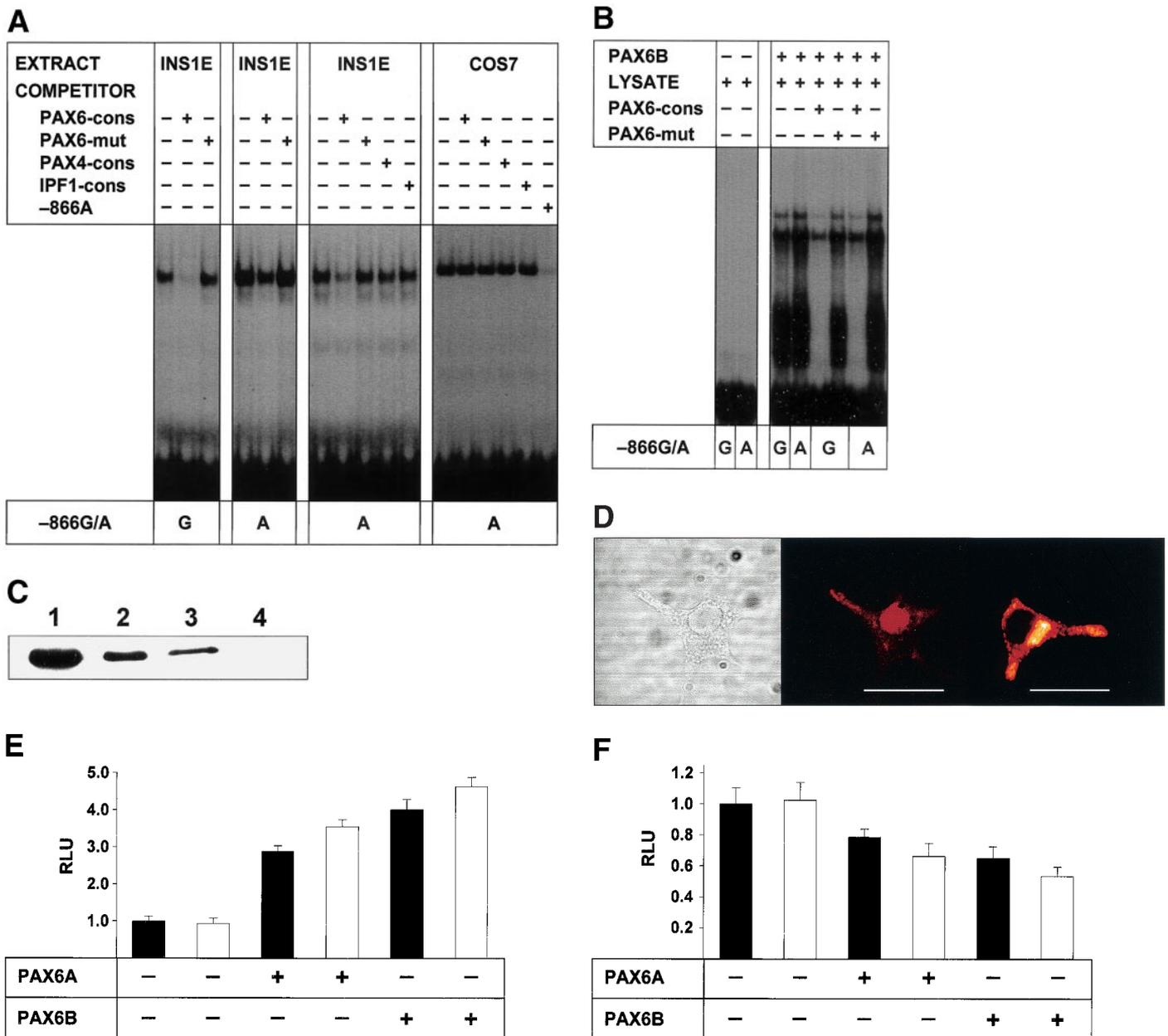
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Additional information for this article can be found in an online appendix at <http://diabetes.diabetesjournals.org>.

AIR, acute insulin response; FSIGT, frequently sampled intravenous glucose tolerance test;  $S_G$ , glucose effectiveness;  $S_I$ , insulin sensitivity index; UCP2, uncoupling protein 2.

The escalating global epidemic of overweight and obesity (“globesity”) that is currently taking over many parts of the world predicts a tremendous increase in type 2 diabetes prevalence in the near future (1). One of the many genes that might be involved in the development of obesity as well as diabetes could be uncoupling protein 2 (UCP2). We recently reported an association of a functional G/A polymorphism in the UCP2 promoter with obesity (2). The variant A allele of the -866G/A polymorphism was associated with enhanced transcriptional activity and decreased risk of obesity. In addition, we observed similar basal glucose, but lower insulin levels in -866G/A than in wild-type subjects. The polymorphism is located in a multifunctional *cis* regulatory site that showed distinct interactions with nuclear proteins in a cell type-specific manner. Computational analyses suggested binding sites for several *trans* factors, including IPF1 and PAX6, both of which have been implicated in pancreatic  $\beta$ -cell development and function (3–6) and were predicted to bind preferentially to the variant -866A allele (2). Other studies performed in animal and cell culture models concluded that enhanced  $\beta$ -cell UCP2 expression reduces the insulin secretory response to glucose (7–10). Taken together, these findings led to an intriguing question: whether enhanced UCP2 activity might act as an “adiposity angel and diabetes devil” (11).

To identify the *trans* factors binding to the respective promoter site in pancreatic  $\beta$ -cells, we performed band-shift assays using the -879/-839 DNA element and nuclear extracts from INS1-E (derived from rat  $\beta$ -cells) and COS-7 cells. Specific DNA-protein complexes produced by the two nuclear extracts displayed distinct electrophoretic mobilities (Fig. 1A) and differed from complexes obtained with PAZ-6 and HeLa nuclear extracts (2) (not shown). In all experiments, nuclear proteins from INS1-E cells showed stronger binding to the probe containing the variant A at -866 (Fig. 1A). For protein identification, we used several DNA probes as competitors: a PAX6 consensus probe used for crystallization of PAX6 in its binding form (12), a mutated PAX6 probe containing two invalid nucleotides, an IPF1 consensus probe from the rat glucokinase promoter (4), and, to exclude binding of another



**FIG. 1.** PAX6 preferentially binds to and more effectively *trans* activates the variant  $-866A$  UCP2 promoter allele. **A:** Electrophoretic mobility shift assays with nuclear extracts prepared from INS1-E and COS-7 cells. Extracts were incubated with  $\alpha^{32}P$ -labeled double-stranded oligonucleotides corresponding to the  $-866G$  and  $-866A$  alleles of UCP2 ( $-866G$ : 5'-TGGCTGTTTACGCGTCAGTTACCCCCACAGG-3';  $-866A$ : 5'-TGGCTGTTTACGCGTCAGTTACCCCCACAGG-3'). Competition studies included the following double-stranded competitor probes: PAX6 consensus sequence probe (PAX6-cons), 5'-GCATtTTCACGCATgAGTgcaCag-3'; mutated PAX6 consensus sequence probe (PAX6-mut), 5'-GcaTtTTCAGcCATgAGTgcaCag-3'; PAX4 consensus sequence probe (PAX4-cons), 5'-GcgcGgTgAtGCGTgtGcagCggC-3'; IPF1 consensus sequence probe from rat glucokinase element 1 (IPF1-cons), 5'-GCCcagtAgaCATtAGcacCtCgC-3'; and  $-866A$ . Upper case letters indicate sequence homology with the UCP2 promoter. The relative location of the  $-866G/A$  UCP2 promoter polymorphism is shown in upper case underlined. Letters in lower case italics indicate mutations introduced into the PAX6 core consensus sequence. Studies with three different extracts showed identical results. **B:** Electrophoretic mobility shift assays with in vitro translated PAX6B and unprogrammed reticulocyte lysate as control. **C:** Anti-PAX6 Western blot showing the presence of PAX6 protein in nuclear extracts isolated from INS1-E (lane 1) and COS-7 (lane 2) cells. Lanes 3 and 4: In vitro translated PAX6B as positive and unprogrammed reticulocyte lysate as negative control. **D:** Indirect immunofluorescence staining of PAX6 and insulin on INS1-E cells with anti-PAX6 (middle) or anti-insulin (right) antibody, detected by a TRITC- or FITC-conjugated secondary antibody, respectively. A phase contrast image is shown on the left. Scale bar = 25  $\mu$ m. **E** and **F:** INS1-E cells (**E**) or COS-7 cells (**F**) cotransfected with UCP2 promoter constructs  $-866G$  or  $-866A$  and, as indicated, PAX6A/PAX6B expression plasmids or the empty pcDNA6/V5-His plasmid backbone. RLU, relative luciferase units, where activity of the  $-866G$  construct was set to 1.0. Results are representative for one of three independent experiments, each with greater than or equal to five replicates. ■,  $-866G$  reporter construct; □,  $-866A$  reporter construct; means  $\pm$  SD.

PAX family member present in  $\beta$ -cells, a PAX4 binding matrix consensus probe (13). Among competitors used, only the PAX6 consensus probe competed for binding of proteins contained in INS1-E cell nuclear extracts, whereas none of the probes competed for binding of COS-7 nuclear proteins. Unlabeled bandshift probe, how-

ever, was a highly effective competitor, implicating other COS-7 nuclear proteins in specific binding. We independently validated binding of PAX6 with in vitro translated human PAX6B. Again, binding of PAX6B was stronger to the variant probe (Fig. 1B). We verified, by Western blotting, the presence of PAX6 proteins in nuclear extracts

TABLE 1  
UCP2 -866 G/A polymorphism and metabolic parameters in obese nondiabetic subjects

	-866G/G	-866G/A	-866A/A	<i>P</i> *	<i>P</i> †
<i>N</i> (F/M)	14/3	13/5	3/1		
<i>S</i> <sub>G</sub> (10 <sup>-2</sup> /min)	1.09 ± 0.97	2.08 ± 1.55	1.34 ± 0.84	0.237‡	0.076‡
<i>S</i> <sub>1</sub> (10 <sup>-4</sup> · min <sup>-1</sup> · mU <sup>-1</sup> · l)	4.052 ± 3.051	2.974 ± 2.966	1.920 ± 1.770	0.115‡	0.124‡
AIR 3-5 (mU/l <sup>-1</sup> )	314.1 ± 145.2	264.3 ± 187.9	233.4 ± 160.2	0.166§	0.117§
<i>S</i> <sub>1</sub> * AIR 3-5 min (10 <sup>-4</sup> /min)	841.8 ± 499.2	578.4 ± 375.4	335.4 ± 202.7	0.015‡	0.029‡

Data are means ± SD. \**P* and †*P*, *P* values for regression models assigning numbers to genotypes of 0, 1, and 2 or 0, 1, and 1, respectively. ‡Adjusted for age, sex, and BMI; §adjusted for age, sex, BMI, *S*<sub>G</sub>, and *S*<sub>1</sub>.

of INS1-E and COS-7 cells, as well as in vitro translation reactions (Fig. 1C), and demonstrated nuclear localization of PAX6 proteins in INS1-E cells by confocal microscopy (Fig. 1D).

To study PAX6-mediated transcriptional effects, we transiently transfected INS1-E cells with variant and wild-type UCP2 reporter constructs and observed similar reporter gene activities (1.00 ± 0.12 vs. 0.97 ± 0.14, NS) (Fig. 1E). Cotransfection of PAX6A-isoform expression vectors increased transcription more efficiently from the variant than the wild-type allele (4.08 ± 0.63 vs. 3.44 ± 0.61 fold induction, *P* = 0.031, two-way ANOVA), and cotransfection of PAX6B (containing the additional exon 5a) induced stronger responses than PAX6A (4.31 ± 0.31 vs. 3.21 ± 0.39, *P* < 0.001). To simulate a different transcriptional environment, we performed transfection studies in COS-7 cells. Basal transcriptions from wild-type and variant UCP2 promoter alleles were similar (1.00 ± 0.10 vs. 1.02 ± 0.12, NS) (Fig. 1F). Cotransfection of PAX6A-isoforms resulted in stronger transcriptional suppression of the variant than the wild-type allele (0.60 ± 0.11 vs. 0.72 ± 0.09 fold induction, *P* = 0.012). Cotransfection of PAX6B reduced transcription to a greater extent than cotransfection of PAX6A (0.59 ± 0.09 vs. 0.73 ± 0.10, *P* = 0.002). As suspected from bandshift studies and computational analyses, PAX6 preferentially bound to and activated transcription from the variant UCP2 promoter in INS1-E cells. In COS-7 cells, overexpression of PAX6 may be required to displace *trans* factors driving UCP2 transcription, thereby inhibiting transcription. The distinct *trans* activations of UCP2 by PAX6A and -6B are consistent with studies showing that insertion of exon 5a may modulate PAX6 DNA binding and *trans* activation properties (14).

Next we ascertained possible effects of the -866G/A polymorphism on the acute insulin response (AIR) to glucose and performed frequently sampled intravenous

glucose tolerance tests (FSIGTs) in 39 obese nondiabetic subjects. Age, sex, BMI, and fasting values for glucose, insulin, cholesterol, triglycerides, and leptin did not differ among -866 genotype groups (online appendix, <http://diabetesjournals.org>). Glucose effectiveness tended to be lower in wild-type subjects, whereas insulin sensitivity and AIR tended to be higher (Table 1). Since a hyperbolic relationship exists between AIR and insulin sensitivity in normal subjects (15,16), we compared the product of AIR and insulin sensitivity, termed disposition index (16), among genotype groups. The disposition index was highest in wild-type subjects (Table 1). In sex-specific analysis, disposition index differences were also observed in the larger group of women (*P* = 0.030).

Since both insulin resistance and impaired compensatory upregulation of insulin secretion play essential roles in the pathogenesis of type 2 diabetes (17), we tested whether the -866G/A polymorphism is associated with type 2 diabetes in obese subjects. Hardy-Weinberg expectations were fulfilled in all study populations (Table 2). Genotype distributions differed between obese diabetic and control subjects, irrespective of contrasting differences in age of control subjects and age of patients at diabetes diagnosis. Estimated diabetes risks of G/A and A/A subjects were similar in both comparisons, consistent with a recessive effect of the G allele (Table 3). Thus, the G/G genotype, identified as the at-risk genotype for obesity, was associated with a twofold reduction of type 2 diabetes risk in obese subjects. Sex-specific comparisons of diabetic patients with control subjects combined from both populations revealed differences in genotype distributions in women (*P* = 0.031) but not in the smaller group of men (*P* = 0.111). Using 50 years as cutoff level for age of diabetes diagnosis, we observed differences in genotype distributions in both the younger (*P* = 0.025) and the older age-groups (*P* = 0.025).

TABLE 2  
Characteristics of obese study subjects with type 2 diabetes and obese control populations

	Type 2 diabetes	Control group 1	Control group 2
Sex* (M/F)	96/105	56/235¶	52/48
Age† (years)	56.2 ± 12.9	41.1 ± 7.4¶	53.5 ± 5.2#
Diabetes age at onset† (years)	49.9 ± 10.1	—	—
BMI‡ (kg/m <sup>2</sup> )	36.9 ± 6.0	40.7 ± 6.9¶	34.6 ± 2.8#
Glucose§ (mmol/l)	8.46 ± 3.26	5.17 ± 0.89	5.22 ± 0.65
Cholesterol§ (mmol/l)	5.38 ± 1.57	5.23 ± 1.13	6.03 ± 1.00¶
Triglyceride§ (mmol/l)	3.18 ± 4.72	2.66 ± 3.20	1.90 ± 0.84#
HbA <sub>1c</sub> § (%)	8.1 ± 1.7	n/d	5.7 ± 0.4¶

Data are means ± SD. \* $\chi^2$  test; †ANOVA; ‡adjusted for age and sex; §adjusted for age, sex, and BMI. ||*P* < 0.001, diabetes age versus age of control group 1 or 2; ¶*P* < 0.001, type 2 diabetes versus control group 1 or 2; #*P* < 0.05, type 2 diabetes versus control group 1 or 2; Tukey post-hoc honestly significant difference test. n/d, not determined.

TABLE 3  
Association results of the -866G/A polymorphism with type 2 diabetes in obese study populations

	<i>n</i>	%	<i>P</i> <sup>*</sup>	Odds ratio (95% CI) univariate analysis	<i>P</i> <sup>†</sup>	Multivariate analysis	<i>P</i> <sup>‡</sup>
Type 2 diabetes							
-866G/G	65	0.32					
-866G/A	106	0.53					
-866A/A	30	0.15					
Control group 1							
-866G/G	137	0.47		1.00		1.00	
-866G/A	117	0.40		1.91 (1.29–2.84)	0.001	2.10 (1.29–3.41)	0.003
-866A/A	37	0.13	0.004	1.71 (0.97–3.01)	0.063	2.12 (1.05–4.26)	0.035
Control group 2							
-866G/G	49	0.49		1.00		1.00	
-866G/A	39	0.39		2.05 (1.22–3.45)	0.007	2.14 (1.24–3.66)	0.006
-866A/A	12	0.12	0.019	1.88 (0.88–4.05)	0.105	1.88 (0.85–4.13)	0.118

\* $\chi^2$  test for overall comparison between diabetes population and control group 1 and control group 2 populations. <sup>†</sup>*P* values and their odds ratios for genotypes were calculated by logistic regression analysis. <sup>‡</sup>*P* values for multivariate analyses were adjusted for sex, age (age at onset of type 2 diabetes), and BMI.

This study demonstrates an association of the -866G/A UCP2 polymorphism with age-adjusted risk of type 2 diabetes in obese patients. This result is supported at the physiological level by an influence of the polymorphism on the disposition index. At the molecular level, we show that PAX6, a *trans* factor essential for pancreatic islet function (3), influences transcription in an allele-specific manner. That a polymorphism with modest functional effects increases the risk of obesity, but guards against its metabolic complication, may be surprising. Our selection of obese study subjects may have facilitated ascertainment of genotype differences in  $\beta$ -cell function, since obesity is invariably associated with some degree of insulin resistance, thereby promoting compensatory hypersecretion of insulin by  $\beta$ -cells (15).

Our results in humans are consistent with concepts that emerged from studies in animal and cell culture models (7–10,18). Allele-specific enhancement of UCP2 expression in  $\beta$ -cells would decrease mitochondrial coupling and, as a consequence, ATP synthesis and acute insulin secretion in response to glucose. Moreover, the early insulin secretion has been shown to be essential for priming insulin-sensitive tissues and facilitating their glucose uptake (19,20). Hence, the UCP2 promoter wild-type allele, owing to its reduced transcriptional activity in  $\beta$ -cells, may be conducive for adipose tissue expansion, thereby aggravating the obesity risk that may result from reduced UCP2 expression in adipose tissue (2). If our findings hold in other genetic backgrounds and environments, UCP2 would indeed play the contrasting role in adiposity and diabetes that has been considered (11). However, the situation could be even more complicated because UCP2 may support  $\beta$ -cell function and integrity by protecting against oxidative damage (21,22), while inhibiting insulin secretion (7–10). Whether any of these UCP2-mediated effects confers an advantage or becomes harmful may depend on modifier genes and environmental influences.

#### RESEARCH DESIGN AND METHODS

**Study subjects.** A total of 201 unrelated patients with type 2 diabetes were recruited from the diabetes outpatient clinics of the Landeskliniken Salzburg, the Krankenhaus Hallein, and the University Hospital Innsbruck, all located

within 120 miles of each other. Patients who were obese (BMI >30 kg/m<sup>2</sup>) and <70 years of age at the time of diabetes diagnosis were included. Control population 1 comprised 291 subjects who were referred for weight-reducing surgery or a conservative weight-reducing program, and population 2 comprised 100 nondiabetic obese (BMI >31 kg/m<sup>2</sup>) participants of the population-based SAPHIR (Salzburg Atherosclerosis Prevention Program in Subjects at High Individual Risk) Study (2,23). In addition, 39 obese subjects without type 2 diabetes were recruited for intravenous glucose tolerance testing. Subjects were classified as diabetic if they were using hypoglycemic medications or had fasting plasma glucose concentrations  $\geq$ 7.0 mmol/l. Study populations comprised only white Europeans, mainly of Bavarian or Austrian German descent. Participants provided informed consent, and the study was approved by the local ethics committee.

**Laboratory analyses.** Plasma glucose, insulin, cholesterol, triglyceride, and leptin were measured as described (2). HbA<sub>1c</sub> levels were determined by ion-exchange chromatography. Typing of the -866G/A UCP2 promoter polymorphism and cloning of reporter constructs was performed as described (2). Primer sequences for cloning of full-length PAX6A and -6B into pcDNA6/V5-His (Invitrogen, Carlsbad, CA) were based on GenBank NM 000280 (forward 5'-CCAGCCAGAGCCAGCATGCAG-3', start codon in italics; reverse 5'-TTACTGTAATCTTGGCCAGTATTGAGAC-3', stop codon in italics).

**DNA binding studies.** Nuclear extracts from INS1-E and COS-7 cells were prepared, and bandshift assays were performed as described (2). PAX6B protein was generated with the TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI). Competitor probes were used at 30-fold molar excess.

**Western blotting.** Nuclear extracts (10  $\mu$ g protein) or 2  $\mu$ l of the PAX6 in vitro translation were separated and blotted using standard procedures. Binding of PAX6 antibody (1:5,000) (PAX6-C20; Santa Cruz Biotechnology, Santa Cruz, CA) was detected using horseradish peroxidase-conjugated secondary antibodies (1:50,000) and Pico-West substrate (Pierce, Rockford, IL).

**Cell culture and transfection.** INS1-E cells were cultured as described (10). COS-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were transfected with UCP2 reporter constructs and a constant amount of 1  $\mu$ g DNA by adding PAX6 expression plasmids or empty pcDNA6/V5-His plasmid backbone (2).

**Immunohistochemistry.** INS1-E cells were fixed, blocked, and stained with anti-PAX6-C20 (1:400) and anti-insulin (1:2,000). Binding was visualized with TRITC- and FITC-coupled secondary antibodies using the MRC-600 confocal scanning system (Bio-Rad, Hercules, CA).

**FSIGT.** After an overnight fast, a needle was inserted in an antecubital vein at 8:00 A.M. Blood samples were collected at -10 and -1 min before a glucose bolus of 30 g was administered into a contralateral vein at time zero. Blood samples were collected at 3, 4, 5, 6, 8, 10, 14, 19, 22, 25, 30, 40, 50, 60, 70, 80, 100, 140, and 180 min.

**Statistics.** Two-way ANOVA was used to compare *trans* activation of the variant and wild-type UCP2 promoter by PAX6A- and PAX6B-isoforms. Group differences of continuous variables were ascertained by ANOVA. Logarithmic transformations were made if equal variance and normality assumptions of ANOVA were rejected. Measurements were adjusted by multiple regression

for effects of sex and BMI as indicated. A contingency  $\chi^2$  test was used to compare categorical variables.

The minimal model of glucose disappearance was calculated using the SAAM II Minimal Models of the Glucose-Insulin System provided by Cobelli et al. (<http://courses.washington.edu/rfka/minmod/manual.html>) and the SAAM II software (SAAM Institute, Seattle, WA) (24).

The acute insulin response (AIR) to glucose was calculated as the area above the basal and under the time-dependent insulin level curve between 3 and 5 min after the glucose load using the trapezoidal rule. Insulin sensitivity index ( $S_i$ ) and AIR were multiplied to obtain the disposition index (16).

As studies in animal models (7–9) and our promoter studies provided an a priori hypothesis for an association of –866 G/A genotypes with FSI<sub>GT</sub> model parameters, we used multivariate regression models with glucose effectiveness ( $S_G$ ),  $S_p$ , AIR, or the disposition index as dependent variable and genotype (given codes of 0, 1, and 2 or 0, 1, and 1 in the additive or recessive models, respectively) as independent variable. Adjustments were made for age, sex, BMI,  $S_G$ , and  $S_i$  as indicated. Outcome variables  $S_G$ ,  $S_p$ , and AIR were not normally distributed and therefore logarithmically transformed for analyses.

Hardy-Weinberg equilibrium was tested using a  $\chi^2$  goodness of fit test.  $P$  values for differences in genotype frequencies between obese type 2 diabetic patients and obese control subjects were determined using a  $\chi^2$  distribution with 2 degrees of freedom. To determine associations of genotypes with type 2 diabetes among obese subjects, odds ratios with CIs were estimated by logistic regression analyses as described (2).

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